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Functional organization of vestibulospinal inputs on thoracic motoneurons responsible for trunk postural control in *Xenopus*

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ABSTRACT

In vertebrates, trunk postural stabilization is known to rely mainly on direct vestibulospinal inputs on spinal axial motoneurons. However, a substantial role of central spinal commands ascending from lumbar segments is not excluded during active locomotion. In the adult *Xenopus* a lumbar drive dramatically overwhelms the descending inputs onto thoracic postural motoneurons during swimming. Given that vestibulospinal fibers also project onto the lumbar segments that shelter the locomotor generators, we investigated whether such a lumbo-thoracic pathway may relay vestibular information and consequently, also be involved in the control of posture at rest. We show that thoracic postural motoneurons exhibit particular dendritic spatial organization allowing them to gather information from both sides of the cord. In response to passive head motion, these motoneurons display both early and delayed discharges, the latter occurring in phase with ipsilateral hindlimb extensor bursts. We demonstrate that both vestibulospinal and lumbar ascending fibers converge onto postural motoneurons, and that thoracic motoneurons monosynaptically respond to the electrical stimulation of either pathway. Finally, we show that vestibulospinal fibers project to and activate lumbar interneurons with thoracic projections. Altogether, our results complete the scheme of the vestibulospinal control of posture by illustrating the existence of a novel, indirect pathway, which implicates lumbar interneurons relaying vestibular inputs to thoracic motoneurons, and participate in global body postural stabilization in the absence of active locomotion.

KEY POINTS

- Vestibulospinal reflexes participate in postural control. How this is achieved has not been investigated fully.
- We combined electrophysiological, neuroanatomical and imaging techniques to decipher the vestibulospinal network controlling the activation of back and limb muscles responsible for postural adjustments.
- We describe two distinct pathways activating either thoracic postural motoneurons alone or thoracic and lumbar motoneurons together, the latter coordinating specifically hindlimb extensors and postural back muscles.

INTRODUCTION

Postural adjustments are essential to compensate body perturbations and perform trunk and limb movements that need to be spatially and temporally well coordinated (Massion et al., 2004). This stabilization of posture relies on the integration of multi-modal sensory inputs deriving from proprioceptive, tactile and visuo-vestibular signals (Amblard et al., 1990) together with voluntary and locomotor-induced intrinsic feed-forward commands. In particular the vestibular system, which encodes head motion, is known to interact with posturo-locomotor information to maintain body balance in space (Medrea and Cullen, 2013; Cullen, 2012; Bagnall and Schoppik, 2018).

Posture stabilization is achieved principally by trunk and limb skeletal muscles innervated by thoracic and lumbar motoneurons (Deliagina et al., 2008; Beliez et al., 2015). In mammals such motoneurons (MNs) receive uncrossed monosynaptic and crossed polysynaptic vestibulospinal inputs, both originating from the lateral vestibulo-spinal tract (LVST; Shinoda et al., 2006; Kasumacic et al., 2010, 2015). Previous studies suggested that segmental and intersegmental spinal neurons receiving also vestibular inputs could be the neural substrate for motor coordination between thoracic and lumbar, and right and left skeletal muscles during vestibular-driven postural control of trunk and limbs (Krutki et al., 2003; Kasumacic et al., 2015). Beyond this basic description, very little is known about how vestibular inputs act on spinal motor circuits in order to produce appropriate counteractive body movements in response to unexpected head motion. This lack of information is probably due in most animal models to the combined complexity of distributed multifunctional muscles and segmental spinal networks that achieve postural adjustments. In the juvenile *Xenopus laevis*, axial muscles *dorsalis trunci* are located dorsally along each side of the vertebral column and are supposedly implicated particularly in body orientation (Vallois, 1922). On the one hand, during swimming *dorsalis* MN activity is rhythmically driven by the hindlimb movements-organizing spinal network, through the activation of an ascending lumbo-thoracic propriospinal circuitry. This results in the bilateral contraction of *dorsalis* muscles in synchrony with the bilateral contraction of hindlimb extensor muscles, leading to the vertebral axis stiffening responsible for posture stabilization during forward propulsion (Beyeler et al., 2008). On the other hand, vestibular signals directly control the left/right balance activation of *dorsalis* muscles as shown by the occurrence of both postural alteration and rolling swimming behavior after the unilateral destruction of the vestibular

sensory organs in the post-metamorphosis *Xenopus* (Beyeler et al., 2013). Therefore, vestibulospinal and propriospinal influences might interact to ensure the trunk stabilization during both active and passive motion. So far the functional organization of vestibulospinal inputs on thoracic MNs and its coordination with propriospinal circuits remains barely unknown.

We thus took advantage of the *Xenopus* model to investigate whether vestibular descending and lumbar ascending pathways somehow act conjointly to shape the postural command produced by thoracic MNs. We report anatomical and physiological evidence that *dorsalis* MNs receive both direct and lumbar-relayed vestibular inputs from vestibulospinal nuclei, and that lumbo-thoracic propriospinal neurons receive direct vestibular inputs too. Our findings further suggest that direct and lumbar-relayed vestibulo-thoracic pathways respectively ensure trunk only or trunk/hindlimb coordinated reflex responses according to passive head motion physical components.

MATERIALS AND METHODS

Ethical approval

Experiments were conducted on the South African clawed toad *Xenopus laevis* obtained from the *Xenopus* Biology Resources Centre in France (University of Rennes 1; http://xenopus.univ-rennes1.fr/). Animals were maintained at 20–22°C in filtered water aquaria with a 12:12h light/dark cycle. Experiments were performed on juveniles from stage 64-66, according to external body criteria (Nieuwkoop and Faber, 1956). All procedures were carried out in accordance with, and approved by, the local ethics committee (protocols #2016011518042273 APAFIS #3612), and conform to the principles and regulations as described in the Editorial by Grundy (2015).

Neuronal retrograde and anterograde tracing

Application of neuronal anterograde or retrograde tracer crystals was performed following injection procedures already used in previous publications (Straka et al., 2001; Beyeler et al., 2008; Lambert et al., 2013). Wavelength-dependant dextran amine fluorescent dyes [10 kD Alexa dextran amine (AD) 488, 568 and 647nm; 3 kD tetramethyl-rhodamine dextran amine (RDA); Life Technologies] used in each type of tracing experiments are specified in corresponding figure legends.

Thoracic MNs were backfilled from identified dorsal trunk muscles (*dorsalis trunci*). Briefly, consecutive to anesthesia in a 0.05% MS-222 water solution, the skin covering the dorsal trunk muscles was dried and a tiny incision was performed allowing the intramuscular application of tracer crystals. Excess dye was washed out with cold Ringer's saline (93.5 mM NaCl, 3 mM KCl, 30 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 2.6 mM $CaCl_2$, 1 mM $MgCl_2$, and 11 mM glucose, pH 7.4). After recovering from anesthesia, juvenile *Xenopus* were kept in a water tank for at least 48h to allow tracer migration into MN cell bodies and dendrites (*e.g.*, Fig. 1D-E).

Vestibulospinal or lumbo-thoracic propriospinal pathways that project onto *dorsalis* MNs were anatomically deciphered using isolated whole CNS preparations obtained after dissection according to surgery procedures described in Beyeler et al., 2008. Bilateral vestibulospinal descending neuronal groups were first identified with retrograde labeling (Fig. 3). After a tiny unilateral incision, fluorescent dye was applied in thoracic segment 2 (T2) on one side, in a ventro-medial position that corresponds to *dorsalis* MNs location in the thoracic cord (Fig. 1D), and tracer migration was allowed overnight in circulating Ringer's saline at 15-16°C at dark (Lambert et al., 2013) to label brainstem vestibulospinal cell bodies. To distinguish thoracic-projecting vestibular neurons from cervical-projecting vestibular neurons (Fig. 3), a second fluorescent dye, with a different emission wavelength, was applied in the ipsilateral first cervical hemi-segment (C1) the morning after T2 injection, followed by a 4-5h migration. Similar double dye injection was performed to label lumbar neurons projecting to T2 but the dye injection in C1 was perform prior to that in T2 (Fig. 5A). Concomitant fluorescent labeling of either the VIIIth cranial nerve or the first lumbar ventral root (L1) allowed the identification of rostro-caudal anatomical markers (vestibular afferents and L1 MNs, respectively) on cross-sections during confocal imaging.

Vestibulospinal terminals onto backfilled thoracic MNs (Fig. 4C-E) were revealed with overnight anterograde labeling. Following tiny unilateral incision in brainstem dorsal surface on one side, dye crystals were applied either in rhombomere 4 at the VIIIth nerve level in order to label LVST projections, or in rhombomere 5-6 at the Xth nerve level to label tangential nucleus (TAN) projections, respectively. Lumbar propriospinal terminals onto thoracic MNs (Fig. 5D) were labeled with a similar procedure, where crystals were applied in a small incision performed at the ventral surface of a L1 spinal hemi-segment and close to the midline.

Immunofluorescence labeling

After fluorescent tracer migration, isolated CNS preparations were fixed in 4% paraformaldehyde at 4°C overnight, then incubated in a 20% [in phosphate-buffered saline (PBS) 0.1%] sucrose solution for 24h before being embed in Tissue-Tek (VWR-Chemicals) and frozen at -45°C in isopentane (for detail procedure see Lambert et al., 2018). Fluorescence immunohistochemistry on thoracic or lumbar spinal 20-µm cross-sections obtained with a cryostat (CM 3050, Leica) was performed with the same protocol as extensively described in Bougerol et al., 2015, and Lambert et al., 2018. Samples were incubated with the primary antibody for 48 h at 4°C. After rinsing, samples were incubated for 90 min at room temperature with a fluorescent dye-coupled secondary antibody. After washing, cross-sections were mounted in a homemade medium containing 74.9% glycerol, 25% Coon's solution (0.1 M NaCl and 0.01 M diethyl-barbiturate sodium in PBS), and 0.1% paraphenylenediamide. The mouse primary antibodies anti-synapsin (1:200; Synaptic Systems) were used to label presynaptic terminals and revealed with donkey secondary anti-mouse IgG coupled to Alexa Fluor 488 (1:200; Thermo Fischer).

Image acquisition and processing

Cross-sections labeled with fluorescent materials were imaged using an Olympus FV1000 confocal microscope equipped with 488, 543 and 633 nm laser lines. Multi-image confocal stacks with 0.6 µm z-step intervals were generated using a 20x/0.75 oil objective and with 0.3 µm z-step intervals using a 60x/1.4 oil objective. Cell population images were obtained by orthogonal projection from multi-image stacks while synaptic apposition images were drawn from single confocal planes. Final images were processed with artificial fluorescent colors using Fiji (https://fiji.sc/) and Photoshop (Adobe Systems Inc.) softwares.

Brainstem and lumbar cord 3D reconstruction

Following neuronal tracing, consecutive brainstem or lumbar spinal cord 40-µm slices were imaged with the confocal microscope using a 10x air objective to obtain multi-channel image z-stacks. In all preparations used in 3D reconstruction, every consecutive z-stack was subsequently used to generate a multi-channel orthogonal projection view. These latter images were then separated channel-by-channel to be reassembled in a rostrocaudal sequence of images for each wavelength channel. As a result, two rostrocaudal image sequences were produced for single brainstem or lumbar cords, displaying the segmental distribution of two

retrogradely labeled populations of neurons (*i.e*., T2 vs. C1-projecting vestibulospinal neurons, or T2 vs. C1-projecting lumbar propriospinal neurons, respectively). As explained above, labeled vestibular afferents or L1 MNs were used as a rostrocaudal anatomical marker in 3D reconstructions. Every single-channel image sequences were computed in the Fiji *TrackEM2* plug-in to realign all single images and generate new sequences of aligned images. Thereafter, all brainstem and lumbar cord cross-sections were manually outlined from aligned image sequences, and the borders of vestibular afferents or L1 MNs acquired and stored similarly. Every single labeled neuron was then manually pointed, image after image, its coordinates being recorded automatically in a ROI tally sheet (Fiji *ROI Manager* plug-in). Subsequently, a final rostrocaudal image stack including consecutive cross-section borders with all neuron positions was generated with a homemade Fiji macro (Imagys; http://www.incia.u-bordeaux1.fr/spip.php?article629). This final stack was applied a segmentation process to restore tissue volume from single plane images and implemented thereafter into the ParaView 5.5.0-RCA software to generate and export 3D visualizations (Fig. 3D, Fig. 5B). Brightness and contrasts were adjusted for illustration using Photoshop.

In vivo EMG recordings

Procedures used for *in vivo* EMG recordings of dorsal trunk and hindlimb muscles were as described previously (Beyeler et al., 2008). Briefly, the EMG activity of bilateral trunk (*dorsalis trunci*) and ankle extensor (*plantaris longus*) muscles was recorded using pairs of 50 µm insulated wire electrodes connected through a grounded cable to a differential AC amplifier (Model 1700; AM-Systems) and digitized at 10 kHz with a CED 1401 interface (Cambridge Electronic Device). Data were displayed and stored using Spike2 software (Cambridge Electronic Device).

In vitro semi-intact and isolated preparations

Animal dissection for *in vitro* preparations and nerve recording procedures were conducted as described extensively in Beyeler et al., 2008. Under anesthesia and after viscera and forebrain removal, the brainstem-spinal cord together with the thoracic ventral roots (Vr) and the motor nerves innervating ankle flexor (Flex; *tibialis anterior*) and extensor (Ext; *plantaris longus*) on both sides were dissected out from stage 64-66 juveniles. *In vitro* experiments were performed in carbogen-bubbled saline, either normal Ringer's (see above)

or a solution enriched in divalent cations (74.8 mM NaCl, 3 mM KCl, 30 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 10.6 mM $CaCl_2$, 4 mM $MgCl_2$, and 11 mM glucose, pH 7.4) in order to raise neuronal spiking threshold and preserve only monosynaptic connections (Berry and Pentreath, 1976). In semi-intact preparations, the two otic capsules were kept intact and attached to the brainstem with the VIIIth nerve. T2 Vr extracellular activity was recorded with borosilicate glass suction electrodes (tip diameter, 100 nm; Clark GC 120F; Harvard Apparatus) filled with Ringer's solution. Extracellular activity from Ext/Flex motor nerves was recorded using stainless *en passant* wire electrodes. Combined T2 Vr and Ext motor nerve activities were recorded in response to either vestibular nuclei or lumbar cord electrical stimulation in isolated preparations and in response to natural stimulation of vestibular endorgans in semi-intact preparations. Electrical stimulation of vestibular nuclei or lumbar regions consisted of single pulses delivered with a Grass stimulator S88 through a 2 MΩ monopolar stainless electrode (Micro Probe, Inc.). Stimuli (10-15 µs; 5-10 V) were always set with the minimal voltage amplitude capable of eliciting stable motor responses in normal saline. In some vestibular nuclei stimulus experiments, a ventral hemi-section was performed at C1 level to prevent composite vestibulospinal commands to activate downstream motoneurons; no differences in motor response were ever observed in this condition compared to intact isolated spinal cords, and data were pooled in the results. Natural stimulation of vestibular endorgans was performed using a computer-controlled, motorized stimulation apparatus (Turn-table and Sled; Technoshop COH@BIT, IUT de Bordeaux, University of Bordeaux). The animal was centered in the horizontal rotation and translation axes in order to provide natural activation of horizontal semicircular canals and utricles (Fig. 2; see also Lambert et al., 2008). 10 to 15-cycle sequences of sinusoidal stimulation were performed at 0.1-1.0Hz, with amplitudes of ±5-60 °/s for rotation and ±80-470 mm/s for translation, respectively. In this apparatus, T2 Vr and Ext motor nerve discharges were recorded with adjusted suction electrodes using EXT 10-2F amplifiers (NPI Electronics). Data were digitized, displayed as described for EMG recordings, and analyzed relative to the table position sinewave.

Calcium imaging

In CNS isolated preparations Calcium Green-1 Dextran Amine crystals (3 kD CGDA; Invitrogen) were injected unilaterally in a T2 hemi-segment (same procedure as described

above for neuronal tracing) in order to backfill T2-projecting lumbar neurons. The CGDA injection was performed after a partial incision of the T2 hemi-segment from the ventro-lateral surface of the cord. This partial incision ensured the labeling of lumbar INs from the thoracic lateral dendrite area but prevented damages of brainstem descending fibers in the ventro-medial cord. After 4-5h of CGDA retrograde migration, the spinal cord was book-opened from the dorsal line and flattened inside-up in the recording chamber, in order to visualize labeled neurons. Optical recording of calcium transients (Fig. 7E-F; n=5 animals) was performed with an epifluorescence microscope (Olympus BX51WI) and a high CCD camera (QImaging OptiMOS) at a 10 fps image rate (time exposure=95 ms; image interval=100 ms) under x40 water immersion objective (Olympus LUMPlanFLN). Calcium transients (fluorescence changes relative to the fluorescence baseline, ΔF/F) evoked in T2-projecting lumbar interneurons in response to vestibular nuclei electrical stimulation (50 µs single pulses) were measured within ROI drawn over individual neuron somata (Fig. 7E) using MetaFluor (Meta Imaging Series 7.8; Molecular Devices). Depending on the quality of the retrograde labeling, 5-12 neurons were recorded per lumbar region at x40 and 1-3 lumbar regions were investigated per preparation. In total, 60 L1 INs projecting to ipsilateral T2 segment and 91 L1 INs projecting to contralateral T2 segment (see Fig. 7E) were optically recorded in response to either LVST or TAN electrical stimulation.

Data analysis and statistics

Electrophysiological extracellular recordings of motor nerve discharges were analyzed offline with Spike2. To analyze the modulation of nerve activity in response to natural vestibular stimulation, discharge rates in individual nerve recordings were measured by setting an amplitude threshold to count all impulses in such multi-unit recordings. Firing rates (in spikes/s) were averaged over 10-15 stimulation cycles. Statistical analysis of electrophysiological data were performed using Prism5 (GraphPad) and OriginPro8 (OriginLab Corporation), and data are given as means and standard deviation (±SD). Note that, as usually the case in studies requiring animal sacrifice, samples used in this study were voluntarily kept low and, consequently, a p value larger than 0.05 indicated only that the data did not give any reason to conclude that the distributions differed. Circular phase analysis was performed using Oriana2 (Kovach Computing Services). The non homogeneity of phase distributions was first tested with the Rayleigh test, and subsequent circular

statistical analysis was performed only on non-homogenous distributions; no distributions in this study were found homogenous. Similarity between two circular distributions was then investigated using the Watson U^2 test, for which the null hypothesis is that the two distributions are identical, and a significant p value indicates rejection of the null hypothesis. Unfortunately, Oriana2 does not provide the exact p value, but the value of the U^2 is given in the text for each statistical test in order to provide the more information about its significance (the lower U^2, the higher similarity between the two compared distributions). Calcium imaging data were analyzed offline; data are given as means±SD, and statistical significance was tested using Prism5.

RESULTS

Morphology of motoneurons innervating dorsal trunk muscles and patterns of postural activity

Dorsalis trunci muscles are located bilaterally along the vertebrae and consist of thin fleshy fascicles inserted dorsally between transverse and spinous processes principally, although the most anterior ones appear to link also ipsilateral ribs (Fig. 1A left). Especially, the third myomeres are inserted between thoracic vertebra T1's spinous process and T2's transverse process (Fig. 1A right). Similarly, the 4^{th} myomeres are inserted between T2 and T3 spinous processes. In this configuration, a contraction of these specific *dorsalis* myomeres on one side would only twist the vertebral column along the body axis, with the rostral vertebra slightly rotating towards the contracted myomeres and the caudal vertebra in the opposite direction. Such vertebral twists create small amplitude rolling angles that allow trunk postural adjustments and body orientation.

A previous study in freely behaving juveniles has shown that the activity of the third myomere of *dorsalis trunci* muscles depends on whether the animal is swimming or not (Beyeler et al., 2008). Bilateral EMG recordings in swimming animals (Fig. 1B) exhibit rhythmic bursts that occur mainly in phase with ipsilateral *plantaris longus* bursts. In this situation, *dorsalis* activity corresponds to pro-active postural adjustments previously shown to be driven directly by the lumbar CPG for hindlimb kicking during swimming. In resting animals (the absence of swimming is corroborated by the absence of coordinated *plantaris* bursts; Fig. 1C), bilateral *dorsalis* activity occurs mainly uncoordinated, likely driven only by reflex sensory-motor circuits.

Morphology and dendritic organization of thoracic MNs innervating *dorsalis trunci* muscles was revealed by retrograde labeling with two distinct fluorescent dyes injected in *dorsalis* third myomere on each side of the vertebral axis (Fig. $1D_1$). Cell bodies of these MNs were located mainly in the second thoracic spinal segment (T2), organized in bilateral ventro-medial motor columns (Fig. 1D and E). These postural T2 MNs exhibited large somata and a particularly extended dendritic arborization that surprisingly projects long branches into the two hemi-cords (Fig. $1D_2$-F). Indeed, in addition to classical dendrites running into the ipsilateral hemi-segment (thereafter called "lateral dendrites") long commissural dendrites were found to project far into the contralateral hemi-cord, ending in the same regions as the lateral dendrites of contralateral MNs. Although both dendrite types were found in a similar proportion (2.7±0.9 vs. 2.6±0.9, respectively; n=14 animals; t-test, p=0.72; Fig. 1G), lateral dendrites tended to show more ramifications (6.6±2.9) than commissural ones (4.5±1.4; t-test, p=0.09). This particular dendritic arrangement suggests that postural T2 MNs are able to gather the same type of information from both sides of the cord, although probably to a different extent.

Dual responses of thoracic motoneurons to natural vestibular stimulation

In the juvenile *Xenopus*, forward translation corresponds to the main motion component of body displacement during swimming. Backward translation can also be observed, especially in startling responses or when the animal retreats before a threat. Bilateral T2 and hindlimb Ext motor nerve activities were recorded in semi-intact preparations in response to natural stimulation of vestibular endorgans (Fig. 2). Utricular maculae, otolithic endorgans detecting head linear accelerations in all body reference plans including gravito-inertial signals (Straka and Dieringer, 2004; Lambert et al., 2008), were activated with antero-posterior horizontal translations (Fig. 2A and B_1). Horizontal semicircular canal ampulae, detecting head horizontal angular accelerations (Straka and Dieringer, 2004; Lambert et al., 2008), were activated with horizontal rotations (Fig. 2A, B_2 and E_1). In both translation and rotation conditions, series of sinusoidal movements (see Methods) were applied in order to record horizontal body plane-specific dynamic responses of, respectively, utricle-driven and canal-driven motor responses in T2 Vr and Ext motor nerves specific to the horizontal body plane.

T2 Vr bursts on both sides always increased simultaneously during forward linear motion, the maximum discharge rate occurring near the table mid-course between its two extreme positions (Fig. $2C_1$; n=5 animals). The circular distribution of phases for both left and right T2 activity was similar ($p>0.5$, Watson U^2 test: $U^2=0.053$), and analysis showed that left and right peak discharges occurred with a mean phase lead of 114±15° and 116±8°, respectively, relative to the maximal forward position of the motion table (Fig. $2D_1$ left, plain light and dark blue arrows). In most trials (81%) backward linear movements bilaterally triggered simultaneous motor bursts in T2 and Ext nerves (Fig. $2B_1$; n=5 animals). In such cases, Ext discharges were related to small amplitude postural movements of the hindlimbs (Suppl. Movie 1) that differed fundamentally from the large amplitude locomotor kicks (see Beyeler et al., 2013). During backward translation, no T2 Vr response was ever observed alone, *i.e*. in absence of Ext burst, suggesting that backward vestibular-induced T2 Vr motor activity required the activation of the lumbar circuitry. Bilaterally simultaneous bursting activity was recorded from both Ext and T2 motor nerves, the maximum of which occurred around backward mid-course. Phase distributions were similar for bilateral Ext ($p>0.5$, Watson U^2 test: $U^2=0.055$) and bilateral T2 activity ($p>0.2$, Watson U^2 test: $U^2=0.116$), but different between homolateral Ext and T2 activity ($p<0.05$, Watson U^2 test). Analysis showed respective mean phase leads relative to the maximal backward table position of 124±10° and 123±20° for left and right Ext (Fig. $2D_1$ left, plain light and dark orange arrows), and 76±7° and 75±15° for left and right T2 (Fig. D_1 left, dashed light and dark blue arrows). Notably, none of the phase relationships was dramatically affected by changing table motion amplitude or frequency (Fig. $2D_1$ right). Phase analysis also demonstrated that Ext discharges systematically preceded T2 bursts (50±8° mean phase shift), which further supported the hypothesis that backward utricle-induced lumbar network activity is mandatory to trigger concomitant vestibular-induced motor response in thoracic motor nerves.

Bilateral T2 Vr discharges were also modulated by horizontal sinusoidal head rotations (n=7 animals). Leftward rotations evoked motor bursts in the left T2 Vr (Fig. $2B_2$), the maximum discharge rate occurring around the table maximum leftward excursion (Fig. $2C_2$), with a mean phase shift of -4±8° relative to the maximal leftward position of the table (Fig. $2D_2$); conversely, the right side T2 Vr discharge was modulated in a similar way by rightward rotation. Thus, left and right T2 discharges showed very opposite phase distributions (Fig. $2D_2$ left; $p<0.001$, Watson U^2 test: $U^2=0.542$) but none was significantly

affected by table movement frequency or amplitude (Fig. $2D_2$ right). In addition, in less than 20% of the preparations exposed to horizontal rotation, T2 Vr activity demonstrated extra motor bursts coupled with ipsilateral Ext discharges during horizontal rotation, around the mid-course of the round-table in the homolateral direction (Fig. $2E_{1\text{-}2}$), as akin to what was observed as a general rule for frontal backward translation (see above). Similarly too, the circular distribution of both Ext and T2 activity on either side was not clearly affected by table rotation frequency or amplitude, and T2 Vr responses systematically followed Ext bursts with a mean phase shift of 34±15° (Fig. $2E_3$).

Taken together these results showed the existence of two temporally distinct vestibular-evoked reflex responses in postural thoracic MNs, suggesting the potential existence of two distinct parallel functional pathways. A first vestibulospinal command seems to activate only thoracic motor networks, without related Ext motor nerve activity, and may be related to small postural adjustments restricted to the trunk only. A second pathway activates conjointly thoracic and lumbar MNs, and could be involved in a broader vestibular-evoked control of the body balance that would necessitate the coordination of trunk and hindlimbs postural adjustments. This latter vestibular-evoked reflex suggests that the lumbar motor network could constitute a neuronal relay in some vestibulospinal pathways projecting to thoracic motor networks.

Brainstem location of vestibulospinal neurons projecting on thoraco-lumbar spinal segments

Vestibulospinal neurons projecting on thoraco-lumbar spinal cord were located in the brainstem by performing retrograde labeling from spinal segments. Two sequential dye applications were made from homolateral T2, then C1 hemi-segments (see Methods), in association with anterograde labeling of the VIIIth nerve anterior ramus in order to visualize its entry in the brainstem as an anatomical marker of rhombomere 4 (Fig. 3A). Cell bodies were labeled on the two sides of the brainstem, in the regions where vestibular afferents contact central secondary vestibular neurons. A first cluster of cell bodies was found ipsilateral to the injection sites, located laterally and dorsally in the alar plate, and corresponded to neurons of the lateral vestibulospinal tract (LVST; Straka et al., 2001; Fig. $3B_1$). A second cluster was found contralaterally, located slightly more ventrally, and corresponded to neurons of the tangential nucleus (TAN; Straka et al., 2001; Fig. $3B_2$). Most neurons in both vestibulospinal nuclei showed double labeling (insets in Fig. 3B). Cell

counting revealed that neurons projecting to thoraco-lumbar segments represented the main proportion of the labeled LVST (86±4%) and TAN (80±3%) population neurons (Fig. 3C$_1$).

3D reconstruction from brainstem slice series (see Methods; Fig. 3D) demonstrated that C1- and T2-projecting vestibulospinal neurons were intermingled and distributed within the entire LVST and TAN nuclei, spanning from 200 µm rostral to 750 µm caudal to the VIIIth nerve for the LVST, and from 100 µm rostral to 600 µm caudal to the VIIIth nerve for the TAN (Fig. 3C$_2$). Combination of double retrograde labeling from the first lumbar (L1) and T2 segments on two other groups of juveniles (not illustrated) further showed that LVST neurons preferentially project into the lumbar spinal cord (76±8%) while TAN neurons projected more comparably into both thoracic and lumbar segments (T2: 43±5%; L1: 57±5%). Altogether, these results showed a rhombomeric organization of vestibulospinal neurons in *Xenopus* that was comparable to what had been previously described in other vertebrates (Straka et al., 2001; McCall et al., 2017), and further revealed that most of the vestibulospinal neurons project to thoraco-lumbar segments without rostrocaudal somatotopic arrangement within the two brainstem nuclei.

Thoracic postural motoneurons receive direct vestibulospinal inputs

Retrograde labeling from T2 segment indicated that a large part of the TAN and LVST vestibular neurons might project onto T2 postural circuitry. In normal saline, electrical stimulation of either vestibulospinal nucleus evoked compound action potentials recorded extracellularly from T2 ventral roots. Stimulation magnitude was adjusted in order to record minimal responses, which typically consisted of fast and short duration compound response (Fig. 4A-B; n=15 animals) rarely followed a few tens of milliseconds later by longer lasting bursts (not shown). In these conditions, single stimulation pulses applied in the LVST nucleus consistently evoked compound action potentials only on the ipsilateral T2 Vr with a 8.1±6.9 ms delay (Fig. 4B top). These extracellular potentials persisted in high divalent cation saline, although occurring with a significantly increased delay (14.6±9.4 ms; Wilcoxon paired test, p=0.002). Single minimal stimulation pulses in the TAN consistently evoked contralateral compound action potentials in T2 Vr (delay: 10.3±2.9 ms), sometimes accompanied by compound action potentials in the ipsilateral T2 Vr (59% of the cases; delay: 9.9±0.3 ms). However, whereas fast contralateral responses always persisted in high divalent

cation saline with a slightly but non-significant longer delay (12.6±2.1 ms; Wilcoxon paired test, p=0.19), ipsilateral responses persisted in only few preparations (<20%; e.g., Fig. 4B bottom), with a slightly increased delay (11.3±0.2 ms; Mann-Whitney test, p=0.002).

Compound action potentials evoked in T2 ventral roots in response to TAN and LVST electrical stimulation under high divalent cation saline suggested that T2 MNs received monosynaptic inputs from both vestibulospinal nuclei. Unilateral retrograde labeling of T2 MNs was combined with anterograde labeling from the ipsilateral TAN, the contralateral TAN or the ipsilateral LVST nucleus, together with subsequent immuno-detection of the presynaptic protein synapsin (Fig. 4C-E). Because TAN and LVST neurons are partly intermingled, a hemisection at the first spinal cervical segment was performed either ipsilateral to the TAN tracer injection (to avoid LVST terminals labeling; see injection scheme in Fig. 4C and D) or contralateral to LVST tracer injection (to avoid TAN terminals labeling; see injection scheme in Fig. 4E). Sites of close apposition between LVST or TAN terminals and MN dendrites or somata that co-localized with synapsin were found on all labeled thoracic MNs. Synapsin appositions were found between terminals labeled from TAN area and commissural dendrites of ipsilateral thoracic MNs (Fig. 4C), and somata and lateral dendrites of contralateral MNs (Fig. 4D), respectively. Terminals labeled from LVST location presented synapsin apposition with both soma and lateral dendrites of ipsilateral thoracic MNs (Fig. 4E). Although the mean repartition of synapses appeared similar on somata and dendrites (2.0±1.0 vs. 2.7±1.3, respectively), careful analysis from three animals demonstrated heterogeneous distributions since vestibulospinal terminal/synapsin appositions were primarily distributed in the proximo-medial part of both commissural and lateral dendrites (see Fig. 6F).

The anatomical observation of vestibulospinal terminals onto T2 MNs (Fig. 4C-E) is consistent with T2 motor activities evoked in response to vestibular stimulation (Fig. 2 and Fig. 4A-B). Such a combination of electrophysiological and anatomical evidence of direct vestibulospinal inputs on thoracic MNs supports the idea of a direct vestibulospinal pathway to thoracic motor network that would command for specific trunk reflexive postural control in response to head movements as shown in Figure 2. In contrast, the existence of concomitant bursts in hindlimb Ext and T2 motor nerves, occurring with a different stimulus/response phase relationship than T2 responses alone, might require the involvement of another dedicated vestibulospinal circuit.

Thoracic motoneurons receive direct inputs from ipsilateral L1 ascending interneurons

The natural stimulation of vestibular endorgans triggered coordinated bursting activity in both hindlimb extensor and thoracic motor nerves (see above). These vestibular-evoked coordinated thoraco-lumbar reflex responses occurred in a stereotyped sequential manner, the lumbar Ext bursts always preceding T2 ones, which suggested some vestibulospinal commands conjointly activate both thoracic and lumbar motor networks and a foreseeable lumbar interneuronal relay to achieve such coordinated postural adjustments of the trunk and hindlimbs.

The existence of lumbar interneurons (INs) putatively projecting into segment T2 was investigated by performing a sequential retrograde labeling within the spinal cord, which consisted of two consecutive, 12 h-delayed injections of two distinct fluorescent dyes within a cervical hemi-segment and its ipsilateral T2 hemi-segment, respectively (see scheme on Fig. $5A_1$). Such an approach allowed the anatomical segregation of lumbar INs projecting only into T2 segments (single labeling) from INs projecting more rostral (double labeling) (Fig. $5A_2$). Notably, singly-labeled cell bodies were found on both sides of the entire lumbar cord (Fig. 5A-B and Fig. 6A) and located more medially than hindlimb MNs (identified by retrograde tracing from lumbar ventral roots; *e.g.*, Fig. $5B_1$ and Fig. 6A). Moreover, T2 projecting INs were found with a decreasing rostro-caudal gradient along the three lumbar segments (Fig. $5B_2$).

Given their location, the more dorsal group of lumbar INs are likely specialized in processing and dispatching proprioceptive sensory information towards other spinal segments (Lu et al., 2015). In contrast, we hypothesized that the more ventral group of INs could play a major role in coupling motor functions of both lumbar and thoracic segments and, thus, operate the combined vestibular activation of Ext and T2 MNs. To test this hypothesis, unilateral electrical stimulation of minimal magnitude was applied in the rostral part of L1 lumbar segment (see scheme in Fig. 5C; n=12 preparations), in the ventro-medial region where the highest density of T2-projecting INs was found. Motor responses were reliably triggered (99.8%; n=162 trials) in the ipsilateral T2 Vr, although the response delay varied a lot among preparations (mean delay of 5.9±8.6 ms; Fig. 5C), generally followed by delayed long-duration bursts. Perfusion of high divalent cation saline did not prevent the occurrence of the initial T2 Vr discharge, which occurred with non-significantly increased

delay (mean delay of 8.1±14.8 ms; Wilcoxon paired test, p=0.15), whereas the secondary discharge consistently disappeared. Although we could not rule out the additional participation of polysynaptic pathways or the possibility of an antidromic activation of thoracic INS likely projecting to the lumbar cord, the persistence of the fast T2 Vr response in high divalent cation saline strongly suggested the existence of direct monosynaptic inputs between ipsilateral rostral L1 INs and T2 MNs. Unilateral retrograde labeling of T2 MNs was then performed with concomitant dye injection in ipsilateral rostral L1 ventral hemicord (see inset in Fig. 5D). Synapsin immuno-detection was performed on T2 cross sections presenting MN labeled from dorsal muscle and L1 ascending terminals. Numerous close appositions of synapsin signal and ipsilateral L1 ascending IN axons were found on every labeled T2 MN commissural and lateral dendrites (Fig. 5D). Apposition analysis suggested that L1 INs connected in seemingly comparable proportions to commissural (through axon collaterals crossing the midline at the thoracic level; not shown) and lateral dendrites (2.60±0.89 vs. 2.58±1.08; n=5 animals; t-test, p=0.95), although the distribution of L1 INs putative synapses was differing between the two dendritic regions (Fig. 6F). Thus, our electrophysiological and anatomical results highlighted direct excitatory connections between rostral L1 ascending INs and T2 MNs that could robustly couple lumbar Ext and axial MN motor activities during vestibular-controlled postural adjustments.

It is notable that only the stimulation of the rostral L1 segment triggered exclusively ipsilateral lumbar-evoked T2 motor response. These T2 responses were often accompanied by a fast motor burst on hindlimb motor nerves, followed or not by repeated rhythmic-like bursts (*e.g.*, Fig. 6C-E).Yet, the electrical stimulation of more caudal lumbar regions (not illustrated) could also trigger responses in T2 and hindlimb motor nerves from either the same or opposite side of the cord, and synapsin appositions suggesting direct synaptic contacts were sometimes observed between contralateral INs and T2 MN soma/lateral dendrites (e.g., Fig. 6B). Nevertheless, such synapsin appositions were more rarely identified anatomically, and T2 motor responses to electrical stimulation of more caudal lumbar regions were much more labile than rostral L1-evoked ones. In addition, more than half of them were prevented in high divalent cation saline (not illustrated). Hence, T2-projecting INs located in the different lumbar segments could participate in coordinating the discharges of both hindlimb extensor and trunk MNs in response to vestibular signals. However, such a coordinating function would require lumbar INs to receive vestibular inputs at first.

549

550 *T2-projecting lumbar interneurons receive vestibular inputs*

551 Anterograde labeling of vestibular terminals projecting to lumbar segments was
552 performed by dye injection in either vestibulospinal area, while retrograde labeling of T2-
553 projecting lumbar INs was obtained from unilateral dye injection in a T2 hemi-segment.
554 Synapsin immunodetection was then realized on fixed L1 spinal cord slices to detect putative
555 synaptic contacts from either ipsilateral LVST (Fig. 7A-B) or contralateral TAN vestibulospinal
556 fibers and ascending L1 INs (Fig. 7C-D). Several close appositions were found between
557 vestibulospinal terminals of either nucleus and L1 IN somata and identified proximal
558 dendrites that co-localized with synapsin. Careful apposition counting performed on four
559 distinct animals showed that 56% of the labeled L1 INs received LVST synapses and 68%
560 received TAN synapses, with a respective mean number of synaptic contacts of 2.1±0.2 and
561 2.2±0.2. These observations suggested that ascending lumbar INs could receive synaptic
562 inputs from vestibulospinal axons of the two nuclei.

563 Optical recordings were undertaken to investigate the potential activation of T2-
564 projecting L1 INs by central vestibular inputs (Fig. 7E-F). Crystals of Calcium-Green Dextran
565 Amine (CGDA) were applied laterally in a T2 hemi-segment to fill retrogradely ascending L1
566 INs on both sides (see scheme in Fig. 7E). Calcium transient activity from such backfilled L1
567 INs and extracellular motor nerve activity from T2 Vr were recorded simultaneously in
568 response to either ipsi- or contralateral vestibular nuclei electrical stimulation. Single pulse
569 stimulation of a LVST nucleus bilaterally evoked calcium transient in T2-projecting L1 INs that
570 was coupled to the vestibular-evoked burst discharge in the T2 Vr motor nerve (Fig. $7F_1$,
571 upper traces). 100% of recorded neurons (60 ipsilaterally and 91 contralaterally projecting L1
572 INs; n=4 preparations) responded to LVST stimulation as exemplified in Fig. $7F_2$ (left traces)
573 with a ΔF/F peak of 2.4±2.1% and 4.0%±3.2% (Fig. $7F_3$) for ipsi- and contralaterally T2-
574 projecting L1 INs, respectively. The same pattern of response was observed after TAN
575 electrical stimulation in 100% of T2-projecting neurons L1 INs on the two sides (e.g., Fig. $7F_1$,
576 bottom traces; Fig. $7F_2$, right traces) with a ΔF/F peak of 2.6±1.6% and 3.4%±1.7% (Fig. $7F_3$)
577 for ipsi- and contralaterally T2-projecting L1 INs, respectively. No statistical differences were
578 found between either groups (Kruskal-Wallis test, p=0.15). Sometimes, a single pulse (either
579 in LVST or TAN) elicited repeated rhythmic-like transients, consecutive to the first optical
580 response, over several seconds (3-10s) in all recorded neurons (Fig. $7F_2$, see black arrow on

left traces), suggesting a possible involvement in the generation and/or maintaining of locomotor-like activity (as also suggested above by the observation of rhythmic bursts on hindlimb motor nerves in response to L1 IN electrical stimulation). These optical recordings thus demonstrated that T2-projecting lumbar INs are responsive to vestibulospinal inputs and, thus, might be able to relay vestibulospinal commands to postural thoracic MNs.

DISCUSSION

This study reports anatomical and physiological evidence that the thoracic MNs responsible for *dorsalis trunci* 3rd myomere postural activity receive a dual command from brainstem vestibular nuclei at rest, one conveyed by direct descending vestibulo-thoracic pathways, the other mediated by yet undescribed vestibulo-lumbo-thoracic pathways involving a lumbar interneuronal relay (Fig. 8). We propose that the former pathway is involved in the treatment of sensorimotor signals resulting exclusively in postural adjustments of the trunk while the latter is rather engaged in the balance of the entire body by coordinating trunk and hindlimbs reflexive movements.

Due to their peculiar dendritic arbor, notably characterized by long commissural dendrites, thoracic postural MNs on both sides are likely allowed to gather information from the same sources, even though those sources project unilaterally in the spinal cord. Comparable anatomo-functional organization was found in the lateral line system of teleost fishes, where octavolateralis efferent neurons integrate bilateral sensory inputs and their dendrites extent both ipsi- and contralaterally (Roberts and Meredith, 1989). MNs with dendrites crossing the ventral commissure have been observed in many vertebrate species, including lampreys (Wallen et al., 1985), mudpuppies (Jovanovic and Burke, 2004), terrestrial frogs (Szekely, 1976; Erulkar and Soller, 1980; Bacskai et al., 2010), turtles (McDonagh et al., 2002), rodents (Rose and Collins, 1985; Allan and Greer, 1997; Tarras-Wahlberg and Rekling, 2009), and cats (Light and Metz, 1978; Cameron et al., 1983). However, commissural dendrites were not found in all motoneuronal populations (Ulfhake and Cullheim, 1981; Ulfhake and Kellerth, 1981; Rosenthal and Cruce, 1985). Crossing dendrites may be specific to axial MNs, the ventro-medial position of which allowing them to extend dendrites towards their contralateral counterparts. Initially, commissural dendrites in the adult frog were proposed to play a major role in the reciprocal activation of dorsal muscles on the two sides, each side exciting the other (Szekely, 1976), although there was no proof of such

reciprocal excitation. In contrast such central MN-MN co-excitation exists in the larval zebrafish, although it remains homolateral and involves central axon collaterals (Menelaou and McLean, 2012; Bello-Rojas et al, 2019). The present study in *Xenopus* juvenile rather suggests that crossing dendrites allow MNs on both sides to integrate the same sets of inputs, whether descending from the brainstem or ascending from the lumbar spinal cord, the result of which is a coordinated activation (perhaps synchronization) of bilateral dorsal muscles.

Thoracic MNs appear to play a particularly pivotal role in the spinal control of posture. Indeed, in addition to lumbar swimming CPG-generated postural commands (Beyeler et al., 2008) both direct and lumbar-relayed vestibular commands converge onto T2 MNs. In normal saline, minimal electrical stimulation of brainstem vestibular nuclei consistently elicited fast T2 ventral root responses that mostly persisted in high divalent cation saline. While LVST stimulation activated only ipsilateral T2 MNs, TAN minimal stimulation triggered responses in both ipsi- and contralateral T2 motor nerves. However, only TAN-evoked contralateral responses always persisted under high calcium/high magnesium saline, which could suggest TAN-triggered ipsilateral response relies totally on local interneuronal relay. But, because motor bursts on both sides occurred with roughly the same delay and possible synaptic contacts were observed between TAN projections and ipsilateral T2 MNs, it was more likely that T2 motor responses on the two sides resulted from monosynaptic vestibulospinal inputs. Due to the existence of long motoneuronal dendrites laying in the two hemicords, it is highly conceivable that fibers from a given TAN nucleus activate contralateral MNs through synaptic contacts on lateral dendrites and ipsilateral MNs through contacts on commissural dendrites. High divalent cation saline is used to conserve only monosynaptic responses because it stabilizes neuronal membranes and, hence, limits signal propagation along inactive neurites (Berry and Pentreath, 1976). Given that TAN synapses were found farther from the soma on commissural than lateral dendrites, perfusion of high divalent cation saline should impede more TAN signal transmission along commissural dendrites and, as a consequence, prevent MNs to reach firing threshold in response to ipsilateral TAN minimal stimulation. In support to this assumption that high divalent cation saline disturbed signal transmission along MN dendrites was the concomitant observation of an increased delay of LVST-evoked T2 compound action potentials and the

tendency, although non-significant in our experiments, of TAN-evoked bursting responses to occur also with an increased delay in the contralateral T2 motor nerve.

Postural T2 MNs are driven by the lumbar circuitry during locomotion, subjugating the dynamic control of posture to the hindlimb kicking command (Beyeler et al., 2008). In this context, each Ext burst is accompanied by a concomitant motor burst in the ipsilateral T2 ventral root during fictive swimming, and similar coordination is observed between back and Ext muscle activity during actual swimming. Here, we describe an ascending lumbo-thoracic connection that involves lumbar INs making possible connections onto T2 postural MN dendrites. Minimal stimulation of these INs evoked motor responses in T2 ventral roots that for the most part persisted in high divalent cation saline, confirming the existence of functional synapses with T2 MNs. Often, lumbar IN stimulation simultaneously triggered responses on hindlimb motor nerves (Fig. 6C-E). Taken together, these results suggest that such lumbar INs may be responsible for the strict coupling between hindlimb and dorsal MNs that was observed during swimming (Beyeler et al., 2008). Beyond, our results also demonstrate T2-projecting lumbar INs to respond to vestibular nuclei activation, as previously reported for other spinal INs (Miller et al., 2009; Kasumacic et al., 2015). In addition, we show that coordinated discharges of both hindlimb Ext and postural T2 MNs are produced in response to passive head motion, with Ext activity systematically preceding T2 discharge by 150-200 ms (Fig. 2). Taken together, our results suggest that ascending lumbar INs dispatch a part of the passive head movement-induced vestibulospinal commands towards hindlimb and thoracic postural MNs. As a consequence, in addition to its essential function during swimming the lumbo-thoracic circuitry appears to play a major role in the control of posture in response to passive motion too. Our results further suggest that the dual vestibular-driven thoracic motor response, either independent or coupled to vestibular-driven hindlimb extensor motor activity, reflects a differential central sensory-motor processing of head motion signals arising from inner ear sensory organs. Such a functional organization of sensory-motor transformation was already proposed for vestibulo-ocular pathways (Straka et al., 2009; Beraneck and Straka, 2011; Straka et al., 2014). All neuronal relays of the vestibulo-ocular arc, from vestibular afferents to extraocular MNs, demonstrate neural integrative properties that tune vestibulo-ocular pathways in parallel frequency-tuned channels. Such a temporal treatment leads to transform head acceleration sensory signals into motor output signals with different levels of integration, like velocity and

676 position (Straka et al., 2009, 2014). In our experiments, during natural translation or rotation
677 of the head in the horizontal plane (Fig. 2) the peaks of all motor responses always occurred
678 when the stimulus acceleration changed, i.e. either when the table approached extreme
679 positions or around turn-table/sled velocity peaks between two maximal positions.
680 Therefore, we hypothesize that the dual vestibular-driven thoracic response could also result
681 from a differential transformation of the acceleration input signals in some position- or
682 velocity-related motor output signals, involved in either a trunk-restricted postural
683 adjustment or a whole-body balance control, respectively.

684 In mammals, projections from vestibulospinal neurons on one side of the brainstem
685 are organized in two main tracts, one projecting medially in the two sides of the spinal cord
686 and the other lateral, projecting exclusively ipsilaterally (see Shinoda et al., 2006). In the
687 larval frog in contrast, medial projections originate only from contralateral second order
688 vestibular neurons (Straka et al., 2001). We described here two similar pathways in the
689 juvenile *Xenopus*, which originated respectively from TAN and LSVT nuclei. Such
690 vestibulospinal nuclear organization appears to be a common feature in early vertebrates
691 and prefigures vestibular nuclei organization in higher vertebrates (Straka and Baker, 2003).
692 However, whereas medial projections classically stop within the cervical spinal segments
693 while lateral projections run through the entire spinal cord (*e.g*, cat: Kuse et al., 1999;
694 Kushiro et al., 2008; mouse: Kasumacic et al., 2010, 2015; primate: Boyle and Johanson,
695 2003; terrestrial frog: Fanardjian et al., 1999) we report here long projections running down
696 to the lumbar segments from both groups of vestibulospinal nuclei. Indeed, only a small
697 amount of vestibulospinal neurons stop their course into the cervical cord, and TAN neurons
698 project almost equally into the thoracic and lumbar segments while the majority of LVST
699 neurons project into the lumbar cord. Long ago, vestibulospinal projections were associated
700 with their functional effects on animal postural control (see Keshner and Cohen, 1989),
701 medial cervical projections being mostly dedicated to neck control and head stabilization
702 (Boyle, 1993) and lateral projections mainly controlling axial and limb muscles for body
703 postural adjustment and antigravity extensor tone (Cottingham and Pfaff, 1987; Ali et al.,
704 2003). In juvenile *Xenopus*, we found a relatively small proportion of vestibulospinal neurons
705 projecting only in cervical segments. This might be explained by the absence of head
706 movement with respect to the trunk and by the thinness of forelimbs compared to the body
707 size, which excludes any significant forelimb-mediated postural adjustment. In contrast,

thoracic and lumbar segments were the favorite targets of vestibulospinal neurons, where they could activate directly axial and extensor MNs respectively, the two main postural effectors in the juvenile *Xenopus*. Direct connections between vestibulospinal fibers and spinal MNs were already described in various species, at various spinal levels (Grillner et al., 1970; Magherini et al., 1974; Wilson et al., 1970). As a totally unexpected result, we described two distinct pathways to convey vestibular signals to thoracic MNs. Indeed, we found that direct vestibular inputs reached T2 MNs directly, whereas indirect vestibular information visibly involved a relay in the lumbar spinal cord from where it activated simultaneously T2 MNs through ascending INs and hindlimb Ext MNs. The existence of such two distinct pathways may be explained by the gross morphology of the two major postural effectors in the juvenile *Xenopus* and their likely involvement in two distinct types of postural responses. When activated alone, axial back muscles allow only a small amplitude and precise buoyancy control which would require only accurate positional information. In contrast, involving the hindlimbs mediates larger postural adjustments that occur in response to stronger disequilibrium, the strength of which would be more reliably encoded as disturbance velocity. Simultaneously, and as shown previously during swimming (Beyeler et al., 2008), adequate trunk stiffening would be generated during hindlimb extension in order to stabilize the whole body. Thus, in the juvenile *Xenopus* the vestibulospinal system is organized in such a way that the main postural effectors are in direct link with precise vestibulospinal projections, and maybe vestibular sensitivities, in order to generate the most efficient postural adjustment.

In most animal models, postural muscles are also involved in locomotor movements. In terrestrial quadrupeds for instance, the vertebral column is long and flexible, and virtually all back muscles are also engaged in propulsion during walking and running (Alexander et al., 1985; Muybridge, 1957). In the post-metamorphosis *Xenopus*, on the contrary, the vertebral column is short (8 vertebra, from atlas to urostyle), and its stiffness does not allow the trunk to produce any lateral undulation or antero-posterior flexion/extension that could participate in propelling the animal. The anatomical arrangement of *dorsalis trunci* myomeres suggests that contracting unilaterally the 3rd and/or 4th myomere results in a slight twist of the vertebral column towards the side of the contracted muscles, so creating a small amplitude rolling angle between anterior (skull/scapula) and posterior (ilio-sacrum/urostyle) more rigid bony ensembles. A previous modelization study demonstrated

740 that the degree to which this part of the column is twisted has strong impact on the animal
741 static posture, and suggested that controlling such a twist may be one essential function of
742 the dynamic control of posture during swimming (Beyeler et al., 2013). The present
743 description of the convergence of direct and lumbar-relayed vestibulospinal influence on T2
744 MNs strongly reinforces the idea of a general role of *dorsalis trunci* muscles in postural
745 control, both during swimming and in response to passive motion. In addition, the
746 conjunctional *dorsalis* and hindlimb extensor MNs activation in response to vestibular head-
747 motion signals implicates lumbar INs that likely also participate in coupling these two motor
748 entities during swimming. This further suggests that proactive and reflex postural responses
749 share common propriospinal pathways in vertebrates.
750
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Additional Information

Competing Interests

The authors declare no competing interests.

Author contributions

All experiments were performed at the Institut de Neurosciences Cognitives et Intégratives d'Aquitaine. All persons who participated in this work are listed as authors and all authors have significantly contributed to this work: Conception: FML and DLR; data acquisition and analysis: AOB, RG, LC, EC, FML and DLR; drafting and revising critically: AOB, FML and DLR. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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FIGURE LEGENDS

Figure 1. Dorsal postural system of *Xenopus laevis* trunk. A. Dorsal (left) and lateral (right) views of *dorsalis trunci* third myomere (3rd myo; light green) insertion (arrowheads in bottom right image) between thoracic vertebra (Vert.) 1 and 2. Bottom right schematic illustrate better the myomere insertion on vertebra. Scale bars=3 mm for left and top right images. Curved arrows in right panel illustrate the vertebral leftward twist induced by the left 3rd myomere contraction. T1 Vr to T3 Vr=thoracic ventral roots 1 to 3. Dotted green lines delineate vertebra. OC=otic capsule; C=caudal; D=dorsal; R=rostral. **B-C.** In vivo EMG recordings from *dorsalis trunci* (dt) and *plantaris longus* (pl) on left (le) and right (ri) sides during (**B**) and in the absence (**C**) of active swimming. **D**. Confocal images orthogonal projection of a T2 cross-section after dextran (Dex.) retrograde labeling of *dorsalis* 3rd myomere MNs on left (AD 647; cyan) and right (AD 568; magenta) sides (**D_1**), and left side MNs extraction illustrating *dorsalis* MNs lateral (Lat.) and commissural (Com.) dendritic morphology (**D_2**). **E**. Confocal images orthogonal projection of MNs from a whole-mount open-book T2 spinal segment. **F**. Schematic representation of a T2 MN. c.c=central canal. **G.** Quantification of primary and secondary dendrite branching in MN lateral and commissural dendrites (Lat. d. and Com. d., respectively). Error bars are SDs. ns=non-significant.

Figure 2. Thoracic and lumbar motor responses to vestibular endorgans natural stimulation. A. Schematic of the semi-intact preparation used to record bilateral ventral root activity from the second thoracic spinal segment (le T2 Vr, dark blue, and ri T2 Vr, light blue) and bilateral hindlimb extensor motor nerves (le Ext Vr, red, and ri Ext Vr, orange), in response to angular (green) and linear (purple) head displacements and the respective activation of intact semi-circular canals (green) and utricles (purple). Black dot in the brainstem indicates the center of rotation. **B-C**. Examples of motor responses evoked by a 1-Hz sinusoidal forward/backward translation (**B_1**) and a 0.5-Hz sinusoidal 30° left-right rotation (**C_1**) in the horizontal plane, and corresponding mean discharge rates (**B_2** and **C_2** respectively) in response to sinusoidal variations of the stimulating motion table horizontal position (continuous sinusoids). Broken sinusoids indicate stimulus instantaneous acceleration, as derived from motion table position. Alternate pink and white rectangles in **B_1** and **C_1** highlight the sinusoidal stimulus alternating phases. **D.** Phase analysis from all experiments in horizontal translation (**D_1**) and horizontal rotation (**D_2**) configurations for all

recorded motor nerves. Left panels: circular diagrams illustrating the mean distribution vector for each nerve activity. Right panels: mean phase relationship compared to the maximal frontal (**D_1**) and maximal lateral (**D_2**) table position for T2 and Ext activities as a function of stimulus frequency. **E.** Thoracic and lumbar motor responses to horizontal angular rotation. Examples of motor bursts (**E_1**) and corresponding mean discharge rate (**E_2**) in response to 25-Hz, 30° sinusoidal horizontal rotation of the motion table (continuous sinusoids). Broken sinusoids indicate the stimulus instantaneous acceleration, as derived from motion table position. **E_3.** Circular phase analysis from all experiments for all recorded motor nerves. Top panel: circular diagram illustrating the mean distribution vector for each nerve activity. Bottom panel: mean phase relationship compared to the maximal lateral position of the stimulating motion table for T2 and Ext activities as a function of stimulus frequency. Error bars are SDs.

Figure 3. Brainstem spatial distribution of vestibulospinal neurons projecting in cervical and thoraco-lumbar spinal cord. **A**. Scheme of isolated (brainstem-spinal cord) CNS preparation illustrating the sequential, homolateral injection of dextran dyes in the 2nd thoracic segment (RDA in T2, red) and 1st cervical segment (AD 647 in C1, cyan). Dyes are retrogradely transported to the cell bodies (dots in the hindbrain, Hb) of neurons that project in injected spinal cord (SC) segments. A third dye injection in ipsilateral cranial nerve eight (VIIIth N., green) allowed antero-posterior localization in the brainstem. **B**. Confocal images orthogonal projection of a hindbrain cross-section showing LVST (**B_1**) and TAN (**B_2**) vestibulospinal neurons labeled from cervical (cyan) and thoraco-lumbar (red) segments (scale bar=100µm). Insets **a** and **b** show single labeled neurons from thoraco-lumbar (red) and from cervical (blue), as well as double labeled neurons in both LVST (**a**) and TAN (**b**) nuclei (merged channels images; scale bar=20µm). IVth=fourth ventricle. **C**. Ratio (**C_1**) and rostro-caudal repartition (**C_2**) of LVST and TAN neurons projecting (proj.) respectively to cervical (C1, blue) and thoraco-lumbar (T2, red) spinal cord. Error bars are SDs **D.** 3D reconstruction from confocal image stacks showing spatial distribution of LVST and TAN vestibulospinal neurons retrogradely labeled from C1 (blue) and T2 (red) segments, (scale bar=500µm). D=dorsal, L=lateral, C=caudal.

Figure 4. Electrophysiological and anatomical characterization of direct vestibular inputs to thoracic motoneurons. A. Left: CNS scheme illustrating LVST site stimulation and right T2

ventral root recording (ri T2 Vr). Right: Superimposed examples of discharge evoked in a T2 ventral root by minimal electrical stimulation of the ipsilateral LVST nucleus. **B**. Left: LVST and TAN minimal stimulation-evoked responses in ipsilateral and contralateral T2 ventral roots respectively, in control saline (black), high divalent cation saline (high Ca2+/Mg2+, red) and during wash (gray). Mean responses are illustrated: raw traces were firstly aligned on stimulation artifact initiation; thereafter, stimulation artifacts were truncated, and raw traces were averaged. Little arrows on mean traces point to the initiation of the mean compound burst in control and high divalent cation solution, respectively. Right: Box charts of delays from all preparations in control and under high divalent cation saline for LVST-evoked ipsilateral T2 responses (top) and TAN-evoked ipsilateral (ipsi) and contralateral (contra) T2 responses (bottom). Error bars are 95% confidence intervals. **C**. Left panel: CNS scheme depicting retrograde labeling of *dorsalis trunci* (d.t.) MNs (AD 647, magenta), ipsilateral TAN neurons anterograde labeling (RDA, cyan), and cervical spinal cord hemisection (C1 hsct; see Methods). Middle panel: Confocal images orthogonal projection of a T2 cross-section illustrating retrogradely labeled T2 motoneurons (magenta) and anterogradely labeled vestibulospinal fibers (cyan). Scale bar=100µm. Right panel: magnification of square area (a) in middle panel, illustrating vestibulospinal fiber terminals (vest.fib), T2 MN commissural dendrites, and synapsin (Syn.) immunofluorescence labeling. Merged image illustrating fluorescent signals close apposition is shown at the bottom right. Scale bar=20µm. **D-E.** Protocol schematic and merged magnifications for contralateral TAN and ipsilateral LVT nucleus, respectively. Greater magnifications of areas "a" are shown below as separated channels to better illustrate apposition zones. Scale bar=20µm; com. d.=commissural dendrites; lat. d.=lateral dendrites.

Figure 5. Electrophysiological and anatomical characterization of L1 interneuronal ascending inputs to thoracic motoneurons. A. CNS scheme illustrating injection sites for dextran (Dex.) retrograde labeling of lumbar ascending interneurons from the 1st cervical (RDA, orange) and 2nd thoracic segments (AD 647, cyan), and cross section scheme of the 1st lumbar segment. Insets show ipsilateral and dorsal (**a**), and contralateral and ventral (**b**) lumbar ascending interneurons projecting to T2 hemi-segment (**A_1**), as well as interneurons projecting to C1 hemi-segment (**A_2**, orange). **A_1** and **A_2** from two distinct preparations. **B**. 3D reconstruction of lumbar spinal segment from confocal image stacks depicting spatial

organization of interneurons projecting into the 2nd thoracic segment (blue dots) in transversal (**B_1**) and dorso-lateral (**B_2**) views. Retrograde labeling of L1 MNs (light brown) allowed rostro-caudal localization within the lumbar cord. L1 Vr=L1 ventral root. Scale bars=100µm. **C.** Top left: CNS scheme showing L1 electrical stimulation site and T2 ventral root (T2 Vr) recording. Top right: Typical example of recordings in ipsilateral (top) and contralateral (bottom) T2Vr in response to L1 IN minimal stimulation in control saline (black), high divalent cation saline (red) and during wash (gray). Bottom: Box charts of responses delays from all preparations in control and under high divalent cation saline illustrating the large variation in delays of L1 IN-evoked ipsilateral T2 responses. Error bars are 95% confidence intervals. **D.** Confocal images orthogonal projection of a 2nd thoracic cross-section illustrating retrogradely labeled *dorsalis* MNs (AD 647, magenta) and lumbo-thoracic fibers ascending from ipsilateral L1 hemi-segment (asc. ipsi. LIN fib.; RDA, cyan). Merged magnifications on the right illustrate fluorescent signal close appositions (arrowheads) on MNs lateral dendrites (lat. d.) together with synapsin immunofluorescence (green). Inset in left image depict neuronal labeling protocol.

Figure 6. Variety of lumbar INs and IN stimulation-evoked motor responses. A. Confocal images orthogonal projection from a 2nd lumbar segment cross-section showing ascending ipsilateral and contralateral INs (aIIN and aCIN, respectively; RDA, cyan) retrogradely labeled from left T2 hemi-segment together with L2 motoneurons and afferent fibers (L2 MN and L2 af, respectively; AD 488, orange) labeled from L2 ventral root (L2 Vr). **B.** Retrograde labeling of T2 *dorsalis* MNs (AD 647, magenta) and anterograde labeling (RDA, cyan) of contralateral L1 IN terminals coupled with synapsin immuno-detection (green). Arrowheads point to close apposition between the three fluorescent signals, near the MN soma and on lateral dendrites (lat. d.). **C.** CNS scheme illustrating the electrical stimulation of L1 INs (stim) while recording simultaneously from hindlimb flexor (Flex) and Extensor (Ext) nerves and from the 2nd thoracic ventral roots (T2 Vr) on both sides (le=left; ri=right). **D.** Example of motor nerves early responses to the minimal stimulation left L1 INs in control (black), high divalent cation (red) and wash (gray) conditions. **E.** Example of rhythmic locomotor-like bursting activity recorded from most of the motor nerves in response to a left L1 IN single electrical stimulation. **F.** Schematic summary of the mean distribution on T2 postural MN soma, lateral (lat.) and commissural (com.) dendrites of putative synaptic contacts from LVST (black bars),

TAN (grey bars) and L1 IN (empty bars) terminals. Error bars are SDs. dist.=distal; med.=medial; prox.=proximal.

Figure 7. LVST inputs onto T2-projecting L1 interneurons. A. CNS scheme showing injection sites for LVST fibers anterograde labeling (RDA, magenta) and ipsilateral L1 INs retrograde labeling from contralateral T2 hemi-segment (AD647, cyan). Spinal hemisection was performed at C1 level contralaterally to the injected LVST. **B.** Confocal images orthogonal projection of a L1 cross-section illustrating T2-projecting lumbar INs (T2 proj. IN; cyan) and LVST terminals (vest.fib; magenta). Scale bar=100µm. Insets **a** and **b** illustrate two examples of fluorescent signal appositions (arrowheads) together with synapsin immunofluorescence (green) on IN somata and dendrites; scale bar=20µm. **C-D.** Same arrangement as A for contralateral TAN-L1 INs synaptic contacts. **E.** Scheme of the isolated brainstem-spinal cord preparation used to record LVST stimulation-evoked calcium transients (Δ*F/F*) from L1 INs (orange) retrogradely loaded from a T2 hemi-segment with Calcium Green Dextran Amine ($CGDA^+$), and examples of CGDA-labeled INs. **F.** Representative examples of calcium transients recorded from 11 L1 INs in response to LVST and TAN stimulation (single pilse = 50 µs). **F_1.** Superimposed responses illustrating the similarity of calcium response in all recorded INs to LVST (top) and TAN (bottom) stimulation. **F_2.** Averaged calcium transients recorded from the same 11 distinct L1 INs projecting into contralateral T2 segments in response to repeated ipsilateral LVST (left) and contralateral TAN (right) stimulation. Arrow on left panel indicates rhythmic-like bursting response to the first LVST stimulation. **F_3.** Δ*F/F* means for each preparations (top; error bars are SDs), and box charts (bottom; error bars are 95% confidence intervals) illustrating for all preparations the distribution of calcium response amplitudes for ipsilateral (black) and contralateral (grey) L1 INs to both LVST and TAN electrical stimulation. ns=non-significant.

Figure 8. Direct and indirect vestibulo-thoracic pathways involved in *dorsalis* motoneurons activation. A-B. Summary of postural responses during forward (**A_1**) and backward (**A_2**) linear translations, and during rightward **(B_1)** and leftward **(B_2)** angular rotations. Dark colors: T2 response alone; light colors: coupled T2 and Extensor motor responses. **C.** Schematic organization of direct and lumbar-relayed LVST and TAN pathways to T2 *dorsalis* MNs. Bs=brainstem; Lumbar Sc=lumbar spinal cord; iIN=ipsilateral interneuron; cIN=contralateral interneuron.

Supplemental movie 1. Hindlimb reflex movements induced by forward/backward repetitive natural stimulation of vestibular endorgans. Sliding bar on the left part of the movie indicates sled motion. During each backward displacement, observe the small amplitude paw movements that correspond to electrophysiological motor bursts recorded from *plantaris longus* motor nerves in the same condition (e.g, Fig. 2B1). Such movements, corresponding to postural adjustments, have amplitude much smaller than full hindlimb extension characteristic of swim kicking (see Beyeler et al, 2008, 2013).