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Particle motion is broadly represented in the vestibular medulla of the bullfrog across larval development

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Abstract

In their shallow-water habitats, bullfrog (*Rana catesbeiana*) tadpoles are exposed to both underwater and airborne sources of acoustic stimulation. We probed the representation of underwater particle motion throughout the tadpole's dorsal medulla to determine its spatial extent over larval life. Using neurobiotin-filled micropipettes, we recorded neural activity to z-axis particle motion (frequencies of 40–200 Hz) in the medial vestibular nucleus, lateral vestibular nucleus, dorsal medullary nucleus (DMN), and along the dorsal arcuate pathway. Sensitivity was comparable in the medial and lateral vestibular nuclei, with estimated thresholds between 0.016 and 12.5 μm displacement. Neither best responding frequency nor estimated threshold varied significantly over larval stage. Transport of neurobiotin from active recording sites was also stable over development. The DMN responded poorly to z-axis particle motion, but did respond to low-frequency pressure stimulation. These data suggest that particle motion is represented widely and stably in the tadpole's vestibular medulla. This is in marked contrast to the representation of pressure stimulation in the auditory midbrain, where a transient “deaf period” of non-responsiveness and decreased connectivity occurs immediately prior to metamorphic climax. We suggest that, in bullfrogs, sensitivity to particle motion and to pressure follows different developmental trajectories.

Keywords

Auditory; Bullfrog; Medulla; Tadpole; Vestibular

Introduction

Anuran tadpoles undergo considerable structural and functional modification over the course of metamorphic development (Shi 2000). External morphological changes include emergence and differentiation of limbs, broadening of the mouth, and migration of the eyes from lateral to more dorsal positions on the head. Internal changes include restructuring of the lungs, gut, and skull. Feeding behaviors transform from filter-feeding of vegetative matter during tadpole stages to active capture of live prey in post-metamorphic froglets and adults. The drastic and rapid nature of these transformations highlights the usefulness of the anuran model for understanding neural control of development.

The bullfrog (*Rana catesbeiana*) is a particularly appropriate model for the study of the development of neural systems related to hearing and balance. This species transforms from a totally aquatic, swimming, mute larval form to an amphibious, jumping, vocal adult, thereby experiencing, during a short time span, two different biophysical environments for processing of auditory and vestibular signals. This change in environment is preceded, during larval stages, by the progressive differentiation of auditory and vestibular sensory organs in the inner ear, the sequential emergence of first the opercularis and then the tympanic transduction pathways, and the appearance of the external tympanum in the early froglet period. The progression from aquatic to amphibious life is also reflected in the degeneration of the peripheral lateral line neuromasts and their central projections by the completion of metamorphic climax (Simmons and Horowitz 2006).

Central octavolateralis systems also undergo considerable developmental changes over larval life. Waves of neurogenesis in the medulla and auditory midbrain (torus semicircularis; TS) occur, peaking during metamorphic climax (Chapman et al. 2006; Simmons et al. 2006). The dorsal medullary nucleus (DMN; homolog of the mammalian cochlear nucleus) migrates from a more lateral to a more dorsal-medial position in the medulla, expanding into the region previously occupied by the lateral line neuropil (LLnp) (Fritzsch et al. 1988). During the “deaf period,” immediately prior to the onset of metamorphic climax, there is a temporary disruption of afferent and efferent connectivity between the superior olivary nucleus (SON) and the ipsilateral TS, which is reflected in a transient reduction in midbrain sensitivity to airborne pressure stimulation (Boatright-Horowitz and Simmons 1997). Metamorphic climax witnesses both the anatomical and physiological restoration of this connectivity and the emergence of additional ascending projections from other medullary nuclei (Horowitz et al. 2007b). By the completion of climax, medullary-midbrain connectivity is overall similar to that in post-metamorphic bullfrogs, providing the structural basis for hearing in air.

Because bullfrog tadpoles inhabit shallow bodies of water, underwater sound sources and water flow stimulation are likely as important biologically as are airborne sounds. Yet, in spite of the considerable anatomical changes that occur over larval life, little is known about the central processing of these stimuli across the metamorphic transition (Boatright-Horowitz and Simmons 1997; Boatright-Horowitz et al. 1999). In an earlier study (Horowitz et al. 2007a), we recorded neural responses from the tadpole’s medial vestibular nucleus (MVN) and adjacent LLnp to low-frequency (<70 Hz) stimulation produced by z-axis (vertical) vibrations that simulate underwater particle motion (Fay and Edds-Walton 1997). We found that robust responses in the MVN to this low-frequency stimulation could be recorded at all larval stages, including during the deaf period. These data suggest that particle motion information remains available to the animal during the deaf period for processing of airborne sounds, thus preventing a total loss of auditory/vestibular input that would be deleterious to survival in a time period when processing of pressure information is impaired.

Here, we probe in more detail the representation of z-axis particle motion in the dorsal medulla in bullfrog tadpoles, both by using a broader range of frequency stimulation and by sampling medullary areas outside of the MVN/LLnp region. We test the hypothesis that z-axis particle motion is widely represented throughout the dorsal medulla during larval development. We also examine the pattern of neurobiotin transport from active recording sites to determine if there is a disruption in connectivity in the dorsal medulla during the deaf period, analogous to that observed in our earlier work.

Materials and methods

Animals

Rana catesbeiana tadpoles ($n = 53$) were obtained from a commercial supplier (Dozier Lester, Duson, LA, USA). Animals used for physiological experiments were in late larval ($n = 30$) and metamorphic climax ($n = 8$) stages of development. An additional 15 animals (early larval, $n = 7$; late larval, $n = 8$) were used for tracing projections of the peripheral endorgans via neurobiotin injections directly into the inner ear or the eighth nerve (nVIII). Stages of development are defined by the Gosner (1960) criteria on the basis of external morphological criteria and categorized into groups based on criteria outlined by McDiarmid and Altig (1999). Early larval stages (Gosner 25–30) are defined by the state of development of hind limb buds. Late larval stages (Gosner stages 31–41) are defined by the extent of emergence and differentiation of hind limbs and toes. The end of the late larval period (stages 38–41) was characterized by Boatright-Horowitz and Simmons (1997) as a deaf period for pressure stimulation, based on internal blockage of the oval window by the growing opercularis muscle and by the extreme loss of sensitivity of the TS to airborne sounds. External markers of metamorphic climax (Gosner stages 42–46) include the emergence and differentiation of fore limbs, changes in head shape, and the absorption of the tail.

Tadpoles were group-housed in clear aquaria containing aerated distilled water with added salts (pH 7–7.5) and were fed cooked unsalted spinach and commercial goldfish flakes. The colony room was maintained on a 12:12 light:dark cycle at temperatures ranging from 22 to 25°C. Experiments were conducted between the months of April and September to minimize any seasonal effects on neural responding.

Surgical procedures

Each animal was anesthetized by immersion in 0.1% buffered 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222; pH 7.0; Sigma, St Louis, MO, USA) for 1–3 min or until reflexes disappeared. It was then loosely wrapped in moist gauze to facilitate cutaneous respiration. Using sterile technique, the medulla was exposed by a dorsal incision through the skin and cartilaginous skull, and the choroid cleared. The wound was covered with sterile Gelfoam (Upjohn, Kalamazoo, MI, USA) moistened with sterile 0.9% saline, and its margins were swabbed with lidocaine ointment. The animal was placed in shallow aerated water until normal swimming behavior resumed (1–2 h). It was then immobilized with an intramuscular injection of 0.06% (w/v) D-tubocurarine chloride (0.010 mL/g, Sigma) into the tail region. Local anesthetic was swabbed on the proximal end of the tail, and a metal hook was inserted here to serve as a ground electrode. To stabilize the animal during recordings, its ventral surface, up to the level of the inner ear, was embedded in a solution of 10% ultra-low gelling temperature agarose (Type IX, Sigma), as adapted from methods used to stabilize larval zebrafish (Liao 2010). We adopted this modification of our earlier method of stabilization (Horowitz et al. 2007a) so as to more firmly and rigidly affix the animal to the recording platform. To examine the influence of the agarose embedding on neural responses, we recorded, using tungsten electrodes, thresholds to low-frequency z -axis stimulation from the MVN of four embedded animals (2 late larval, 2 metamorphic climax), and compared these data with those from unembedded animals (data from Horowitz et al. 2007a). Thresholds to similar stimulating frequencies were lower (mean 0.83 μm displacement) in experiments with embedding than those without (mean 4.48 μm displacement). We presume that the agarose decreased any movements of the animal that could have induced unintended water displacement and thus influenced peripheral transduction and neural sensitivity.

Stimulus control and calibration

A shaker table system, as previously described (Fay 1984; Horowitz et al. 2007a), was used to simulate underwater particle motion in the z (vertical) plane. Briefly, z -axis motion was produced by a Brüel & Kjaer (Norcross, GA, USA) Type 4809 vibration exciter, attached by brass screws to the bottom center of a cylindrical (18 cm diameter) water-filled aluminum dish. A thin perforated Lucite platform rigidly attached across the diameter of the dish was used to hold the animal in its agarose block. For recordings, the dish was filled with room temperature tank water up to the level of the surgical opening. The dish and shaker were positioned on a bench top isolation table (Newport Corp, Irvine, CA, USA), which also supported a three-dimensional hydraulic micromanipulator (Narishige, East Meadow, NY, USA) and the headstage of a microdrive (Trent Wells Mark 3, Coulterville, CA, USA). The vibration table-top rested on a solid platform made of alternating layers of bricks, wood, and neoprene mats. The entire system was located within a single-walled sound-attenuating booth (Industrial Acoustics, Bronx, NY, USA), with stimulus generation and recording equipment located outside the booth. The booth was maintained at room temperature (22–25°C) during experiments.

A Tucker-Davis Technologies (TDT, Alachua, FL, USA) System 3 hardware/software system was used to produce signals and collect neural responses. The z -axis shaker was driven by sinusoidal signals (frequency 10–250 Hz) which were digitally generated at 10 kHz sampling rate and 16 bit D-to-A conversion accuracy. The driving signals were attenuated (TDT PA5 programmable attenuator), amplified (Crown D-75 amplifier), and re-attenuated by –25 dB using a custom-made attenuator connected directly to the shaker. This reduced electronic noise from the power amplifier so that lower stimulus amplitudes could be employed. The actual motion of the dish in the x (lateral), y (lateral), and z (vertical) planes (Fay 1984) was monitored by three accelerometers (model 333B50, PCB Piezotronics, Depew, NY, USA) mounted on the dish. The accelerometers were powered and their output monitored by a PCB Piezotronics Model 482A22 ICP sensor signal conditioner. Signals from the accelerometers were amplified, filtered, and passed to the TDT real-time processor. Custom-written MATLAB scripts (v. 6.5, MathWorks, Natick, MA, USA) calculated the displacement and acceleration of the dish based on the frequency and nominal voltage of the driving stimulus. Figure 1a shows the frequency responses of the x , y , and z axes in response to stimulation of the z -axis shaker at a nominal attenuation of 10 dB. This nominal attenuation was chosen because it is the attenuation level used for the search stimuli; for actual data collection, most stimuli were attenuated by at least 15 dB. The frequency response of the dish shows a prominent resonance between 70 and 80 Hz, with responses falling off substantially at both lower and higher frequencies. Because of this peak, amplitudes of stimulation at all frequencies were adjusted and calibrated with respect to this frequency response. In addition, frequencies in the resonance range were not presented during experiments. Figure 1b shows the relationship between stimulation in the z plane and cross-talk in the x and y planes by plotting the percentage of z plane activation measurable in the x and y planes. At the resonance peak, there was substantial cross-talk. This cross-talk was diminished (to <20%) at lower frequencies and even more diminished (to <5%) at higher frequencies. We conclude from these measurements that neural responses to z -axis stimulation, at stimulus levels lower than 10 dB attenuation, do not reflect significant contribution from motion in the x and y planes over the most critical range between 90 and 200 Hz.

Airborne pressure stimuli [noise bursts (80–1,000 Hz), single sinusoids (80–300 Hz), duration 1 s, rise-fall time 10 ms] were digitally generated (10 kHz sampling rate, 16 bit) by the TDT system, amplified, filtered, attenuated, and presented free-field via a loudspeaker (Paradigm, Ontario, Canada; frequency range 70 Hz to 20 kHz, flat ± 2 dB) suspended 1 m

above the tadpole's head at an angle of 11.3° from the vertical position. This angle of incidence was used previously in characterizing midbrain responses to pressure stimulation in tadpoles (Boatright-Horowitz and Simmons 1997) and it permits transmission of acoustic energy across the air–water interface. Cross-calibrations between in-air (20 μ Pa) and underwater signal levels (0.1 Pa; Nelson 1996) were performed using a Brüel & Kjaer Type 4165 condenser microphone (38.3 dB re: 1 V/Pa) placed directly above the tadpole's head and a hydrophone (−28 dB re: 1 V/Pa; FishFone, BioAcoustics, Woods Hole, MA, USA) positioned at approximately the same location as the tadpole's inner ear. Acoustics of the dish were relatively flat (± 5 dB) from 80 to 3,500 Hz. Stimulation through the loudspeaker at sound pressure levels of 100 dB SPL or greater produced some vibrations of the shaker dish, but at levels within the noise floor of the system (0.0225 μ m). We conclude from these measurements that the pressure stimulus at levels lower than 100 dB SPL did not produce significant dish vibrations or particle motion that could influence the neural response. Voltage signals from the microphone and hydrophone were processed by the TDT real-time processor and analyzed with a custom-written MATLAB script.

Recording procedures

Neural responses from the dorsal medulla were recorded using low-impedance (250–1,000 K Ω) micropipettes back-filled with a solution of 4% Neurobiotin (SP1120, Vector Laboratories, Burlingame, CA, USA) in 1.0 M potassium chloride. Neurobiotin travels in both anterograde and retrograde directions and across gap junctions (Huang et al. 1992). The micropipette was placed on the dorsal surface of the medulla and lowered in 1 μ m steps while searching for a response. Initial electrode placements were made in the lateral medulla (area of the DMN). If no response could be evoked at depths down to 300 μ m, then in successive passes, placements were moved progressively more medially. Search stimuli were sinusoidal signals varying in frequency (20–65 or 90–150 Hz) at a nominal attenuation of 10 dB. To differentiate presumed single units, the spike discriminator was set to pick out only the largest amplitude responses (signal/noise ratio of at least 2:1). When a responsive site was encountered, z-axis sinusoidal stimuli (frequency range 15–250 Hz, rise-fall 10 cycles) were presented at a duty cycle of 10 s (3 s on, 7 s off) for 10 sweeps. The order in which different frequencies were presented was pseudorandom. Using a modified tracking procedure, stimulus amplitudes at each frequency were progressively lowered in steps of 5–10 dB to bracket the relative threshold of response. After responses to z-axis stimulation were recorded, sensitivity to airborne pressure stimuli (duty cycle 4 s, presented in random order) was probed. Neural responses were amplified, filtered (300–3,000 Hz), digitally recorded (10 kHz sampling rate), discriminated, and monitored in real time as dot rasters and post-stimulus time (PST) histograms. Individual neuronal traces were not stored due to the configuration and limits of the TDT software system. Because there is extensive bilateral connectivity in the dorsal medulla (Grofova and Corvaja 1972; Feng 1986), only one responsive site per animal was marked by iontophoresis. Each site could be held between 5 and 15 min, and entire recording sessions typically lasted 1–2 h.

Determination of recording sites

At the end of each recording session, a constant current source (Model 51413, Stoelting, Wood Dale, IL, USA) was used to deposit neurobiotin at the recording site (0.7 μ A alternating current for 3 min). The micropipette remained in place for 5 min after termination of the current in order to allow the tracer to permeate the brain region. The site was then thoroughly rinsed with 0.9% sterile saline, and moistened sterile Gelfoam was placed in the wound. Animals lived for 4 h after injection to allow for dye transport. They were then terminally anesthetized in 0.6% MS-222 (20 min) and transcardially perfused with heparinized 0.9% saline and 4.0% paraformaldehyde (pH 7.4). The head was post-fixed in

paraformaldehyde for 24 h at 4°C. The brain was removed, cleaned of meninges, embedded in 5% agarose (ISC Bioexpress, Kaysville, UT, USA), and sliced coronally (50 µm thickness) on a vibratome (Pelco 1000 Plus, Redding, CA, USA). Sections were mounted on gelatin-subbed slides, rinsed for 45 min in 1× phosphate-buffered saline (pH 7.4) with 1% Triton-X 100 (Sigma) at room temperature, and then incubated in phosphate-buffered saline and streptavidin conjugated to Alexa Fluor 488 (1:200 dilution, Invitrogen, Eugene, OR, USA) for 2 h at room temperature. Neurobiotin thus fluoresces green. Following incubation, sections were rinsed in phosphate-buffered saline and cover slipped with non-fluorescent mounting medium (Aqua Poly/Mount; Polysciences, Warrington, PA, USA).

Analysis of neural responses

Physiological responses were analyzed off-line using TDT Open Explorer and Brainware software and custom-written MATLAB scripts. Analyses included compilation of PST and period histograms and calculation of relative spike rates (spike rate in the stimulus interval compared to the interstimulus interval) and vector strength (VS) for quantification of phase locking to the stimulus waveform. The Rayleigh test was used to test the statistical significance of VS (Rayleigh statistic >12.8 , $P < 0.001$). Best frequency of response (BRF) was defined as that stimulus frequency producing the best driven response (relative spike rate in the stimulus compared to the interstimulus interval) at the lowest stimulus amplitude tested. Because thresholds could not be reliably estimated at all recording sites, these values do not necessarily reflect best excitatory frequency as typically defined. Estimated threshold at BRF was defined as a 20% increase in spike rate in the stimulus compared to the interstimulus interval. In cases where no spontaneous activity could be recorded, threshold was estimated as that stimulus amplitude producing responses in one-half of the trials. Because thresholds could not be precisely defined at all sites (activity was lost before all amplitudes could be tested), we consider the threshold values presented here to be conservative estimates. Thresholds of response to z-axis stimulation are expressed as peak-to-peak displacement in microns (µm), corrected for the frequency response of the shaker dish. Thresholds of responses to underwater pressure stimuli are expressed as dB re: 0.1 Pa (Nelson 1996). Statistical analyses were performed using SPSS v. 15 (SPSS, Chicago, IL, USA) and graphs plotted using SigmaPlot 8.0 (Jandel, Point Richmond, CA, USA).

Endorgan tract tracing

For endorgan tracing studies, tadpoles were anesthetized, wrapped in moist gauze and placed on a surgical block. The skin over the otic capsule was reflected, and the dorsal surface of the capsule slit open and reflected rostrally. A 2 mm borosilicate micropipette (tip diameter 20 µm) was backfilled with oil and front loaded with 15% neurobiotin in phosphate buffered saline. Extracellular injections were made using a Nanoject II (Drummond Scientific, Broomall, PA, USA) injector. The tip of the micropipette was placed in specific branches of nVIII or directly into the otolith organs (sacculus, lagena, utricle). Injections were typically 23–46 nL of tracer. Tadpoles were allowed to survive for 1–4 h before being euthanized and perfused. Brains and endorgans were removed and processed for neurobiotin reaction product, as described above.

Visualization of label

Sections were visualized and imaged using an Olympus BX-60 microscope (Melville, NY, USA) equipped with custom-made fluorescence cubes and an Olympus DP72 camera. Images were acquired using a Dell Pentium IV 2.4 GHz computer running image acquisition and analysis software (Olympus) and were stored as 24-bit RGB TIFF images. Sections were also imaged using a Leica TCS SP2 confocal microscope system (sequential scan mode, Leica Microsystems, Bannockburn, IL, USA). High power z stack images were taken

through each section and viewed in orthogonal planes using Leica confocal software. Anatomical divisions and nomenclature are based on our previous work (Horowitz et al. 2007a, b) and are consistent with those used by Birinyi et al. (2001) and Straka et al. (2003) in adult *Rana temporaria*. The caudal nucleus as defined by Christensen-Dalsgaard and Walkowiak (1999) is considered here as part of the MVN (a caudal nucleus could not be unambiguously defined in the larval tadpole brain). The saccular nucleus as defined by Matesz (1979) encompasses the ventralmost portion of the DMN.

Results

Physiological responses to *z*-axis particle motion were collected from 34 sites using neurobiotin-filled pipettes. Centers of neurobiotin deposits at 31 sites are illustrated schematically in Fig. 2. The neurobiotin reaction was not performed for two additional animals whose physiological data are included in this report. One recording site in the medial raphe was excluded from analysis. Of the recovered recording sites, many ($n = 12$) are located medially and dorsally in the medulla, within the MVN and the adjacent LLnp. More laterally, one site was located within the boundaries of the DMN and four others were located near the medullary entry point of nVIII. Six sites were located within or around the medial boundary of the lateral vestibular nucleus (LVN) or around the border between the LVN and the descending vestibular nucleus (DVN). Eight sites were located ventral to the MVN, along the dorsal arcuate pathway (DAP) in the reticular gray. Because neurobiotin traveled to the MVN, LVN or DMN even from the most ventral of these sites, physiological data from these sites are included in this report. Seven sites (4 in the MVN, 1 in the DMN, and 2 in the LVN; the remaining LVN sites were lost before these stimuli could be presented) also responded to pressure stimulation. No active sites were located within the lateral line nucleus.

A broad range of BRFs, extending from 38 to 214 Hz, was recorded to *z*-axis stimulation. Mean BRFs in late larval and metamorphic climax animals were similar (104 and 112 Hz, respectively; Fig. 3a), and there was no significant correlation between animal stage and BRF ($r^2 = 0.03$). Five animals in the deaf period (stages 38–41) had BRFs between 66 and 150 Hz (mean 103 Hz), not significantly different from BRFs in earlier or later larval stages. Estimated thresholds at BRF (thresholds could not be determined at two sites) ranged widely, from a low of 0.016 to a high of 12.5 μm displacement (overall mean 1.74 μm displacement; Fig. 3b). There was no significant correlation between estimated threshold and larval stage ($r^2 = 0.003$). Estimated thresholds for the five deaf period animals were 0.12, 0.28, 1.45, 2.1, and 12.5 μm displacement, spanning the entire range of thresholds seen in earlier larval stages. Four sites in early larval animals had among the highest thresholds observed, ranging from 3.7 and 12 μm displacement. Estimated thresholds were overall lower during metamorphic climax (range 0.16–1.95 μm displacement), but this trend was not statistically significant. Spontaneous activity varied among electrode sites, with an overall mean of 10.3 spikes/s (median 3.6 spikes/s).

Z-axis sensitivity in the MVN/LLnp

Sites in the MVN and in the adjacent LLnp ($n = 12$; Fig. 4) responded vigorously to *z*-axis stimulation, with BRFs ranging from 38 to 132 Hz and thresholds from 0.016 to 4.7 μm displacement (mean 0.99 μm ; threshold could not be estimated at one site). Most frequency-threshold curves (Fig. 4a) showed broad tuning over the frequency range of stimulation, with one presumed single unit site, with a BRF of 65 Hz, showing a clear V-shaped response curve. Synchronization response curves (Fig. 4b) and period histograms (Fig. 4c, center and right) indicated that there was little significant phase-locking above about 100 Hz. PST

histograms to BRF stimuli were phasic in shape, with a strong onset peak (Fig. 4c, left). The phasic nature of the response likely contributed to the low values of VS.

Examination of neurobiotin label suggests that recording sites located more dorsally in the MVN ($n = 8$; Fig. 5a–c) were more likely to show significant phase-locking to equivalent low frequencies than those located more ventrally ($n = 3$; Fig. 5c–e). A representative injection into the dorsal MVN near the LLnp border labeled cells around the injection site, large fiber bundles passing through the LLnp back to the site of entry of nVIII fibers (Fig. 5a), and a small bundle of fibers projecting rostrally to the superior cerebellar nucleus (data not shown). Distant projections were limited to the contralateral MVN (Fig. 5b) via transport along the DAP and through the contralateral LLnp. This site showed good phase-locking to low frequency stimuli (Fig. 4c, top right). A small injection in the dorsal MVN of a deaf period animal (Fig. 5c) produced strong label of nVIII fibers traversing the dorsal medulla, but more limited label around the injection site. A large injection more ventrally in the MVN (Fig. 5d) resulted in substantial cell label in the ipsilateral DMN and relatively sparser label in the ipsilateral dorsal LVN. Fiber label was visible along the DAP, with both fiber and terminal label present in the contralateral MVN and SON (Fig. 5e). Fibers traveling bilaterally to the principal nucleus of the TS were also observed (data not shown). A large injection in the ventral MVN of a deaf period animal (Fig. 5f) also resulted in considerable fiber label along the DAP and cell label in the contralateral DMN. Sparser cell label was observed in the contralateral dorsal MVN, but there was no label of the LLnp. These two sites showed no significant phase-locking even to frequencies lower than 100 Hz.

Z-axis sensitivity in the LVN

Strong responses to z-axis stimulation were recorded in the LVN, with BRFs in the narrow range of 90–124 Hz, with estimated thresholds ranging from 0.018 to 1.07 μm displacement. Frequency-threshold curves typically showed broad tuning above about 80 Hz (Fig. 6a), with some sites showing increased thresholds above about 150 Hz. Synchronization response curves (Fig. 6b) were variable, with some sites showing considerable and others showing more restricted phase-locking (Fig. 6c, center and right columns). PST histograms to frequencies at and around BRF were typically phasic in shape, with a strong onset peak (Fig. 6c, left column).

Neurobiotin transport patterns from deposits in the LVN generally produced ipsilateral cell and fiber label in the ventral DMN with more restricted label in the MVN. Label of fibers extending toward and through the DAP to the contralateral LVN was also typically observed. Patterns of transport from similarly sized injections did not vary between animals at different developmental stages. In particular, strong transport from the LVN was observed in a deaf period animal (Fig. 7). Here, a deposit in the caudal LVN resulted in label of cells in the bordering DVN, with more limited label in the ventral DMN (Fig. 7a) and only sparse label more dorsally in the MVN. There was extensive fiber label of nVIII (Fig. 7b; transport ended at the nerve ganglion, so there was no label of inner ear endorgans). Caudal projections to the ipsilateral inferior olive and along the vestibulospinal tract to the contralateral raphe nucleus and the inferior reticular nucleus were also observed (Fig. 7c).

Z-axis sensitivity in the dorsal arcuate pathway

Responses to z-axis stimulation were recorded along the DAP extending contralaterally from the dorsal MVN through the reticular gray. BRFs at these sites ranged from 44 to 152 Hz, with thresholds ranging from 0.26 to 12 μm displacement (mean 2.59 μm). PST histograms at and around BRF were phasic in shape, and there was little phase-locking even to low frequency stimuli, and then only at high stimulus amplitudes (2 μm displacement and

above). Neurobiotin deposited at these sites, even in a deaf period animal, labeled cell bodies in the ipsilateral MVN, LVN, DMN and reticular gray, as well fibers traveling along the DAP to the contralateral dorsal medulla (not shown).

Z-axis sensitivity in the DMN

Only one recording site, in a stage 35 animal, was located within the boundaries of the DMN, in spite of numerous attempts to record from this area. This site showed a broad response range over frequencies from 59 to 250 Hz (higher frequencies were not tested) and BRF of 214 Hz (threshold 0.10 μm displacement). PSTs were phasic in shape, and responses to frequencies at and close to BRF were phase-locked (Fig. 8a). This site also responded to pressure stimulation over a similar frequency range (Fig. 8b), but with less synchronized activity. Neurobiotin traveled from the recording site ventrally and medially through the LLnp to the DAP (Fig. 8c). Considerable terminal label was visualized in the ipsilateral MVN (Fig. 8c), while only sparse terminal and fiber label of the contralateral MVN was visible (not shown).

Four recording sites were located laterally in the medulla, between the DMN and the entry point of nVIII. BRFs at these sites ranged from 93 to 115 Hz, with estimated thresholds from 0.73 to 12.5 μm displacement. Labeled fibers traveling from these sites toward the DMN and the MVN were observed, even in a deaf period animal. This pattern of transport suggests that these recordings may reflect activity from incoming nVIII fibers.

Responses to pressure stimulation

Responsiveness to pressure stimulation was evaluated at 21 recording sites. Fourteen sites, including all those located along the DAP, did not respond to airborne sounds, even at high stimulus amplitudes. Seven sites in the MVN, DMN and LVN were sensitive to low frequency pressure stimulation at stimulus levels ranging from +10 to -17 dB SPL re 0.1 Pa (these values are those that evoked responses, and do not necessarily indicate threshold). Characteristics of responses to pressure and to particle motion are shown in Fig. 8 (DMN) and Fig. 9 (MVN and LVN). As in the DMN, PST histograms to both kinds of stimulation in the MVN (Fig. 9a, particle motion; Fig. 9b, pressure) and in the LVN (Fig. 9c, particle motion; Fig. 9d, pressure) were typically phasic in shape. In contrast, the extent of synchronization varied with stimulus modality, even at the same site. Both the DMN site (Fig. 8a) and the MVN site (Fig. 9a, b) showed significant phase-locking to low frequency z-axis stimulation but not to pressure stimulation at the same frequency, while the LVN site (Fig. 9c, d) showed significant synchronization to both particle motion and pressure. The greater synchronization to equivalent low-frequency stimulation was consistent across all sites that responded to both particle motion and pressure.

Endorgan afferent sources to medullary target nuclei

Neurobiotin injections directly into nVIII bundles innervating the otolith organs (saccul, utricle, lagena) of early and late larval tadpoles demonstrated discrete but overlapping patterns of projections to the dorsal medulla (Fig. 10). Injections into the saccul or the saccular branch of nVIII resulted in heavy label of the ipsilateral LVN and DMN, more restricted label in the MVN, and occasional label of fibers extending toward and through the DAP. Extent of label in the MVN and in the DVN (not shown) varied at different planes of section. Injections into the lagenar branch labeled the LVN almost exclusively, with less extensive label of the ventral DVN and only very sparse label of the ventral DMN. Injections into the utricle also labeled the LVN, but with no label of the DMN. Neither the lagena nor the utricle contributed significant fiber or terminal projections to the MVN or to the LLnp.

Discussion

Our data show that responses to z-axis particle motion are widely represented in two medullary vestibular nuclei—the MVN and the LVN—in the bullfrog tadpole at all stages of larval development. In contrast, there is very limited responsiveness to z-axis particle motion in the DMN at the same stages of development. Importantly, our data show robust responsiveness to particle motion in the MVN, LVN and DAP during the deaf period for pressure stimulation. Even though our sample size of deaf period animals is small ($n = 5$), there is no evidence of the severe reduction in sensitivity to pressure as observed in the TS during this developmental time (Boatright-Horowitz and Simmons 1997). Within this limited sample, only one site, located laterally in the medulla in the cell-sparse region between the DMN and the entry point of nVIII, exhibited a very high threshold to particle motion. The other sites exhibited thresholds within the range of variability in the entire sample, with one site in the LVN of a deaf period animal showing among the lowest thresholds in our sample. Moreover, results of neurobiotin label show, contrary to data on dye transport between the SON and the TS (Boatright-Horowitz and Simmons 1997; Horowitz et al. 2007b), no failure of transport from these medullary nuclei during the deaf period. Specifically, brains of all deaf period animals in which neurobiotin was deposited (either in the MVN, LVN or along the DAP) showed label of appropriate medullary targets, with the extent of label related to the size of the injection rather than to the animal's developmental stage. Transport to the auditory midbrain was limited in all animals, regardless of developmental stage, likely related to the short survival time after iontophoresis. We suggest that the anatomical disconnection previously observed during the deaf period effect is not a widespread phenomenon in the tadpole brain, but is restricted to the SON-TS, and perhaps purely pressure-sensitive, pathways.

Representation of particle motion in the medulla of developing and adult frogs

The stable representation of particle motion observed in this study is consistent with our earlier report (Horowitz et al. 2007a) of low-frequency sensitivity to particle motion in the MVN. Data from that study showed a range of BRFs from 18 to 46 Hz with an overall increase during metamorphic climax stages, consistently strong phase-locking to BRF stimuli, and thresholds in the range of 1.5–12 μm displacement (mean 6.92 μm). In contrast, MVN sites in the present study, in which a broader frequency range of stimulation was used, had BRFs ranging from 38 to 132 Hz with mean thresholds of 0.99 μm displacement. Even within the same low frequency (<70 Hz) range, estimated thresholds were overall lower (mean 3.61 μm displacement) in the present study. We interpret these differences to the more secure way in which the tadpole was held in position during recordings, which presumably reduced any extraneous movements between the animal and the surrounding fluid.

There are no published data on the functional properties of medullary nuclei in any species of anuran tadpole with which to compare our results, and there are few physiological data on responses to z-axis stimulation in adult frogs of any species. Christensen-Dalsgaard and Walkowiak (1999) recorded from the saccular and caudal nuclei of adult *R. temporaria* in response to dorsal–ventral vibrations at frequencies from 10 to 80 Hz. They observed thresholds in the dorsal medulla as low as 0.01 cm/s^2 (acceleration), but population data were not presented. The lowest thresholds in our data, converted to units of acceleration, are 0.4 cm/s^2 , with an upper limit of 1.47 cm/s^2 . PST histograms in that study showed several shapes (although shape was not related to recording site), from tonic to phasic with a sharp onset. Our data show no evidence of tonic PST histogram shapes in response to z-axis stimulation. Elliott et al. (2007) recorded strong activity to tones and calls in the DMN of

adult *Xenopus laevis*, but did not systematically characterize responses of this area to dorsal–ventral vibrations.

Our data show a great deal of variability in the degree of synchronization to z-axis stimulation. Some sites showed no phase-locking even at low (<100 Hz) frequencies, others synchronized only at high stimulus amplitudes, while still others showed a broad frequency range of phase-locking. This variability appeared to be related to site of injection and projection patterns, with sites in the LVN showing the most consistent phase locking and those in MVN showing more variable responses. In addition, injections into these nuclei that resulted in label of the DMN showed less extensive phase locking than those in which transport to the DMN was absent. In adult ranids (Narins and Lewis 1984; Yu et al. 1991), saccular afferents exhibit strong phase-locking to frequencies within their tuning curves, but variability in synchronization is seen at the first synapse in the medulla (Christensen-Dalsgaard and Walkowiak 1999). Similarly, there is weak or absent phase-locking in the dorsal octaval nucleus of the adult toadfish *Opsanus tau* in response to particle motion stimulation (Edds-Walton and Fay 2005), compared with the strong phase-locking in saccular afferents (Fay and Edds-Walton 1997).

As previously described (Horowitz et al. 2007a), thresholds to z-axis stimulation in tadpoles are overall higher than those recorded in the medulla (Christensen-Dalsgaard and Walkowiak 1999; Elliott et al. 2007) and in nVIII fibers of adult frogs (Koyama et al. 1982; Narins and Lewis 1984; Christensen-Dalsgaard and Narins 1993; Yu et al. 1991), and in saccular afferents (Fay and Edds-Walton 1997) and medulla (Edds-Walton and Fay 2005) of adult toadfish. It is likely that these threshold differences reflect developmental growth in the mass of the inner ear organs responsible for detection of z-axis vibrations.

Inner ear organs mediating particle motion sensitivity

There is very little known about the detailed organization and central projections of any inner ear organ across larval development in any species of frog (Lewis and Li 1973; Hertwig 1987) and how these transduce biologically relevant stimuli across the metamorphic transition. An interesting question raised by our data, then, concerns the identity of the inner ear organ or organs that mediate particle motion sensitivity in the tadpole medulla. Based on results of anatomical tracing with lipophilic dyes (Horowitz et al. 2007a) and by analogy with data on particle motion sensitivity in fishes (Fay 1984; Fay and Edds-Walton 1997), we earlier interpreted sensitivity to particle motion in the MVN as reflecting afferent input from the saccule. The physiological and anatomical results from this study support this interpretation. Furthermore, our data implicate the saccule as at least one source of particle motion sensitivity in the LVN. Although we have limited data on neurobiotin transport from the other otolith organs, we did observe widespread label in the LVN, but not in the MVN, after injections into the lagena and the utricle (Fig. 10). We thus cannot rule out a contribution from either of these other otolith organs in mediating z-axis sensitivity in the LVN. It is possible that the physiological sensitivity to particle motion observed in the vestibular medulla reflects input from more than one otolith organ, depending on the spatial location of the recording site. Analyses of the functional development of these organs are needed to address this question.

On the basis of extensive anatomical mapping of the vestibular rhombomeres in the medulla of *Rana clamitans* tadpoles, Straka et al. (2001) argued for a basic conservation of anatomical patterning in tadpoles and adults. The central representation of the otolith organs in bullfrog tadpoles as derived from our data shows both similarities and differences with previous data from adult frogs. In *R. temporaria*, afferents from the lagena and utricle terminate in an overlapping manner in the LVN and DVN, while saccular projections to

these nuclei are much more restricted (Birinyi et al. 2001). Only very few terminal endings from the utricle or lagena are found in the MVN. Similarly, maps of electrical field potentials from in vitro stimulation of individual nVIII branches show widespread overlap of the utricle and lagena in the LVN and in the DVN, but not in the MVN (Straka et al. 2003). Our anatomical data in tadpoles are generally consistent with these results.

Responses to z -axis stimulation in the DAP could reflect activity from fibers originating in the MVN or LVN projecting to the contralateral medulla, recordings from reticular gray cells themselves, or interconnections between the LVN and the reticular gray, as demonstrated in adult *Rana esculenta* (Matesz et al. 2002). Interestingly, in both larval *R. clamitans* (Straka et al. 2001) and adult *R. temporaria* (Birinyi et al. 2001), direct projections to the reticular gray by nVIII fibers innervating the otolith organs have been observed. We did not observe any transport from the otolith organs to the reticular gray, but this may be related to the small amount of dye deposited and the limited survival time of the animals after iontophoresis.

In adult frogs, the saccule projects to the ventral DMN/dorsal LVN (saccular nucleus), with only limited, restricted projections to the MVN (Kuruvilla et al. 1985; Birinyi et al. 2001). Saccular field potentials recorded from isolated adult hindbrain are similarly restricted to these areas (Straka et al. 2003). The saccule in adult frogs is considered to be a dual-sensitivity organ that responds to both substrate vibrations and low-frequency acoustic stimuli (Narins and Lewis 1984; Yu et al. 1991), although with lower sensitivity to sounds. On these bases, we expected to find robust responses to z -axis particle motion in the tadpole's DMN. The insensitivity of this nucleus to this mode of stimulation, in spite of the extensive saccular innervation of this nucleus even in tadpoles, raises the possibility that separate groups of saccular fibers project to the DMN as opposed to the MVN and LVN and that these fibers are more sensitive to pressure stimulation. Interestingly, the observation that responses to both z -axis particle motion and to pressure stimulation could be recorded at several sites in both the MVN and LVN supports the idea that the tadpole's saccule transduces both pressure and particle motion. It has been proposed that the frog's amphibian papilla also exhibits dual sensitivity to pressure waves and substrate vibrations (Yu et al. 1991; Christensen-Dalsgaard and Narins 1993). The inability to consistently record responses to z -axis particle motion in the DMN, the main site of termination of amphibian papilla fibers, argues against the hypothesis that this organ also has a dual sensitivity (to particle motion and to pressure) in tadpoles. A more extensive survey of DMN sensitivity is needed to further define the function and organization of the dorsal medulla across development.

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Abbreviations

BRF	Best frequency of response
DAP	Dorsal arcuate pathway
DMN	Dorsal medullary nucleus
DVN	Descending vestibular nucleus

LLnp	Lateral line neuropil
LVN	Lateral vestibular nucleus
MVN	Medial vestibular nucleus
nVIII	Eighth nerve
PST	Post-stimulus time histogram
SON	Superior olivary nucleus
TS	Torus semicircularis
VS	Vector strength

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Fig. 1.

Sensitivity of the shaker dish to displacements in the *x*, *y*, and *z* planes. **a** Responses of *x*, *y*, and *z* accelerometers to high-amplitude stimulation (nominal attenuation of 10 dB) of the *z*-axis. Data are presented as displacement (μm) by frequency for *x* (black), *y* (red), and *z* (green) axes. The shaker dish shows a strong resonant standing wave in the 70–80 Hz range. **b** Frequency response of the *x* (closed circles) and *y* (open circles) axes plotted as a percentage of the response of the *z*-axis in response to *z*-axis stimulation. Activation of the *x* and *y* accelerometers (crosstalk) in the frequency range most relevant to this study (90–240 Hz) is <5% of that at the *z* accelerometer

Fig. 2.

Distribution of recording sites in the tadpole's medulla. The *left half* of the image shows a cresyl violet stained section through the rostral/caudal mid-point of the medulla (*dotted line on left insert*) of a stage 34 tadpole. *Black dots* on the right schematic show relative locations of recording/injection sites, all on the same plane of section for illustrative purposes. Some points have been displaced somewhat for clarity. The sulcus limitans of His (*arrow*) was used as the demarcation between MVN sites and those along the DAP. Dorsal is up

Fig. 3.

Best frequency of response and estimated threshold to z -axis stimulation do not significantly vary across larval development. **a** BRF is not significantly correlated with larval stage. The two dashed vertical lines between stages 38–41 represent the boundaries of the deaf period for pressure stimulation. BRFs in the deaf period are within the range of variability seen in both earlier and later developmental stages. **b** Estimated thresholds at BRF are not significantly correlated with larval stage. Note the break in the y-axis between displacement values of 4 and 12 μm

Fig. 4.

Responses to particle motion in the MVN. **a** Frequency-threshold curves from five sites, chosen to show variability in shapes of the curves. **b** Synchronization response curves, plotted as VS, from the same five sites. **c** PST histograms (*left column*) and period histograms (*center and right columns*) from two of the five sites shown in **a** and **b**, chosen to show the variability in phase-locking. PST histograms are plotted over the 10-s duty cycle. The *solid red line* at 3 s shows the stimulus offset. Period histograms are plotted over one period of the stimulus. The histograms in the *center column* show responses at BRF close to threshold, while those in the *right column* show responses at the frequency exhibiting the best phase-locking for that site. The Rayleigh value is indicated in each plot, with statistical significance indicated by the *asterisk*. Data in the *top row* are derived from a stage 35 animal: BRF 105 Hz, threshold 0.59 μm displacement; period histograms at 105 Hz (0.59 μm displacement) and 54 Hz (1.15 μm displacement). Data in the *bottom row* are derived from a stage 43 animal: BRF 149 Hz, threshold 0.28 μm displacement; period histograms at 149 Hz (0.99 μm displacement) and 105 Hz (1.47 μm displacement)

Fig. 5.

Neurobiotin label after injection into the MVN. **a** Injection site (*asterisk*) in the dorsal MVN of a stage 35 tadpole showing heavy cell body and fiber label around the injection site. Labeled fibers (*arrow*) transect the LLnp and traverse toward nVIII. Physiological data from this site are shown in Fig. 4c, top row. Dorsal is up, medial to the right. **b** Label of the contralateral medulla from the same injection. Labeled fibers and terminals (*arrowhead*) are present in the MVN, with short fiber segments (*arrow*) traversing the LLnp. Dorsal is up, medial to the left. **c** Label of nVIII fibers (*arrow*) from an injection into the dorsal MVN of a stage 38 (deaf period) tadpole. The injection site is in the approximate position of the *asterisk*, but is located two sections more rostrally. Dorsal is up, medial is to the right. **d** Recording site in the ventral MVN of a stage 43 tadpole. There is considerable cell body label around the injection site (*asterisk*), as well as more dorsally in the MVN and in the DMN. Sparser label is seen in the dorsal LVN. Physiological data from this site are shown in Fig. 4c, bottom row. Medial is to the right. **e** Contralateral projections from the same injection (*asterisk*) pass through the DAP (*arrow*) and decussate at the midline to label fibers and terminals in the contralateral SON (*arrowhead*). **f** Label of the contralateral DMN (*arrow*) from an injection in the ipsilateral ventral MVN of a deaf period (stage 39) tadpole. There is sparse cell label in the dorsal MVN (*arrowhead*). Medial is to the left. Scale bars **a** 150 μ m; **b** 50 μ m; **c** 50 μ m; **d** 150 μ m; **e** 300 μ m; **f** 150 μ m

Fig. 6.

Responses to particle motion in the LVN. **a** Frequency-threshold curves from four sites. **b** Synchronization (VS) response curves from the same four sites. **c** PST histograms (*left column*) and period histograms (*center and right columns*; significant VS indicated by the *asterisk*) from two of the four sites shown in **a** and **b** chosen to show the variability in phase-locking. The histograms in the *center column* show responses at BRF close to threshold, while those in the *right column* show responses at the frequency exhibiting the best phase-locking for that site. Data in the *top row* are derived from a stage 41 animal: BRF 117 Hz, threshold 0.018 μm displacement; period histograms at 117 Hz (0.10 μm displacement) and 60 Hz (0.10 μm displacement). Data in the *bottom row* are derived from a stage 36 tadpole: BRF 124 Hz, threshold 0.68 μm displacement; period histograms at 134 Hz (0.85 μm displacement) and 93 Hz (1.6 μm displacement)

Fig. 7.

Neurobiotin label and transport after injection into the LVN of a stage 41 (deaf period) tadpole. **a** Injection site (*asterisk*) showing label of multipolar-shaped cells (*arrow*) and of smaller round-bodied cells more medially at the border of the DVN (*arrowhead*). Dorsal is up, medial to the right. **b** Neurobiotin label in nVIII from the same injection. Medial is to the left. **c** Caudal projections from the same injection produce label in the posterior LVN and DVN, inferior olive (*arrow*), raphe nucleus (*arrowhead*) and inferior reticular nucleus (*double-headed arrow*). Scale bars 100 μm

Fig. 8.

Responses to particle motion and to pressure stimulation from the same recording/injection site in the DMN of a stage 35 tadpole. **a** PST (*top*) and period (*bottom*) histograms to particle motion at BRF (214 Hz) and close to threshold (0.10 μm displacement). There is significant synchronization (*asterisk*) to the particle motion stimulus. **b** PST (*top*) and period (*bottom*) histograms to pressure stimulation at BRF (134 Hz) and close to threshold (87 dB SPL). No significant synchronization is seen. **c** Neurobiotin label and transport. *Left* image shows labeled cells around the injection site (*asterisk*) and fibers projecting ventrally to and along the DAP (*arrow*). *Right* image is a higher powered shot showing labeled fibers projecting medially to the ipsilateral MVN (*arrowhead*) and through the LLnp to the DAP (*arrow*). Scale bars 150 μm (*left*), 50 μm (*right*)

Fig. 9.

Comparison of responses to particle motion and to pressure stimulation in the MVN (**a, b**) and in the LVN (**c, d**). **a** PST (*top*) and period (*bottom*, with VS as indicated) histograms to z-axis stimulation of the MVN in a stage 44 tadpole. Stimulus frequency 100 Hz, amplitude 0.07 μm displacement. **b** PST (*top*) and period (*bottom*) histograms to pressure stimulation of the same site. Stimulus frequency 95 Hz, stimulus amplitude 2 dB re 0.1 Pa. Significant synchronization is seen only in response to the particle motion stimulus. **c** PST (*top*) and period (*bottom*) histograms to z-axis stimulation in the LVN of a stage 34 tadpole. Stimulus frequency 80 Hz, stimulus amplitude 0.14 μm displacement. **d** PST (*top*) and period (*bottom*) histograms to pressure stimulation of the same site. Stimulus frequency 80 Hz, stimulus amplitude 3 dB re 0.1 Pa. There is significant synchronization to both modalities of stimulation

Fig. 10.

Overlay of pseudocolored neurobiotin label in the dorsal medulla following injections into individual inner ear organ nerve branches. Transport from the saccule (stage 34 tadpole) is indicated in *green*, transport from the lagena (stage 37 tadpole) is indicated in *blue*, and transport from the utricle (stage 35 tadpole) is indicated in *yellow*. At this plane of section, label in the MVN from saccular injections is more restricted than at more rostral levels. Dorsal is up, medial to the right. *Scale bar* 200 μ m