Innovations in motoneuron synchrony drive rapid temporal modulations in vertebrate acoustic signaling

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Chagnaud BP, Zee MC, Baker R, Bass AH. Innovations in motoneuron synchrony drive rapid temporal modulations in vertebrate acoustic signaling. J Neurophysiol 107: 3528–3542, 2012. First published March 14, 2012; doi:10.1152/jn.00030.2012.—Rapid temporal modulation of acoustic signals among several vertebrate lineages has recently been shown to depend on the actions of superfast muscles. We hypothesized that such fast events, known to require synchronous activation of muscle fibers, would rely on motoneuronal properties adapted to generating a highly synchronous output to sonic muscles. Using intracellular in vivo recordings, we identified a suite of premotor network inputs and intrinsic motoneuronal properties synchronizing the oscillatory-like, simultaneous activation of superfast muscles at high gamma frequencies in fish. Motoneurons lacked spontaneous activity, firing synchronously only at the frequency of premotor excitatory input. Population-level motoneuronal output generated a spike-like, vocal nerve volley that directly determines muscle contraction rate and, in turn, natural call frequency. In the absence of vocal output, motoneurons showed low excitability and a weak afterhyperpolarization, leading to rapid accommodation in firing rate. By contrast, vocal activity was accompanied by a prominent afterhyperpolarization, indicating a dependency on network activity. Local injection of a GABA_A receptor antagonist demonstrated the necessity of electrophysiologically and immunohistochemically confirmed inhibitory GABAergic input for motoneuronal synchrony and vocalization. Numerous transneuronally labeled motoneurons following single-cell neurobiotin injection together with electrophysiological collision experiments confirmed gap junctional coupling, known to contribute to synchronous activity in other neural networks. Motoneuronal synchrony at the premotor input frequency was maintained during differential recruitment of variably sized motoneurons. Differential motoneuron recruitment led, however, to amplitude modulation (AM) of vocal output and, hence, natural call AM. In summary, motoneuronal intrinsic properties, in particular low excitability, predisposed vocal motoneurons to the synchronizing influences of premotor inputs to translate a temporal input code into a coincident and extremely synchronous, but variable-amplitude, output code. We propose an analogous suite of neuronal properties as a key innovation underlying similarly rapid acoustic events observed among amphibians, reptiles, birds, and mammals.

hindbrain; motoneurons; superfast muscles; vocalization

High-frequency, synchronous neuronal firing is implicated in a wide spectrum of behavioral functions ranging from attention, information coding, and memory formation to neurological disorders including epilepsy and Parkinson’s disease (Averbeck and Lee 2004; Axmacher et al. 2006; Baker et al. 1999; Jia and Kohn 2011; Niebur et al. 2002; Uhlhaas and Singer 2006). Recent studies also suggest an essential role for population level synchrony in the motor patterning of acoustic signaling, where rapid temporal modulation in the high-gamma (>80 Hz) range is linked to the actions of “superfast” muscles in several vertebrate lineages, including fishes (sonic swim bladder), reptiles (rattlesnake shaker), birds (syrinx), and mammals (larynx) (Elelams et al. 2004, 2008, 2011; Rome et al. 1996). Considerable progress has been made in identifying ultrastructural (Appelt et al. 1991; Bass and Marchatrete 1989; Fawcett and Revel 1961), oxidative (Walsh et al. 1995), and molecular-contracile properties (Rome 2006) adapted for high-frequency contraction (often >80 Hz under ambient conditions). Although nerve recordings alone suggest an extreme degree of synchrony in fish vocal systems that is not observed in other motor systems, namely, those underlying locomotion (see Discussion), it remains essentially unknown how motoneuron populations pattern rapid acoustic events in fishes and vertebrates in general. We used intracellular recordings in an in vivo preparation to investigate the intrinsic and network properties leading to motoneuron synchrony in the evolutionarily conserved vocal network driving superfast muscle activity among toadfishes (Bass et al. 2008).

Toadfishes, which include midshipman fish, generate acoustic signals for social communication functions (Fig. 1A) by vibrating one set of superfast muscles attached to the walls of the swim bladder (Cohen and Winn 1967; Skoglund 1961; Tavolga 1971). Prior studies in toadfishes (Bass 1985; Bass and Baker 1990; Fine et al. 1984; Pappas and Bennett 1966) identified paired hindbrain vocal motor nuclei (VMN; Fig. 1, B and C), providing ipsilateral innervation to these muscles via the vocal nerve formed by axons exiting the brain through occipital nerve roots likely homologous to hypoglossal roots (Fig. 1, B and C) (Bass et al. 2008). Individual vocal motoneuron responses are matched 1:1 with brief, fast-rising vocal nerve waveforms/spikes representing synchronous motoneuron activity (Fig. 1D) (Bass and Baker 1990; Pappas and Bennett 1966). Both motor nuclei fire in phase (Fig. 1E), with each bilaterally synchronous vocal motor volley (VOC) resulting in simultaneous vocal muscle contraction and one sound pulse (Bass and Baker 1990, 1991; Cohen and Winn 1967). Morphophysiological analyses in distantly related teleosts show a comparable vocal motor system (Bass and Andersen 1991; Bass et al. 2008; Bass and McKibben 2003; Onuki and Somiya 2007), suggesting its key role in the widespread evolution of acoustic communication among fishes (Fish and Mowbray 1970; Lach et al. 2006).
Motoneuron synchrony

In this report, we show that a novel suite of intrinsic and network motoneuronal properties, namely, low somatodendritic excitability combined with especially dense and robust premotor network excitatory and inhibitory inputs, ensure extremely synchronous motoneuron activation at the excitatory input frequency. These mechanisms make certain an extreme degree of temporal fidelity at a population level that, in this case, directly determines natural call frequency while allowing for size-dependent recruitment that contributes to amplitude modulation of motoneuronal output and, hence, natural calls. We propose a similar complement of intrinsic and network properties adapted for motoneuron synchrony in vocal networks among reptiles, birds, and mammals (see above) also dependent on the rapid temporal modulation of acoustic events.

Materials and Methods

Animals. Midshipman fish have two male reproductive morphs (Brantley and Bass 1994). Nest-building, territorial type I males acoustically court females. Only type I males (n = 43, standard length: 9.7–15.8 cm) were used in this study because of their larger repertoire of agonistic and advertisement calls that mainly differ in duration and degree of amplitude modulation (Bass et al. 1999; Brantley and Bass 1994). Type II males neither build nests nor acoustically court females, but instead steal fertilizations from type I males using sneak and satellite-spawning strategies. Type II males, like females, are only known to make agonistic grunt calls in non-nesting contexts. Animals were hand collected from either nests or offshore trawls and housed in isolation in an environmental control room at 17 ± 2°C on a 14:10-h light-dark cycle. Animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee.

Surgery. Surgical procedures followed previously described methods (e.g., Bass and Baker 1990; Kittelberger et al. 2006; Weeg et al. 2005). In brief, fish were anesthetized by immersion in 0.025% ethyl p-amino benzoate (Sigma, St. Louis, MO) in artificial seawater before surgery. Bupivacaine (0.01 g/ml; 0.2 ml) was additionally injected every 4 h near the surgical site for local, long-lasting anesthesia/analgesia. A dorsal craniotomy exposed the brain, rostral spinal cord, and ventral occipital nerve roots innervating sonic muscles. Animals were then given an intramuscular injection of pancuronium bromide (0.5 mg/kg body wt; Astra Pharmaceutical Products, Westborough, MA) for immobilization and transferred to an experimental tank. Recirculated, chilled (17 ± 2°C) seawater was pumped across the gills at all times. The experimental tank rested on a vibration isolation table (TMC, Peabody, MA).

Stimulation. Vocal output was monitored with an extracellular electrode (75-μm diameter, Teflon-coated silver wire with an exposed ball tip 125–200 μm in diameter) placed on an occipital (vocal) nerve root (VN; Fig. 1B). VN recordings were amplified 1,000-fold and bandpass filtered from 300 Hz to 5 kHz with a differential AC amplifier (model 1700; A-M Systems, Carlsborg, WA). Vocal output was evoked by current pulses delivered to previously mapped midbrain vocal sites (Bass and Baker 1990; Goodson and Bass 2002; Kittelberger et al. 2006). Electrical stimulation was delivered using insulated tungsten electrodes (impedance: 5 MΩ; A-M Systems). Current pulses were delivered via a constant current source (model 305-B; WPI) driven by a stimulus generator (A310 accupulser; WPI) producing 1–10 transistor-transistor logic (TTL) pulses at 100–300 Hz per pulse train per stimulus trial (standard settings were 5 pulses at 200 Hz). Intertrial intervals were 0.7–1.4 s.

Neurophysiological recordings. Intracellular electrodes were pulled on a horizontal puller (P97; Sutter Instruments, Novato, CA) and filled with either 5% neurobiotin (Vector Laboratories) in 0.5 M KCOOH (resistance 35–60 MΩ) for intracellular labeling or 2 M KCOOH for recording motoneurons (Bass et al. 1996). In this report, we show that a novel suite of intrinsic and network motoneuronal properties, namely, low somatodendritic excitability combined with especially dense and robust premotor network excitatory and inhibitory inputs, ensure extremely synchronous motoneuron activation at the excitatory input frequency. These mechanisms make certain an extreme degree of temporal fidelity at a population level that, in this case, directly determines natural call frequency while allowing for size-dependent recruitment that contributes to amplitude modulation of motoneuronal output and, hence, natural calls. We propose a similar complement of intrinsic and network properties adapted for motoneuron synchrony in vocal networks among reptiles, birds, and mammals (see above) also dependent on the rapid temporal modulation of acoustic events.

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physiological analysis. Neuronal signals were amplified 100-fold (Biomedical Engineering, Thornwood, NY) and digitized at a rate of 20 kHz (Digidata 1322A; Axon instruments) using the software pCLAMP 9 (Axon instruments). An external clock (Biomedical Engineering) sending TTL pulses synchronized stimulus delivery and data acquisition. A current step applied to the recording electrode was used to monitor electrode resistance during the search for neurons.

For antidromic stimulation, electrodes were implanted in vocal muscles attached to lateral walls of the swim bladder following a ventromedial incision in the body wall. Bipolar silver wire electrodes, insulated with enamel except at their tips (0.15-mm diameter, separated by 0.3 mm), were inserted into each muscle along the midline prior to where the nerve branches. Electrodes were connected via a constant current source (model 305-B; WPI) to a stimulator that delivered single shocks of square pulses. The body wall was sutured shut and sealed with Vetbond (3M).

**Bicuculline injection into vocal motor nucleus.** Micropipettes with diameters of 20-30 μm were fabricated and filled with either 10% bicuculline methiodide (Sigma) in 0.1 M phosphate buffer (PB) or 0.1 M PB alone. The pipette solution was pressure-ejected with a picospritzer (Biomedical Engineering) using a pulse duration of 50 ms at 10 lvpm. Four midline injections were performed sequentially at different rostrocaudal locations of VMN.

**Neurophysiological analysis.** Neuronal data were analyzed using the software IGOR Pro 6 (WaveMetrics), the free software package NeuroMatic (www.neuromatic.thinkrandom.com), and self-written scripts. The pulsatile VOC comprises a series of spikes that directly activate motoneurons. **VOC duration** was measured as the duration between the onset of stimulation and onset of an EPSP's rising phase. Effects were quantified as the ratio of the antidromically evoked action potential's amplitude after and before QX 314 injection, averaged for five stimulus applications. The amplitude of the motoneuronal activity during vocal activity (VOC) was calculated as the distance between the maximum and minimum amplitude of the motoneuronal activity during the evoked VOC.

Motoneuron firing patterns were visualized using a phase-plane plot of the recorded voltage (V) against the difference in voltage over time (dV/dt). The dV/dt trace was smoothed using a Gaussian filter to reduce electrical noise.

**Anatomy.** To visualize intracellularly recorded neurons, positive current (4–10 nA) with a duty cycle of 50% at 2–4 Hz was passed through a neurobiotin-filled recording electrode for 3–30 min. After a 2- to 6-h survival, fish were deeply anesthetized (0.025% benzocaine) and perfused with ice-cold teleost Ringer solution with 10 U/ml heparin (Elkins-Sinn, Cherry Hill, NJ), followed by 3.5% paraformaldehyde-0.5% glutaraldehyde in 0.1 M PB. Brains were postfixed (2–12 h) and then stored in 0.1 M PB (pH 7.2). One day before sectioning, brains were cryoprotected overnight in 30% sucrose solution in PB, embedded in gelatin (15%), and sectioned frozen in the transverse plane (120 μm thick) on a sliding microtome. Floating sections were reacted with an ABC kit (Vector Laboratories), mounted on gelatin-coated slides, and counterstained with cresyl violet. Neurontin-filled neurons were reconstructed using a camera lucida drawing tube (Leitz) attached to a microscope (Leitz Dialuz) at a magnification of ×400. Drawings were scanned and images were further processed with the software Photoshop 7.0 and CorelDRAW. Photographs of sections were taken using a microscope (Nikon) and a digital camera, and whole images were later processed with Photoshop 7.0. Image stacks of light microscopy pictures were used to generate photographs with high contrast and sharpness using Zerene Stacker (http://zerenesystems.com/stacker/).

Images of either ipsilaterally labeled VMN (Fig. 1C) or transneuronally colabeled hindbrain vocal pacemaker nuclei (VPN)-VMN circuit (Fig. 2A) were from previously unpublished images of an earlier study (Bass et al. 1994).

**GABA immunohistochemistry.** The hindbrain vocal motor network was transneuronally labeled via biocytin application to a single vocal nerve at the level of the swim bladder (methods directly adopted from Bass et al. 1994). After 4-5 days, animals were deeply anesthetized and perfused as described above except that the fixative was 4%
Paraformaldehyde-0.2% glutaraldehyde in PB. Brains were postfixed for 2 h at room temperature before storage in PB at 4°C. Brains were cryoprotected (30% sucrose PB) overnight at 4°C and then sectioned (50 µm, transverse plane) on a freezing microtome and immersed in blocking buffer [10% normal goat serum in 0.01 M PB saline (PBS) with 0.1% Triton X-100] for 4 h at room temperature. After blocking, sections were incubated with 1:50 monoclonal anti-GABA (GABA93) produced and characterized by Holstein et al. (2004) for use in closely related toadsfish. All primary and secondary antibody incubation steps were performed overnight at 4°C. After several washes in PBS, sections were preincubated in blocking buffer and then incubated with 4 µg/ml of a fluorescent-tagged goat anti-mouse secondary antibody (IgG-rhodamine red; Molecular Probes). After another series of PBS washes, sections were preincubated in a second blocking buffer (2% BSA and 0.1% gelatin in PBS with 0.1% Triton X-100) and then incubated with 5 µg/ml of a fluorescein-streptavidin conjugate (Vector Laboratories). Sections were washed in PBS, mounted onto gelatin-subbed slides, dried overnight, and coverslipped using a fluorescent mounting medium (Vecastash; Vector Laboratories). Photographs were taken on a fluorescence microscope, and image stacks on a confocal microscope.

Cluster analysis of motor nerve volley. Twelve different motor nerve volleys were used in a cluster analysis. Recordings were kindly provided by A. Berkowitiz from the University of Oklahoma (turtle hip flexor, knee flexor, knee extensor), S. Kishore from Northwestern University (zebrafish, spinal root), G. von Uckermann from Bordeaux University (Xenopus spinal nerve), C. Guschlbauer from University of Cologne (lamprey spinal root), and E. Zornik from the University of Utah (Xenopus glottal and laryngeal nerves). Vocal occipital nerve records from midshipman fish and toadsfish were acquired by us as detailed above; sea robin laryngeal nerves). Vocal occipital nerve records from midshipman fish and toadsfish were acquired by us as detailed above; sea robin laryngeal nerves). Vocal occipital nerve records from midshipman fish and toadsfish were acquired by us as detailed above; sea robin laryngeal nerves). 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in response to the combination of depolarizing current injection and synaptic input, whereas peak 2, which was coincident with the eVOC spike, represented the VMN network component (Fig. 4A, inset at middle trace shows magnification of a single response and corresponding vocal nerve activity, gray and black traces, respectively; gray arrow indicates second peak). Hyperpolarizing current injection during eVOCs revealed strong rhythmic activity (Fig. 4A, bottom trace) with a brief, fast-rising component coincident with the eVOC spike (Fig. 4A, inset at bottom trace, gray arrow), followed by a slower decaying component (black arrow).

A second body of evidence for a VMN network component came from a significant four- to fivefold increase in amplitude of the motoneuron afterhyperpolarization (AHP) during eVOCs (Fig. 4A, top trace, paired black arrows) compared with responses induced by positive current injection alone (Fig. 4A, top trace, gray arrow) \((n = 6 \text{ neurons}; \text{average AHP amplitude during eVOCs: } -5.07 \pm 2.28 \text{ mV}; \text{average AHP during current injection: } -1.19 \pm 0.82 \text{ mV;} \text{Mann-Whitney } U\text{-test: } P < 0.001\). This difference in AHP amplitude, despite similar depolarization levels, indicates an additional (extrinsic) contribution to the activity of the recorded motoneuron.

Further evidence of a network contribution to individual motoneuron responses during VOCs came from early action potentials occasionally generated by the recorded neuron that were not coincident with a VOC spike (Fig. 3C, red trace). Early, smaller amplitude action potentials did not show the characteristic AHP seen during responses coinciding with VOC spikes (Fig. 3C, red trace and inset), further suggesting that the prominent AHP coincident with a VOC spike was induced by the VMN network component. The amplitude of those spikes was significantly lower than the ones correlated with an eVOC spike \((n = 8 \text{ neurons}; \text{average amplitude of uncorrelated spikes: } 20.4 \pm 7.7 \text{ mV}; \text{average amplitude of correlated spikes: } 26.9 \pm 7.6 \text{ mV;} \text{Mann-Whitney } U\text{-test: } P < 0.001\). Support for VMN network activity during vocal responses also came from experiments that recorded intracellular motoneuronal activity \((n = 11\) using electrodes containing the lidocaine derivative QX314 \((100 \text{ mM in 2 M KCOOH})\) that intracellularly inactivates voltage-dependent sodium channels (Connors and Prince 1982). After in vivo intracellular QX314 injections, there was a significant decrease in amplitude of motoneuron responses during eVOCs (Fig. 4, B and C) and of antidromic action potentials (Fig. 4B, insets, and C) (Mann-Whitney U-tests: \(P = 0.001\)). Amplitude decreased to a similar extent for both sets of responses (Mann-Whitney U-tests: \(P > 0.624\)). Despite the decrease in amplitude, motoneurons still displayed rhythmic responses during eVOCs. To control for possible effects due to the current injection alone, we performed control experiments \((n = 9\) with only KCOOH-filled electrodes and applied current pulses as in the QX314 experiments. No significant effect was detected on the amplitude of either the motoneuronal activity during the eVOC or the antidromic action potential (Mann-Whitney U-test: \(P = 0.473\) and 0.970, respectively).

An added indication of a VMN network component came from a positive correlation between motoneuron \((n = 6\) response amplitude during eVOCs and eVOC spike amplitude (Fig. 5A, black and gray traces and arrows; linear regression in Fig. 5B: \(y = 0.58x + 39.77; R^2 = 0.64, P < 0.001\)). The variance in eVOC spike amplitude likely represented changes in the extent of coupling across the motoneuron population, as represented by the VMN network component in the recorded neuron’s activity.

Spontaneous vs. midbrain evoked network activity. Like eVOCs, sVOCs exhibited initial vocal activity superimposed on a membrane depolarization, whereas subsequent spiking activity was superimposed on a membrane hyperpolarization (Fig. 6A, top traces). A phase-plane plot (Fig. 6A, bottom; color-coded with top trace to show corresponding activity) showed highly
sustained oscillatory activity (blue spirals) after the first sVOC spike. At the onset of vocal activity, the membrane potential spiraled out (red spirals) from the baseline resting level (black).

A comparison between eVOC and sVOC activity recorded from the same neuron revealed no apparent effect of midbrain electrical activation on the overall temporal pattern of activity (Fig. 6B), as reflected in their similar firing frequency (n = 6 neurons, t-test: P = 0.977). As in eVOCs, motoneuron responses showed a fast rise time during sVOCs (Fig. 6B, right inset). Thus midbrain electrical stimulation did not distort natural hindbrain vocal motor activity. One difference in motoneuron activity between eVOCs and sVOCs was attributed to the effects of midbrain electrical activation. The summation of EPSPs during eVOCs was not present in sVOCs (Fig. 6B, left inset), reflecting the different time course and activation of hindbrain motor and premotor populations during eVOCs.

Recordings during sVOCs also provided support for a VMN network component. During sVOCs, the majority of motoneurons (17 of 23) consistently generated responses coincident with the first sVOC spike (Fig. 6C). As with eVOCs (see above), motoneurons (6 of 23) occasionally fired responses before the first one coincident with the first sVOC spike (Fig. 6D), again emphasizing similarities between the two VOC responses. On average, these responses were significantly lower in amplitude than responses coinciding with sVOC spikes (n = 6 neurons; average amplitude of uncorrelated spikes: 11.7 ± 4.2 mV; average amplitude of correlated spikes: 18.5 ± 9.4 mV; Mann-Whitney U-test: P = 0.003). Motoneuron activity preceding a sVOC spike was assumed to originate from EPSPs that reached action potential threshold in the recorded motoneuron but did not reach threshold for a large population of neurons in the VMN network, as evidenced by the missing sVOC spike in the VN record and either a weak or absent AHP in the motoneuron record (Fig. 6D). The apparent changes in potential amplitude in the VMN record while sVOCs were being recorded strongly suggested that the smaller amplitude potentials preceding a sVOC spike (see above) were the recorded motoneuron’s action potential, whereas the larger potentials coincident with the sVOC spike reflected the action potential of the recorded neuron and the summation of motoneuron action potentials across the VMN population, i.e., the VMN network component.

As in eVOCs, unsynchronized VMN responses were never observed during sVOCs, i.e., no motoneuron action potentials could be detected between nerve spikes, reflecting the strong coupling of motoneuron activation (synchronicity) during vocalization.

**Individual motoneuron responses to current injection.** Motoneurons (n = 15) responded phasically with one to seven successive action potentials (Fig. 7A), with most (n = 10) firing less than five, during intracellular injections of variable amplitude current steps (100-ms duration, Fig. 7, A and B). Action potential amplitude always decreased within a series of action potentials (Fig. 7A, top trace and inset). Both amplitude decrease and phasic firing of action potentials suggested motoneurons possessed slowly reactivating depolarizing conductances. To test the assumption that these conductances were a limiting factor determining vocal motoneuron activity (see above), we injected a pulse train of positive current during intracellular recordings (n = 7) (pulse duration 2 ms, 10–230 Hz; higher frequency stimuli were not used due to transients in current injection profile) to rapidly reactivate those conductances. Neurons fired repetitively without any sign of inactivation in response to each current pulse for up to 5 min (Fig. 7C). We concluded that slow reactivation of depolarizing conductances was likely a determinative factor in setting the periodicity of rhythmic motoneuron activity.

**Electrical coupling within and between motor nuclei.** Electron microscopy and extensive transneuronal neurobiotin/biotin transport imply gap junction coupling throughout the paired VMN (Bass and Marchaterre 1989; Bass et al. 1994). Consistent with this, numerous motoneurons were labeled following single intracellular neurobiotin injection (see Fig. 2D). We found physiological evidence of electrical coupling by using antidromic activation via the vocal nerve. With increasing antidromic stimulation amplitude, membrane depolarizations appeared and increased in amplitude, likely originating from electrotonically coupled motoneurons differentially activated during antidromic stimulation.
Membrane depolarizations increased until an action potential was elicited (Fig. 7D, red trace). The onset of action potential firing coincided with electrical stimulation and thus was not induced by membrane depolarizations reaching action potential threshold. In contrast to eVOC and sVOC activity, antidromically activated motoneurons did not show a distinct AHP after action potential firing, further suggesting a network-dependent activation of the AHP mediating conductance. Electrotone potential responses remained after action potentials fired (Fig. 7D, inset, red arrow). Electrotone coupling was also found after contralateral antidromic activation in 9 of 10 motoneurons, corroborating the exten-
sive coupling between the paired VMN observed with single-cell labeling.

Electrotonic coupling was further investigated in vivo with collision experiments, a physiological demonstration of gap junctional coupling (Kiehn and Tresch 2002), in which motoneuron action potentials were antidromically activated via the vocal nerve and intracellularly induced with current injection. Electrical coupling was evident in 12 of 13 motoneurons. Antidromically activated action potentials (Fig. 7E, small vertical arrows) were gradually shifted toward the current-induced action potential (Fig. 7E, horizontal arrow) until they disappeared following collision with the intracellularly generated action potential (Fig. 7E, red trace; expanded in inset).

Inhibitory input to vocal motoneurons. Inhibitory input contributes to simultaneous firing of neuronal populations (Gauck and Jaeger 2000; van Vreeswijk et al. 1994). To test the hypothesis that vocal motoneurons receive inhibitory input, we recorded from motoneurons using 3 M KCl-filled electrodes (n = 14). After control recordings, Cl− ions were injected intracellularly by applying negative current to the recording electrode; the resting membrane potential became slightly depolarized (2−6 mV), indicative of tonic inhibitory input. Prominent depolarizing potentials appeared after Cl− injection during either eVOCs (Fig. 8A, red middle trace; black trace is baseline; eVOCs not shown) or sVOCs (Fig. 8B, red trace; black trace is baseline) that could be inverted by positive current injection (Fig. 8A, top trace). Hyperpolarizing current injection following Cl− injections revealed double-peaked responses (Fig. 8A, bottom trace). No change in motoneuron firing frequency was detected after Cl− injections (n = 10 neurons; paired t-test: P = 0.24). A phase-plane plot revealed that motoneuron oscillatory activity remained highly stable after Cl− injection (Fig. 8C) despite the change in repolarizing levels during vocal activity (blue arrow). Prominent depolarizations, present at rest only after Cl− injection, revealed ongoing inhibitory input (Fig. 8D).
Inhibitory input is crucial for motoneuron activity. Supporting inhibitory input to VMN (see above), biocytin labeling of the motor nucleus via bulk labeling of the vocal nerve (Fig. 9A) coupled with immunocytochemistry demonstrated prominent GABAergic innervation throughout the motor nucleus (Fig. 9B and C), likely originating from small neurons located around VMN margins (Fig. 9D).

To further understand how inhibitory input contributes to motoneuron activity, we injected the GABA<sub>A</sub> receptor antagonist bicuculline in vivo at four positions along the rostrocaudal extent of VMN (see MATERIALS AND METHODS). Baseline eVOCs were reduced to one low-amplitude spike following bicuculline injections (Fig. 10A and B, top traces, respectively). Corresponding intracellular records (n = 5) showed equally reduced activity (Fig. 10A and B) and a tonic depolarization with a phasic component of smaller rapid membrane potentials at the sVOC frequency (insets in Fig. 10A and B, show superimposed baseline and postbicuculline responses on different timescales to allow better visualization of smaller potentials after bicuculline injections). Compared with baseline responses (Fig. 10C), bicuculline injections during sVOCs increased the rise time of the onset depolarization (Fig. 10D). Bicuculline did not always completely truncate sVOCs to one spike, although any additional VOC spikes had much lower amplitudes than the first (Fig. 10D, inset), likely reflecting decreased motoneuron recruitment (see below). As in eVOCs, a persistent depolarization coupled with a phasic component of smaller rapid membrane potentials at the sVOC frequency could be detected (Fig. 10D, red arrows). The sustained depolarization likely originated from feedforward vocal midbrain (e.g., see Fig. 3A) and duration-setting VPP excitation and the phasic depolarization from frequency-setting pacemaker neurons (Chagnaud et al. 2011).

Differential motoneuron recruitment does not affect network synchrony. Motoneurons differ in size across the VMN (Bass et al. 1996). To test the potential effect of differential recruitment of variably sized motoneurons on the stability and periodicity of motoneuron firing, we recorded from single motoneuron axons in one of the vocal occipital nerve roots as they exit the hindbrain. Unlike intracellular recordings from motoneuron somata and dendrites, axon activity does not reflect the potentially confounding effects of the VMN network on the contribution of individual motoneurons to the VOC spike amplitude. Motoneuron recruitment threshold was operationally defined as the lowest amplitude eVOC spike coinciding with an intracellularly recorded response (Fig. 11A). Because eVOC onset is influenced by electrical activation (Fig. 6B), the first eVOC spike and motoneuron potential were not included in the analysis (Fig. 11A).

Although recruitment threshold varied widely across the sampled population (n = 64) (Fig. 11B and C), each motoneuron reliably contributed to the eVOC once the eVOC spike amplitude exceeded the neuron’s recruitment threshold (Fig. 11B; VOC spike amplitude normalized to 100%).
Motoneurons were always recruited at the eVOC frequency with no apparent effect on the degree of synchrony (Fig. 11, A and B).

Because motoneurons continued to contribute to the VOC response once their recruitment threshold was reached, we anticipated that they were recruited in accordance with the size principle, namely, that smaller ones are recruited before larger ones (Henneman and Mendell 1981). In 12 fish, motoneuron somata were retrogradely labeled via neurobiotin injection into somata were heavily filled ipsilateral to nerve label, compared with contralateral transneuronally labeled VMN. B and C: same as A but with GABA antibody (red). White arrows in C indicate synaptic boutons apposing motoneuron somata (S). D: GABAergic neurons (white arrows) located next to VMN (unstained VMN somata indicated). Scale bars represent 200 (A) and 20 μm (B–D).

Fig. 9. Immunocytochemistry shows extensive GABAergic input to VMN. A: photomicrograph of transverse hindbrain section showing bilaterally labeled VMN (green) after biocytin application to VN. Vocal tract and VMN are heavily filled ipsilateral to nerve label, compared with contralateral transneuronally labeled VMN. B and C: same as A but with GABA antibody (red). White arrows in C indicate synaptic boutons apposing motoneuron somata (S). D: GABAergic neurons (white arrows) located next to VMN (unstained VMN somata indicated). Scale bars represent 200 (A) and 20 μm (B–D).

DISCUSSION

We have shown that a suite of premotor network and intrinsic motoneuronal properties ensures ongoing, high-fidelity transfer of afferent temporal information to an entire target population, in this case paired hindbrain motor nuclei dedicated to producing rapid modulations in acoustic behavior. Whereas pacemaker input provides a high-frequency timing signal that synchronizes electrotonic and inhibitory-dependent network level activity, intrinsic motoneuronal properties (low somatodendritic excitability, repolarizing conductance, membrane hyperpolarization) translate this temporal code into a highly synchronous, but variable amplitude, output code.

Intrinsic and network properties. Motoneuronal membrane hyperpolarization during vocal nerve activity (VOC) revealed strong rhythmic activity likely originating from premotor vocal pacemaker neurons that densely innervate VMN with chemical and electrotonic synapses (Bass and Baker 1990; Chagnaud et al. 2011). We propose that the motoneurons studied have two essential sets of active conductances with opposite effects. In the absence of vocal network activity, a slow-reactivating depolarizing conductance would account for the observed rapid decrease in action potential amplitude upon intracellular current injection (see Fig. 7A, inset). Upon network activation, a voltage-dependent repolarizing conductance is suggested by the prominent AHP coincident with vocal output, i.e., the VOC spikes (see Fig. 3C). The slow reactivation of the depolarizing conductance would essentially render the motoneuron inexcitable to sustained input, preventing spontaneous action potentials along with misfiring during vocal activity. Hence, the bilateral VMN population only fires at a time determined by the excitatory vocal pacemaker input (Chagnaud et al. 2011). The large depolarizing conductance allows for reactivation of the depolarizing conductance, but only during network activity. Sustained motoneuronal activity would then depend on the repetitive reactivation of these conductances at fixed intervals.

Several experiments support the dependency of highly stable, large-amplitude motoneuron responses during VOCs on VMN network activity. Current injection into single motoneurons led to action potentials/responses with AHPs much smaller in amplitude than those during VOCs. Responses occurring before VOCs were smaller in amplitude than those coincident with VOC spikes and did not show the characteristic AHP. Intracellular injection of the lidocaine derivative QX 314 significantly reduced the amplitude of, but could not abolish, rhythmic depolarizing activity, whereas motoneuron response amplitude directly correlated with eVOC spike amplitude. Further evidence of a VMN network component came from membrane potential modulations during variable levels of vocal output (Fig. 5). The extent of synchronous activation throughout VMN seen in the network component, measured by VOC spike amplitude, leads to predictable changes in the membrane potential of single motoneurons. Passive current spread throughout individual neurons is likely facilitated by the prominent size of motoneuron somata and dendrites (for quantitative measurements see Bass and Baker 1990; Bass et al. 1996).

Electrotonic coupling, known to increase the level of synchrony in neural networks (Velazquez and Carlen 2000), including motor circuitry (Zhang et al. 2009), could contribute to the extent of VMN network activity and, in turn, magnitude of the VN response. Collision tests of intracellularly evoked action potentials with antidromic activation from the vocal nerve revealed large membrane depolarizations indicative of a functional motoneuronal connectivity. This unequivocal demonstration of electrotonic coupling complements the anatomic evidence of gap junctions throughout VMN (Bass and Marchaterre 1989; Bass et al. 1994).

Selective inactivation of gap junctional coupling in VMN is not feasible in the in vivo preparation we studied because of extensive electrotonic coupling within and between vocal central pattern generator (CPG) nuclei (VPP–VPN–VMN). Consequently, we did not experimentally demonstrate the functional
role of electrotonic coupling in VMN. We hypothesize, however, that electrotonic spread of the VMN network component has a major role in generating extreme population-level motoneuron synchrony as displayed in the VOC. Electrotonic coupling throughout the paired motor nuclei is best illustrated physiologically by distinguishing a single motoneuron’s action potential from the network component (see Fig. 4) and membrane potential modulations during variable activity of the vocal network (see Fig. 5). The extent of VMN network synchrony, measured by VOC spike amplitude, leads to predictable changes in the membrane potential of single motoneurons (Fig. 5), likely mediated by passive current spread, that is facilitated by the prominent size of motoneuron somata and dendrites. Measures of coupling coefficients between motoneurons necessitate future in vitro recordings from pairs of motoneurons.

Dense GABAergic input to VMN, putatively from small neurons surrounding VMN (see Fig. 9D), likely serves two functions. First, it synchronizes VMN population activity, a feature well established for other synchronously firing neuronal networks (Bartos et al. 2007; van Vreeswijk et al. 1994; Wang and Buzsáki 1996). Second, it contributes to rapid motoneuron repolarization (see Fig. 10) that, along with a slow-reactivating conductance, prevents tonic firing (see above) and therefore maintains population-level motoneuron activity at the pacemaker frequency. Blocking GABA<sub>A</sub> receptors in VMN with bicuculline resulted in fewer motoneuronal and VOC spikes with remaining VOC spikes having decreased amplitude. GABAergic input is therefore likely crucial to repolarization of the motoneuron’s membrane potential and, thereby, maintenance of network level activity. It is not yet known whether the firing patterns of the inhibitory neurons are phasic or tonic with respect to the pacemaker frequency.

There was no apparent disruption of ongoing oscillatory frequency during either intracellular chloride or VMN bicuculline injection (Figs. 8 and 10), implying that inhibitory input does not play a role in setting firing frequency, which would then be controlled by excitatory pacemaker inputs alone (Bass and Baker 1990; Chagnaud et al. 2011). Inhibition’s major role, then, is to ensure that the large synchronous pacemaker depolarization is completely shunted throughout the soma/dendritic compartment, a mechanism that appears to be essential to continued motoneuron activity.

Differential recruitment does not affect synchronous firing. Vocal motoneuron size varies in midshipman fish (Bass et al. 1996). The size principle predicts that smaller motoneurons with higher input resistance tend to depolarize more for comparable excitatory synaptic currents, and thus fire earlier, than larger ones with lower resistance (Henneman and Mendell 1981). Individual motoneurons were differentially recruited (Fig. 11); smaller neurons reliably contributed to VOC amplitude before larger ones, leading to a net increase in the number of active neurons and VOC amplitude. Motoneuron activity remained highly synchronous and temporally stable during

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**Fig. 10.** GABAergic input to VMN is crucial for vocal activity. A and B: superimposed traces of VN (top) and VMN activity (bottom) during eVOC before (A; blue) and after (B; red) extracellular bicuculline injection into VMN (small black arrowheads indicate stimulus artifact). Inset in A shows higher magnification of single baseline and bicuculline injected records (responses truncated). Note the lack of repolarization after bicuculline injection highlighted by gray shading. Inset in B shows inset in A on expanded timescale but reduced amplitude scale displaying 2 EPSPs (red trace and arrows) riding on top of a membrane depolarization. C and D: same as A and B but for sVOC. Red arrows indicate membrane depolarizations at the sVOC frequency riding on top of membrane depolarization. Insets in C and D show increased resolution of VN record, and black arrows in D indicate the decreasing VOC spike amplitudes recorded from the VN.
Motoneuron synchrony plays a determinative role in natural call AM. Although intrinsic and network properties ensure a reliable encoding of pacemaker network features, rapid temporal modulation of acoustic signals is necessary for speed. For acoustic signaling, speed translates into rapid temporal modulation of the waveform of the signal itself. Superfast muscles, like those of the rattlesnake tailshaker and bat larynx, have spike-like electromyographic records and individual spikes. However, the available data suggest that motoneuron synchrony is a prerequisite for precise temporal modulation of acoustic signals in either the high-gamma (e.g., vocal fish, anurans) or beta-gamma (e.g., frogs) frequency range. Anurans differ, however, from vocal fish and the superfast acoustic systems of bats and rattlesnakes in that motor activity is only in the beta-gamma range, and so they likely do not have superfast vocal muscles.

Unlike vocal fish, laryngeal nerve activity in Xenopus laevis, a fully aquatic anuran amphibian, exhibits a temporally stable pulsatile motor volley (Fig. 12B, top record) coupled one to one with spike-like electromyographic records and individual sound pulses. Anurans differ, however, from vocal fish and the superfast acoustic systems of bats and rattlesnakes in that motor activity is only in the beta-gamma range, and so they likely do not have superfast vocal muscles.

Nerve recordings of motor volleys to nonsuperfast muscles used in locomotion, e.g., zebrafish axial and turtle knee flexor muscles (Fig. 12B, middle and bottom records), show low temporal coincidence, reflecting the noncoincident recruitment of motor units. Anurans differ, however, from vocal fish and the superfast acoustic systems of bats and rattlesnakes in that motor activity is only in the beta-gamma range, and so they likely do not have superfast vocal muscles.

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Both the sVOC and eVOC are reliable representations of natural vocalizations in the time domain and exhibit varying degrees of amplitude modulation (AM) like that of natural calls (e.g., Fig. 1A) (Rubow and Bass 2009). Whereas intrinsic and network properties ensure a reliable encoding of pacemaker frequency by the entire VMN population, motoneuron recruitment likely plays a determinative role in natural call AM. Rapid temporal modulation of acoustic signals. Superfast muscles, unlike other skeletal muscles used for locomotion, are adapted for speed rather than force generation. For acoustic signaling, speed translates into rapid temporal modulation, whether determining the rate of amplitude modulation of a fish grunt (e.g., Fig. 1A) and songbird syllable (Elemans et al. 2008), the interpulse interval of the terminal buzz of an echolocating bat (Elemans et al. 2011), or the rattling frequency of a rattlesnake tailshaker (Schaeffer et al. 1996).

Vocal fish motor volleys reflect highly stable, synchronous motoneuron output, as shown in Fig. 12A for the closely related plainfin midshipman (focus of current study), Gulf toadfish, Opsanus beta (same order, Batrachoidiformes) (Nelson 2006), and the distantly related sea robin, Prionotus carolinus (Scorpaeniformes) (Nelson 2006). Extreme motoneuronal synchrony leads to synchronous activation of muscle fibers across the entire sonic/vocal muscle (Gainer and Klancher 1965; Skoglund 1961). Unfortunately, there are no nerve records available for other known superfast muscles, namely, those of the rattlesnake tailshaker and bat larynx. However, the spike-like electromyographic records of tailshaker (Schaeffer et al. 1996) and bat laryngeal muscles (Durrant 1988) mimic the spike-like vocal nerve records of fish (although lower in frequency) and thus strongly suggest a high degree of motoneuron synchrony.
Fig. 12. Superfast muscle motor command signals (nerve volley) differ from nonsuperfast commands. A and B: motor volley (left, representative motor volley; right, 10-ms excerpt highlighting signal waveform) of superfast vocal muscles (A; midshipman, toadfish, and sea robin) and nonsuperfast muscles (B; frog laryngeal, zebrafish, and turtle) used in the farthest point clustering analysis. C: dendrogram showing the clustering of motor signals into 2 main groups showing clear separation between command signals to superfast muscles (green) and to nonsuperfast muscles (red). The frog laryngeal motor volley (indicated in blue) groups within the nonsuperfast muscles but is distinctly separated from other muscles, reflecting its intermediate character state (also see B). D: shown are 2 of 10 factors used in the cluster analysis (see MATERIALS AND METHODS), interspike interval (ISI) width (left y-axis; black filled circles) and root mean square (RMS) of the signal (right y-axis; orange filled circles). Green (superfast muscles) and red (nonsuperfast muscles) circles indicate the 2 classes found in the clustering analysis. Blue circle indicates the frog laryngeal muscle within the nonsuperfast class. X-axis represents the motor volleys used in the cluster analysis shown in C.
range of motoneuron firing coadapted with the rapid contractile properties of superfast sonic muscles. Motoneuronal intrinsic and network properties like those distinguished in this study for vocal fish are, however, likely typical of any motor system where there is intense selection for precise temporal modulation of behavioral events, exemplified by acoustic communication signals.

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