Bilateral coordination of vocal pathways in African clawed frogs, *Xenopus laevis*

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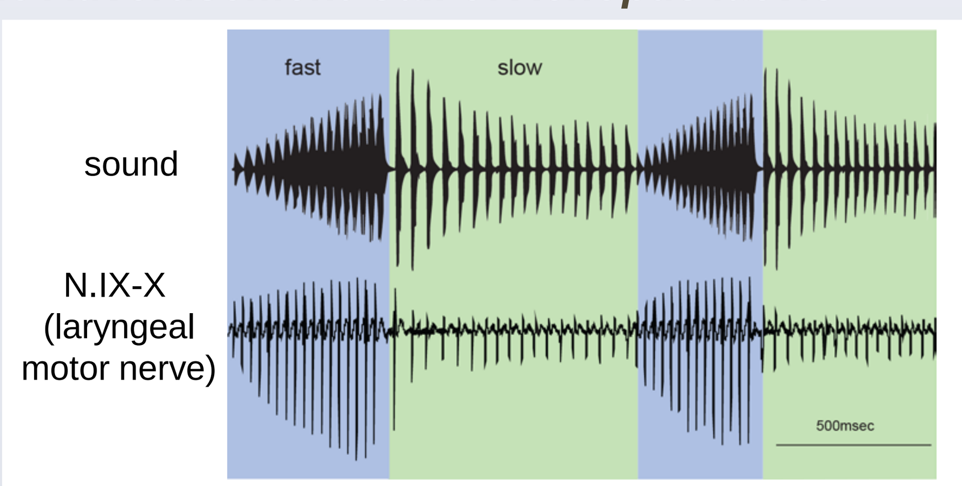
ABSTRACT

Vocalizations of the *Xenopus* are well-suited model system to understand neural basis of behavior because fictive vocalizations can be studied *in vitro*. The central vocal pathways that consist of pairs of premotor (dorsal tegmental area of medulla, DTAM) and motor (n.IX-X) nuclei generate rhythmic motor outputs which in turn contract a pair of laryngeal muscles rhythmically to produce a series of click sounds. Because *Xenopus* sound production mechanisms require simultaneous contraction of the paired muscles, the motor outputs need to be bilaterally synchronous. Here, we explored how bilateral coordination is achieved by the central vocal pathways of *Xenopus*.

Transection experiments revealed that two vocal phases (fast and slow trills) are generated by separate neural mechanisms with distinct regulation of synchronicity. Fast trills rhythms are generated by a neural circuit that includes DTAM and n.IX-X. Bilateral synchrony during fast trills seems to be mediated largely by ascending projections from the n.IX-X to DTAM, and to a lesser extent by the bilateral connections between the DTAMs. Slow trill rhythms, in contrast, seem to be generated by neurons contained in n.IX-X, and bilaterally synchrony is coordinated via projections between the two nuclei. A few synaptic connectivity that may be responsible for the vocal rhythm generation and bilateral coordination were revealed by stimulating experiments.

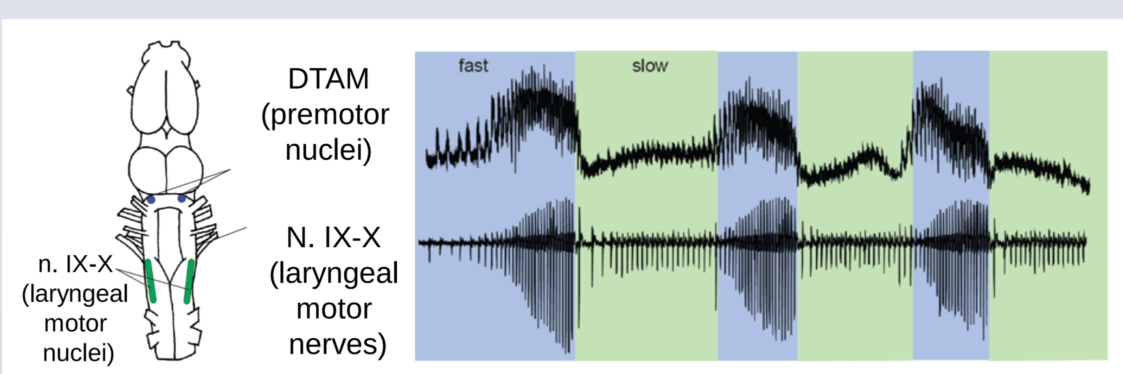
BACKGROUND

The Advertisement Call of *Xenopus laevis*



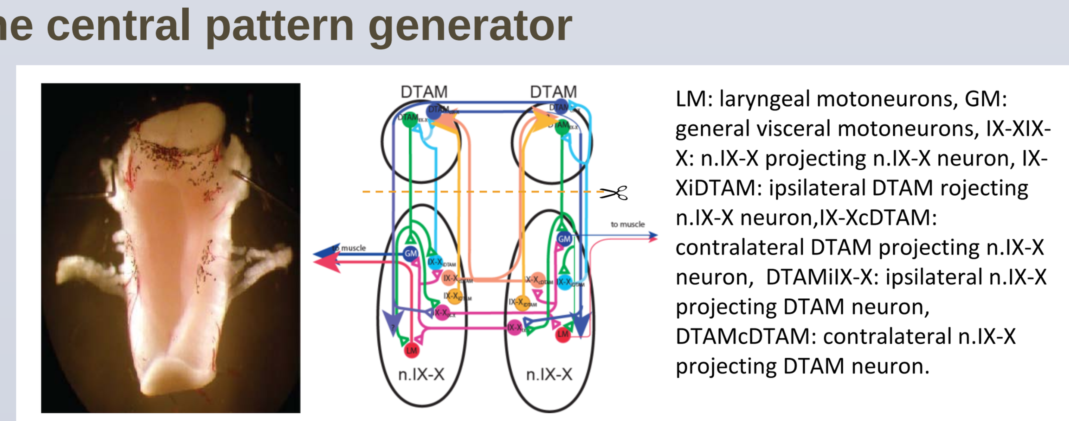
Advertisement calls of male Xenopus consist of repetitive clicks that alternate between fast (70Hz) and slow (30Hz) rates (top trace, sound). The temporal organizations of the call are dictated entirely by motor nerve activity (bottom trace, motor nerve) [1].

Neuronal control of the advertisement call



Motor command is generated by the central pattern generator in the brainstem that consists of the dorsal tegmental area of medulla (DTAM, premotor nucleus, shown in blue) and laryngeal motor nucleus (n.IX-X, shown in green)[2].

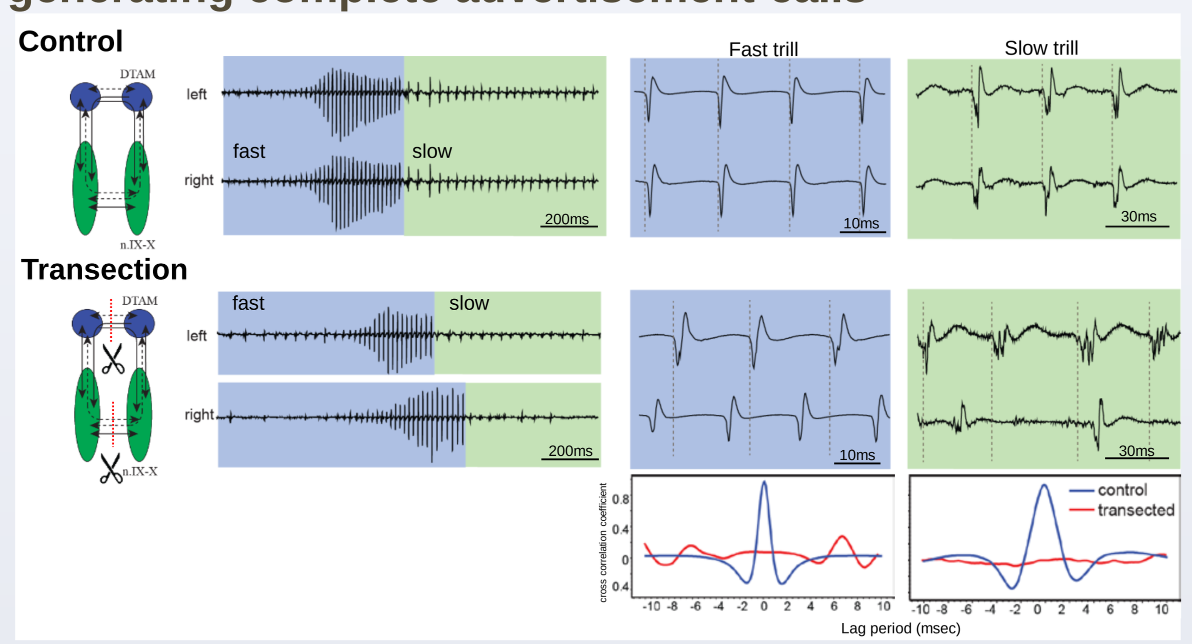
The central pattern generator



Fictive vocalizations can be elicited from an isolated brainstem *in vitro* (left) when serotonin is applied[2]. There are extensive reciprocal connections, including bilateral, descending, and ascending projections, among the DTAMs and n.IX-Xs as shown in the figure above (right)[3]. Ascending/descending connections between DTAM and n.IX-X are critical for the CPG such that bilateral transections of the projections (right, dotted line) abolish fictive vocalizations entirely.

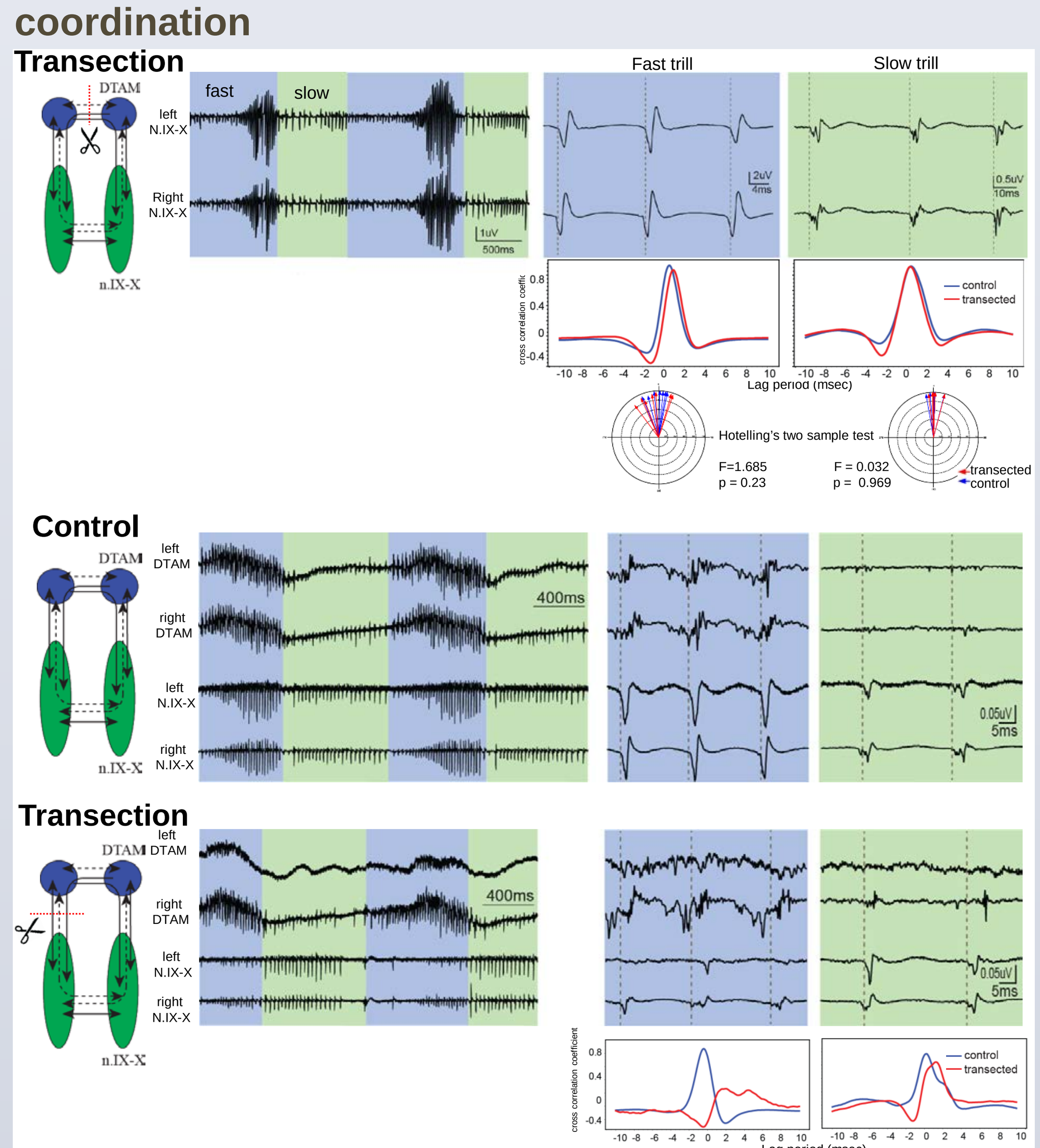
RESULTS

1. Hemi-brain containing a DTAM and a n.IX-X with intact ascending/descending projections is sufficient in generating complete advertisement calls



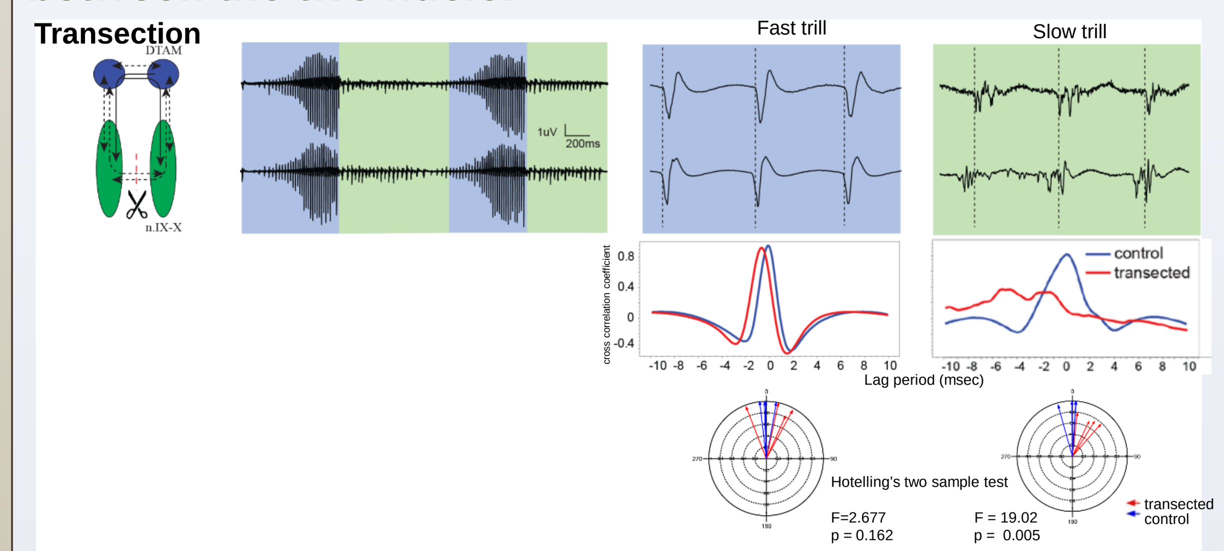
When the connections between left and right DTAMs and left and right n.IX-X are transected, each hemi brainstem generated both fast and slow trills, although the rhythms generated by each half are autonomous, as evident in the low cross correlation coefficient between left and right nerve traces during both fast and slow trills.

2. Fast trill rhythms are generated by the neural circuit including both DTAM and n.IX-X; ascending projections from the n.IX-X seem to mediate bilateral coordination



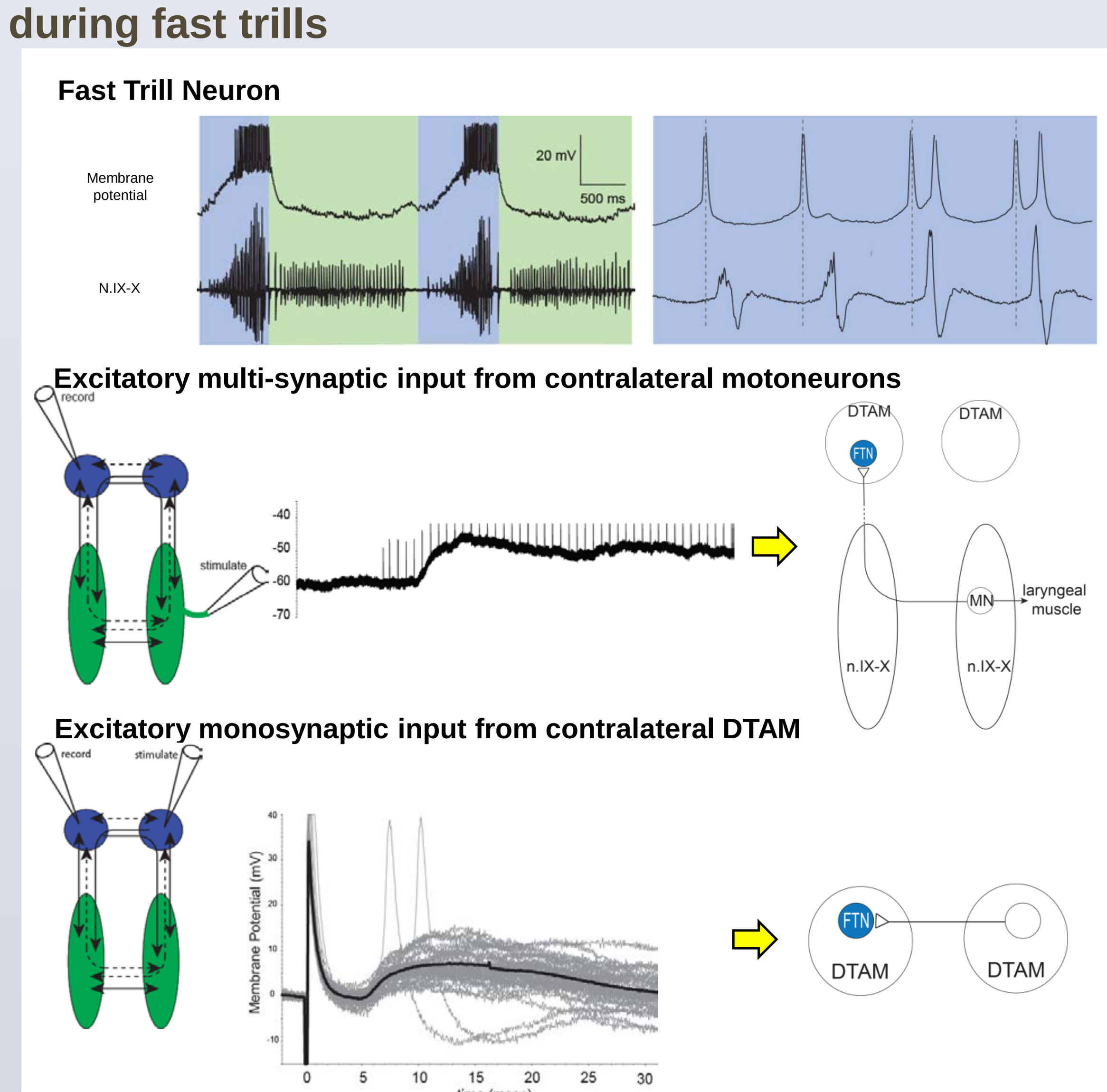
Transections between the two DTAMs had little effect on the temporal organization of the advertisement calls, as evident in cross correlation coefficients and circular statistics (top). However, when the connection between DTAM and n.IX-X is transected unilaterally, fast trills deteriorated drastically (desynchronized CAPs with reduced amplitude and shorter trill duration) while slow trills remained relatively intact. Field potential recordings obtained from transected side of DTAM also showed reduced activity. The difference between the two transection experiments is the ascending inputs received by DTAM from n.IX-X. In the first experiment, both DTAMs receive direct inputs from both n.IX-X, whereas in the second experiment, the transected side of DTAM receives no ascending inputs from either n.IX-X. The results suggest that n.IX-X sends synchronizing inputs to DTAMs that are critical for generating fast trills, but not for slow trills. Comparing the results of the second experiment and those of the hemi-brain experiment (top), we suggest that the transected side of DTAM became dysfunctional and projected its deteriorated activity to the functional DTAM, which in turn disrupted the entire fast-trill generating mechanisms.

3. Slow trills are generated by the n.IX-X, and their activities are coordinated bilaterally by the projections between the two nuclei



Transection between n.IX-X deteriorates the bilateral synchrony between right and left motor nerves during the slow trills, but not during the fast trills. Because both n.IX-X receive direct inputs from ipsi- and contralateral DTAMs in the transected configuration, it is unlikely that the slow trill rhythms are generated by the DTAMs and passed onto n.IX-X. Rather, the slow trill rhythms are generated by the n.IX-X with its activity coordinated via the projection between the two nuclei. Descending inputs from DTAMs to n.IX-X are likely to play a role in initiating the slow trills, as evident in our inability to elicit slow trills when DTAMs are dissociated from n.IX-X bilaterally.

4. Synaptic inputs received by DTAM fast trill neurons (FTNs) from contralateral DTAM and n.IX-X may play an important role in coordinating bilateral synchrony during fast trills



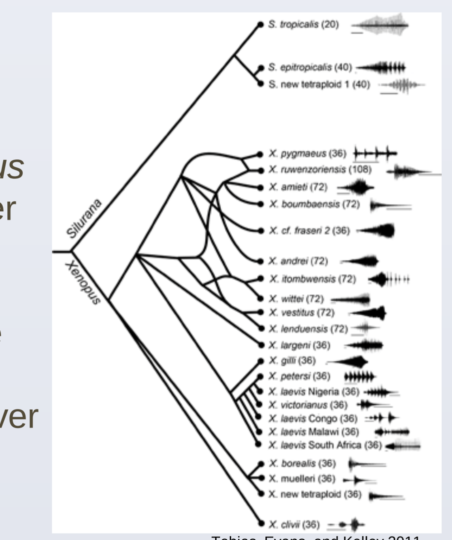
Fast trill neurons (FTNs) in DTAM show NMDAR-dependent long-lasting depolarization during the fast trills, and spike 2-3msec prior to the motor nerve compound action potentials[4]. FTNs project to n.IX-X as evident in antidromic spikes observed in response to n.IX-X stimulation[4]. Based on these firing and projection patterns, FTNs are considered to drive motoneurons during fast trills.

When contralateral laryngeal motor nerve is stimulated with repetitive electrical pulses while whole-cell patch-clamp recordings were obtained from the fast trill neurons, gradual depolarization of the membrane potential were sometimes observed. The depolarization are likely caused by multi-synaptic input from the contralateral population of laryngeal motoneurons. The depolarization did not depend on NMDAR activation, for application of APV had no effect (data not shown). These multi-synaptic inputs ascending from the n.IX-X may enhance the excitability of the FTNs, even though they may not regulate real-time rhythm generation.

In respones to contralateral DTAM stimulation, monosynaptic EPSPs were sometimes observed. These inputs may regulate bilateral coordination of motor activity during fast trill generation.

CONCLUSIONS

- A male Xenopus hemibrain that contains a DTAM and n.IX-X connected by rostral-caudal projections are sufficient to generate complete advertisement calls.
- Two distinct vocal phases of male Xenopus appear to be generated by distinct neural mechanisms, and bilateral coordination is achieved differently.
- Fast trill rhythms are generated by the network of neurons in DTAM and n.IX-X with critical roles played by the ascending and descending projections between DTAM and n.IX-X. Bilateral coordination seems to be achieved, partly by the synchronous ascending inputs projected from n.IX-X to DTAM, and partly by the connections between the two DTAMs including the excitatory synaptic inputs.
- Slow trill rhythms are generated by the neurons contained within n.IX-X, although the initiation of the fast trill phase relies on the descending inputs from the DTAM. Bilateral synchrony during slow trills is achieved via neural connections between the two n.IX-X.
- Evolutionarily, biphasic vocalizations of *Xenopus laevis* are more "derived" trait within the genus *Xenopus*. Many species of *Xenopus* produce monophasic calls that resemble either fast trills or slow trills of *X. laevis* [5]. Understanding how neural circuits of X. laevis accommodate two vocal rhythms may provide us with an insight into the neural mechanisms underlying the acquisition of new behaviors over evolutionary time.



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METHODS

For all the experiments, male X. laevis were anaesthetized with 1.3% MS222, and brains were isolated into ice-cold saline oxygenated with 99% O_2. Fictive vocalizations were recorded from the most caudal root of cranial nerve IX-X (N.IX-X) via suction electrodes placed over each nerve. Local field potentials in DTAM were obtained using 1M ohm tungsten electrode. The same electrode was used to stimulate DTAM for some experiments. Whole-cell patch-clamp recordings were obtained using patch pipettes fabricated from thick-walled (1.5mm od, 0.86mm id) borosilicate capillaries. Electrode resistance were between 6 and 10M ohm. To evaluate the bilateral synchrony, two methods were used. First, cross correlation coefficients were calculated based on left and right nerve traces obtained during fast and slow trills (10 CAPs from each fast and slow trills, 10 songs from each animal were sampled). Second, onset timing of left and right nerve CAPs were used to carry out circular phase analysis. Repetitive CAP activity recorded from one nerve was used to calculate circular phase at which the CAP was activated on the other nerve; synchronous activity will result in mean vector direction of 0°. Based on the vector length and angle, Hotelling's two sample test was used to determine if transection resulted in significant change in the degree of bilateral synchrony.

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