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Temperature-Dependent Regulation of Vocal Pattern Generator

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Yamaguchi A, Gooler D, Herrold A, Patel S, Pong WW. Temperature-dependent regulation of vocal pattern generator. *J Neurophysiol* 100: 3134–3143, 2008. First published October 1, 2008; doi:10.1152/jn.01309.2007. Vocalizations of *Xenopus laevis* are generated by central pattern generators (CPGs). The advertisement call of male *X. laevis* is a complex biphasic motor rhythm consisting of fast and slow trills (a train of clicks). We found that the trill rate of these advertisement calls is sensitive to temperature and that this rate modification of the vocal rhythms originates in the central pattern generators. In vivo the rates of fast and slow trills increased linearly with an increase in temperature. In vitro a similar linear relation between temperature and compound action potential frequency in the laryngeal nerve was found when fictive advertisement calls were evoked in the isolated brain. Temperature did not limit the contractile properties of laryngeal muscles within the frequency range of vocalizations. We next took advantage of the temperature sensitivity of the vocal CPG in vitro to localize the source of the vocal rhythms. We focused on the dorsal tegmental area of the medulla (DTAM), a brain stem nucleus that is essential for vocal production. We found that bilateral cooling of DTAM reduced both fast and slow trill rates. Thus we conclude that DTAM is a source of biphasic vocal rhythms.

INTRODUCTION

Motor patterns expressed by animals are often rhythmic. In many systems, these motor patterns are controlled by a central pattern generator (CPG), a network of neurons that drives motoneurons to spike rhythmically in the absence of sensory feedback. Motor rhythms generated by the CPG depend both on the intrinsic membrane properties of constituent neurons and their synaptic interactions (reviewed by Getting 1989; Marder and Bucher 2007; Marder and Calabrese 1996; Metzner 1999; Ramirez and Viemari 2005; Roberts et al. 1998; Rose 2004). Changes in these cellular and synaptic properties within the CPG network can modify resulting motor rhythms (reviewed by Marder and Bucher 2007; Simmers et al. 1995). Although the identification of interneurons that form the CPG are the critical first steps toward understanding cellular and synaptic bases of rhythm generation, localizing CPG is often difficult because of the complex structure of the CNS (reviewed by Marder and Calabrese 1996). In this study, we attempted to localize the CPG responsible for generating vocal rhythms in African clawed frogs (*Xenopus laevis*) by exploiting the temperature dependence of a vocal pattern generator.

Rhythmic motor patterns of ectotherms typically operate at reduced rates at lower temperatures due to a slowing of their underlying CPG [e.g., locomotion of *Tritonia* (Katz et al. 2004), heart rate of leech (Arbas and Calabrese 1984), communication signals of electric fish (Dunlap et al. 2000; Feng 1976), crickets (Pires and Hoy 1992a,b), grasshoppers (Bauer and von Helversen 1987), frogs (Gerhardt 1978; Yager 1992b)]. These temperature effects have been shown to be due to a slowing of processes at the level of the CPG rather than at the level of the motoneurons or muscles themselves (e.g., Bauer and von Helversen 1987). For example, in a simple rhythmic motor system where a pacemaker neuron serves as a source of the rhythm, the temperature-induced reduction in spike rate of the pacemaker neurons reduces the overall rate of the motor rhythms, but temperature-induced synaptic delay at the level of motoneurons does not. Thus local changes in temperature of the CPG network can alter the motor patterns under its control. This feature provides a useful tool for localizing a CPG network to a particular brain region.

Vocalizations of African clawed frogs, *X. laevis*, are generated by CPGs somewhere in the brain stem (Rhodes et al. 2007). The most common male vocalization, the advertisement call, is a complex biphasic motor rhythm that consists of fast trills followed by slow trills, each containing trains of clicks repeated at fast (~70 Hz) and slow (~30 Hz) rates, respectively (Fig. 1*A*). Neuronal coding of the vocalization is very simple in *X. laevis*: a train of compound action potentials generated by a population of laryngeal motoneurons is faithfully transduced into a series of clicks by a pair of laryngeal muscles (Tobias and Kelley 1987; Yager 1992a; Yamaguchi and Kelley 2000). Thus nerve recordings obtained from the laryngeal nerve serve as an accurate representation of the vocal behavior. Recently we developed an in vitro, whole brain preparation that generates fictive vocalizations in which patterns of nerve activity closely match those recorded from awake, calling animals (Rhodes et al. 2007). Simple neuronal coding of the vocal behavior, together with the development of a preparation where fictive vocalizations can be monitored, make *X. laevis* vocalization an ideal system for the study of pattern generation in vertebrates.

The exact location of the vocal CPG that generates vocal rhythms in *X. laevis* is unclear. Moreover, whether the two rhythms (fast and slow trills) are generated by a single network of neurons that produces two sequential rhythms or by two separate networks is also not known. One of the major candidates for the *X. laevis* vocal CPG is the network of premotor neurons in the dorsal tegmental area of the medulla (DTAM) (Brahic and Kelley 2003; Wetzel et al. 1985; Zornik and Kelley 2007). DTAM plays an essential role in generating advertisement calls: transecting the brain to disconnect DTAM from the rest of the brain stem abolishes fictive vocalizations (Rhodes et al. 2007). Furthermore, patterned electrical stimu-

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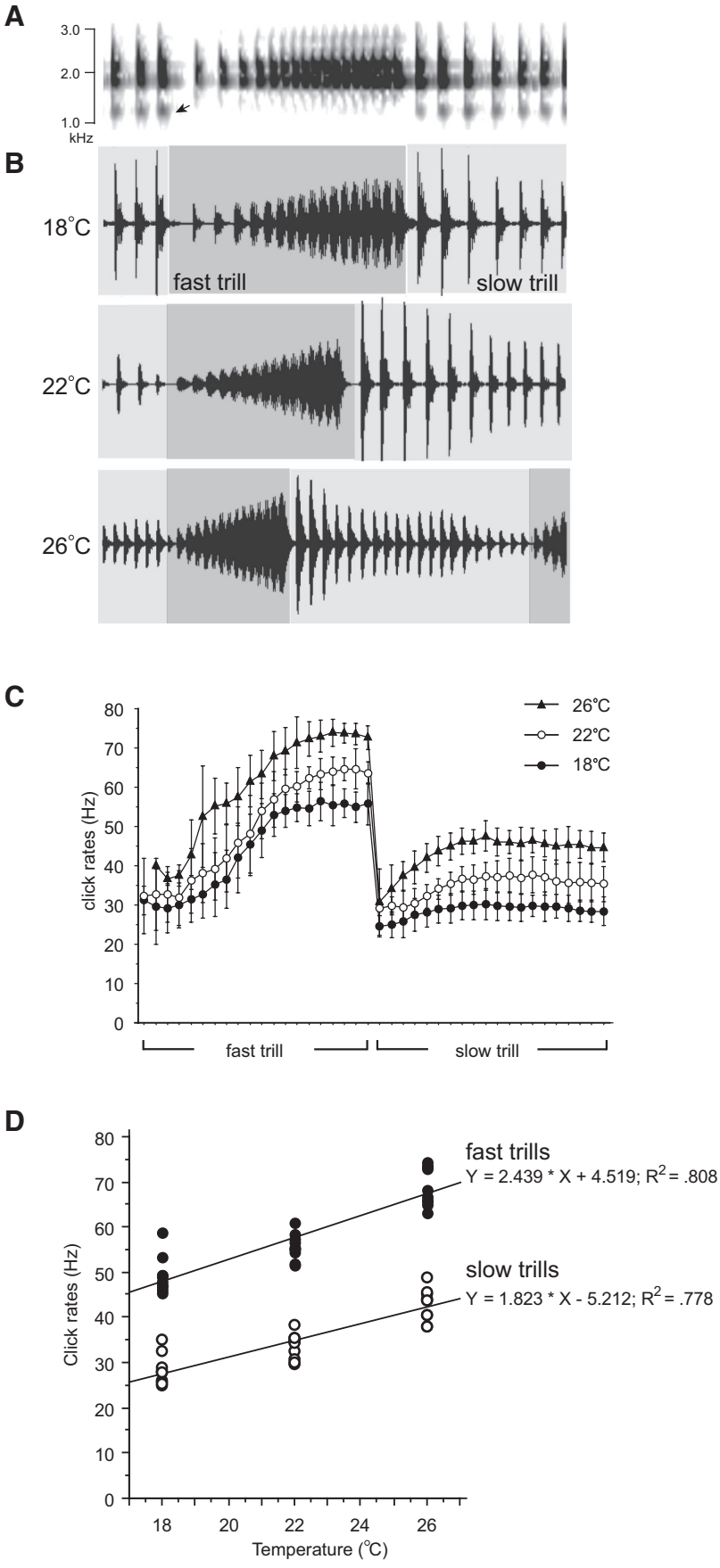

FIG. 1. Temperature-dependent changes in advertisement calls of male *Xenopus laevis*. *A*: sound spectrogram (sound frequency over time) of a male advertisement call recorded at 18°C. Note that low-frequency components of clicks (indicated by ←) are present only in slow trills. *B*: sound waveforms of male advertisement calls produced at 3 different temperatures. Dark and light gray boxes surrounding the waveform indicate fast and slow trills, respectively. *Top*: the waveform of the sound spectrogram in *A*. Click repetition rates increase as the ambient temperature increases. *C*: mean instantaneous click rates of 21 consecutive clicks preceding and following the transition between fast and slow trills recorded at 3 different temperatures for all 10 males. ▲, ●, ○, mean values; error bars, SD. *D*: regression plots of mean instantaneous click rates for slow and fast trills as a function of temperature. ● and ○ mean fast and slow trill rates (average of all 20 intervals before and after the trill transition), respectively, for each individual at each temperature.

lation of DTAM selectively activates fast trills but not slow trills (Rhodes et al. 2007). This latter result led us to hypothesize that neurons in DTAM generate fast trills and that fast and slow trills are controlled by separate networks of neurons.

Here, we first demonstrate the temperature sensitivity of advertisement calls in *X. laevis*. We found that click rates of the advertisement calls are dictated by the ambient temperature as in the closely related species, *X. borealis* (Yager 1992b). We next show that the fictive vocal rhythms generated in isolated brains are also dictated by the ambient temperature but are not dependent on the contractile properties of laryngeal muscles. Thus the temperature-dependent change in click rates derives from a direct effect on the CPG. Finally, we examine the role of DTAM in rhythm generation by selectively and bilaterally cooling the area using cryoprobes. We found that lowering the temperature of DTAM not only decreased the rate of the fast trills but also of the slow trills. We conclude that neurons in DTAM control both rhythms of the male advertisement call in *X. laevis*.

METHODS

Animals

Forty sexually mature male *Xenopus laevis* (6.9 ± 0.1 cm, 37.0 ± 1.9g) purchased from NASCO (Fort Atkinson, WI) were used. They were kept in glass aquaria on a 12/12h light/dark cycle at room temperature. All experimental procedures were approved by the Boston University Institutional Animal Care and Use Committee and were performed in compliance with guidelines published by the National Institutes of Health.

Vocal recordings

Twenty adult males were injected with 600–1,000 IU of human chorionic gonadotrophin (Sigma, St. Louis, MO) subcutaneously to induce advertisement calling (Wetzel and Kelley 1983). The vocalizations were recorded over a 24-h period with a hydrophone (H2; Aquarian Audio Products, Anacortes, WA) suspended in the water using a sound-activated recording system (Syrinx software, www.syrinxpc.com; John Burt). Ten males were used to obtain vocal recordings at 26 and 18°C, and 10 additional males were used to obtain recordings at 22°C. For 26 and 18°C recordings, each male was placed in a 38-l aquarium. The warm temperature (26°C) was maintained using an aquarium heater (VISI Therm type VTH 1000 heater, Aquarium Systems, Mentor, OH), and the cool temperature was maintained by placing an aquarium in a cold room set at 18°C. For these two extreme temperatures, the subject was paired with a stimulus female because the presence of a female is known to promote advertisement calling in males. The order of exposure to different temperatures did not modify the call structure (data not shown). The remaining 10 males were recorded alone in 12-l aquaria at 22°C; we knew from prior experience that male *X. laevis* readily produce advertisement calls at room temperature without stimulus females.

Laryngeal muscle physiology

Six male larynxes were used. The frogs were anesthetized with MS-222 (~7 mg, Sigma) injected subcutaneously. The larynx was isolated and pinned to a petri dish coated with silicone elastomer (Sylgard, Dow Corning, Midland, MI). Laryngeal nerves and muscles were exposed by removing connective tissues and cartilage. The laryngeal nerve was stimulated with a suction electrode. Trains of stimulus pulses (1-ms duration repeated at frequencies ranging from 10 to 100 Hz in 10-Hz increment, 200 mV) were generated using Chart software (AD Instruments, Colorado Springs, CO), and delivered to the electrode via a MacLab/4e D/A converter (AD Instruments) running on a PC. Semi-isotonic tension caused by the nerve shock was recorded using a force transducer (Model 1030, UFI, Morro Bay, CA; frequency response ≤500 Hz) attached to the tendon of the muscle. Force outputs were amplified using BridgeAmp (AD Instruments) and digitized using a MacLab/4e A/D converter with a sampling rate of 1 kHz. Force generated by the laryngeal muscles was recorded at three different temperatures, 18, 22, and 26°C (±0.5°C), and the bath temperature was monitored continuously using a digital thermometer.

In vitro nerve recordings of serotonin (5-HT) induced fictive vocalizations

Fourteen males were used to obtain 5-HT-induced fictive vocalizations via the laryngeal nerve in vitro. Frogs were anesthetized as described in the preceding text and decapitated. The brain was removed from the skull in oxygenated ice-cold saline (containing in mM: 96 NaCl, 20 $NaHCO_3$, 2 $CaCl_2$, 2 KCl, 0.5 $MgCl_2$, 10 HEPES, and 11 glucose, pH 7.8), transferred to a recording chamber, and continually superfused with fresh oxygenated saline at room temperature for 1 h prior to the experiment.

The fourth rootlet of the nerve IX-X contains all the axons of the laryngeal motoneurons (Simpson et al. 1986). A suction electrode was placed onto the nerve rootlet to record the compound action potential of the laryngeal motoneurons (Fig. 2*A*). The signal was amplified with a differential amplifier (Model 1700, A-M Systems, Carlsborg, WA), high-pass filtered at 1 Hz, digitized at 10 kHz (Digidata 1322A, Molecular Devices, Sunnyvale, CA), and recorded on a PC using AxoScope software (Molecular Devices).

Fictive vocalizations were induced by bath application of 5-HT. The temperature of the recording chamber was monitored using a thermocouple microprobe (IT-24P, Clifton, NJ, 250 μm OD) placed within 2 mm of laryngeal motor nucleus with its output connected to a Physitemp digital thermometer (BAT-12, 0.1°C resolution). When the temperature had stabilized at a predetermined value for ≥2 min, superfusion of saline was suspended, and 10 ml of 5-HT stock solution (60 μM) was added to the bath to achieve a final concentration of 30 μM. The temperature of the recording chamber was then maintained for the following 2–5 min by manually adding warmer (>26°C) or cooler (<18°C) saline with 30 μM 5-HT ad libitum. After the 5-HT treatment period, saline superfusion was reinstated at room temperature at a high rate (20 ml/min) for 5 min to completely exchange the solution in the recording chamber. Because most preparations can be induced to produce fictive vocalizations multiple times in response to repeated 5-HT application (Rhodes et al. 2007), we recorded fictive advertisement calls at more than one temperature from most preparations (fictive calls from 5 brains were recorded at all 3 temperatures, those from 7 brains were recorded at 2 lower temperatures, and those from 2 brains were recorded at either 26 or 18°C).

In vitro cooling of DTAM

DTAM was cooled bilaterally by a cryoprobe to evaluate effects of local cooling on the temporal pattern of fictive vocalizations. A cryoprobe was fabricated using an inverted U-shaped silver wire (250 μm) etched to 10 μm in diameter at the tip, soldered to a tungsten tubing that is a part of a closed-circuit loop containing ethanol circulated by a peristaltic pump (Fig. 2*B*). Ethanol within the loop was cooled by passing it through a copper coil surrounded by dry ice (Fig. 2*B*). The temperature of the cryoprobe was adjusted by controlling the speed of the peristaltic pump.

The exact temperature at the tip of the cryoprobe could not be measured during the experiments because of the minute size of the cryoprobe tips (10 μm) together with the relatively large size (250 μm) of the thermocouple probe used to monitor temperature. However, the cryoprobe was calibrated using the thermocouple probe and a digital thermometer (Omega Engineering) so that the estimated tip temperature during the experiments was between 18 and 15°C. The extent of the cooling effect (i.e., distance from the probe where cooling effect could be

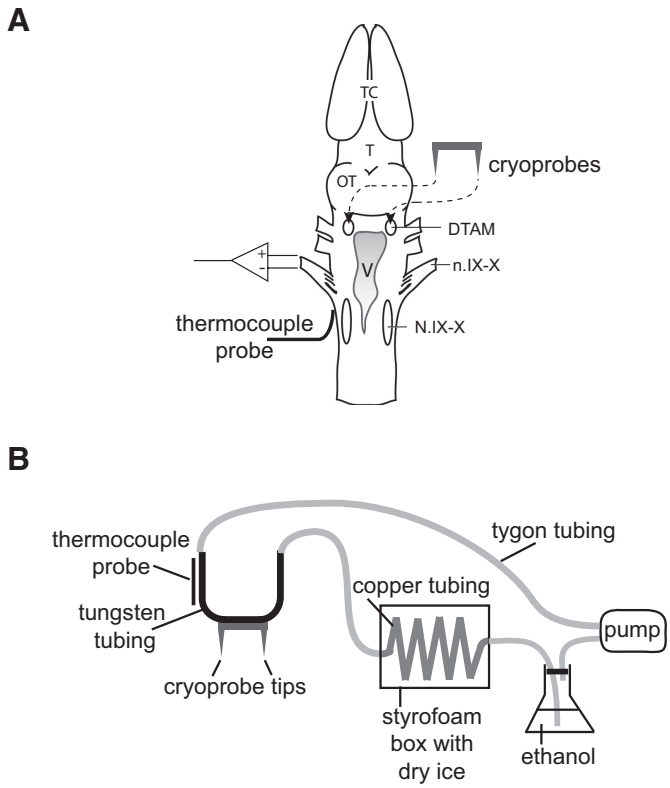

FIG. 2. Whole-brain fictive preparation setup. *A*: cartoon of the isolated *Xenopus laevis* brain illustrating the experimental setup. Nerve recording was obtained from the nerve IX-X to monitor fictive vocalizations. A thermocouple probe was placed against the laryngeal motor nucleus (N.IX-X) to monitor its temperature. For DTAM cooling experiments, an inverted U-shaped cryoprobe was inserted into DTAM bilaterally while the temperature at its shaft was monitored by a thermocouple probe. T, thalamus; TC, telencephalon; V, ventricle; N.IX-X, laryngeal motor nucleus; DTAM, dorsal tegmental area of medulla. *B*: cartoon of the cryoprobe setup. An inverted U-shaped cryoprobe was soldered to a tungsten pipe, which was connected to a closed-circuit of Tygon tubing containing ethanol. The cryoprobe was cooled by pumping ethanol through copper tubing encased in dry ice.

seen) also could not be monitored during the experiments because of the size of the thermocouple probe. However, the temperature at the level of the laryngeal motor nucleus (the rostral end of the nucleus is 3 mm caudal to DTAM) was monitored and confirmed to be 22°C using a thermocouple probe placed against the lateral surface of the laryngeal motor nucleus (Fig. 2*A*).

Six male brains were prepared as described, and the dura and pia mater over the cerebellum were peeled off to allow bilateral insertion of the cryoprobe into DTAM. The cryoprobe tips were inserted 700 μm below the dorsal surface into DTAM bilaterally (Fig. 2*A*) using a motorized micromanipulator (SD instruments). During the control experiment, bath temperature was maintained at 22°C and 5-HT was applied to the bath. During the DTAM cooling experiment, the cryoprobe was first cooled to a preset temperature while the bath temperature was maintained at 22°C by superfusing the recording chamber with warm (>26°C) saline (this was necessary to counteract the effect of cryoprobe decreasing the bath temperature). 5-HT (at 22°C) was then applied to the bath, and the bath temperature was maintained at 22°C during the following 2–5 min by manually adding warm (>26°C) saline with 30 μM 5-HT ad libitum.

Analysis of vocalizations

Ten song bouts from each recording session were randomly selected for further analyses. Each bout of an advertisement call contains a fast and a slow trill with an abrupt and stereotyped transition that is marked by decrease in instantaneous click rates and change in spectral properties of clicks (Fig. 1*A*). To determine whether click rates are influenced by temperature, interclick intervals of 21 clicks preceding and following the transition from fast to slow trills were measured with Raven software (Cornell University of Bioacoustics Laboratory, Ithaca, NY), and their reciprocals were calculated as instantaneous click rates. Mean instantaneous click rates calculated from these 20-click intervals were used for regression analyses.

Analysis of muscle physiology

When laryngeal muscles are stimulated repetitively at frequencies higher than the fusion frequency, they produce a sustained contraction (tetanus). Development of significant tetanic tension obstructs click production by the larynx because such tension prevents the pair of arytenoids discs from contacting each other, a movement critical for sound production (Yager 1992a). To determine whether the fusion frequency at which tetanic tension develops is influenced by temperature, we measured the ratio of fused tension relative to the total tension (the sum of fused and transient tension, Fig. 3*A*). All measurements were made using Clampfit (Molecular Devices).

Analysis of fictive vocalizations

Two to 13 bouts of fictive advertisement calls produced by each preparation at each temperature were selected (mean number of bouts sampled was 8.3 $\pm$ 0.5). Nerve recording traces were rectified and low-pass filtered at 1 kHz using Clampfit 9.2 software (Molecular Devices). Compound action potentials (CAPs; summed action potentials of many axons in a nerve) representing activity of laryngeal motoneurons (Fig. 4*A*) were identified in Clampfit using a threshold search, and inter-CAP intervals were measured. We defined fast trills to be a series of CAPs with progressive increase in rate and amplitude, and slow trills to be a series of CAPs that follow the fast trills that ends with steady rate and amplitude (Fig. 4*A*). When bouts were produced repeatedly, transition from the slow to fast trills is typically marked by a slight reduction in the rate and the amplitude followed by a progressive increase in the rate and amplitude of the CAP (see Fig. 4*A*, transition from slow to fast trills in all 3 traces). As in vocal analyses, 20 instantaneous CAP rates before and after the trill transition were used to calculate mean fast and slow trill rates for each bout.

To evaluate the effect of temperature on the duration of the fast trill compound action potential, traces of nerve recordings were rectified, and the half-width of the first deflection within a CAP was measured (Fig. 4*C*). In addition to six brains used for CAP rate analyses that produced complete fictive advertisement calls (i.e., bouts include both fast and slow trills) at 26°C, two additional brains that produced only fast trills at the temperature was included for this analyses.

Statistic and data analysis

To analyze the relation between temperature and click/CAP rates, a linear regression model was built (temperature as the independent variable, and mean slow and fast trill rates for each animal/brain at each temperature as dependent variables) and regression ANOVA was used to test for the effect of temperature on click/CAP rates. Residual plots were later examined to verify the appropriateness of each model. To determine whether temperature influenced the fused tension generated by the laryngeal muscles, repeated-measures ANOVA (temperature as between factor and frequency as within factors) was used. To determine whether the rates of slow and fast fictive trills generated during the selective DTAM cooling experiment significantly differ from those generated during control condition at 22°C, a Wilcoxon signed-rank test was used.

The temperature coefficient, $Q_{10,}$ was calculated for slow and fast trill rates of in vivo advertisement calls and fictive advertisement calls using the equation: $Q_{10} = (X_2/X_1)^{(10/(T2-T1))}$ where X_1 and X_2 are either click/CAP rates or half-width of the CAP at the lower temper-

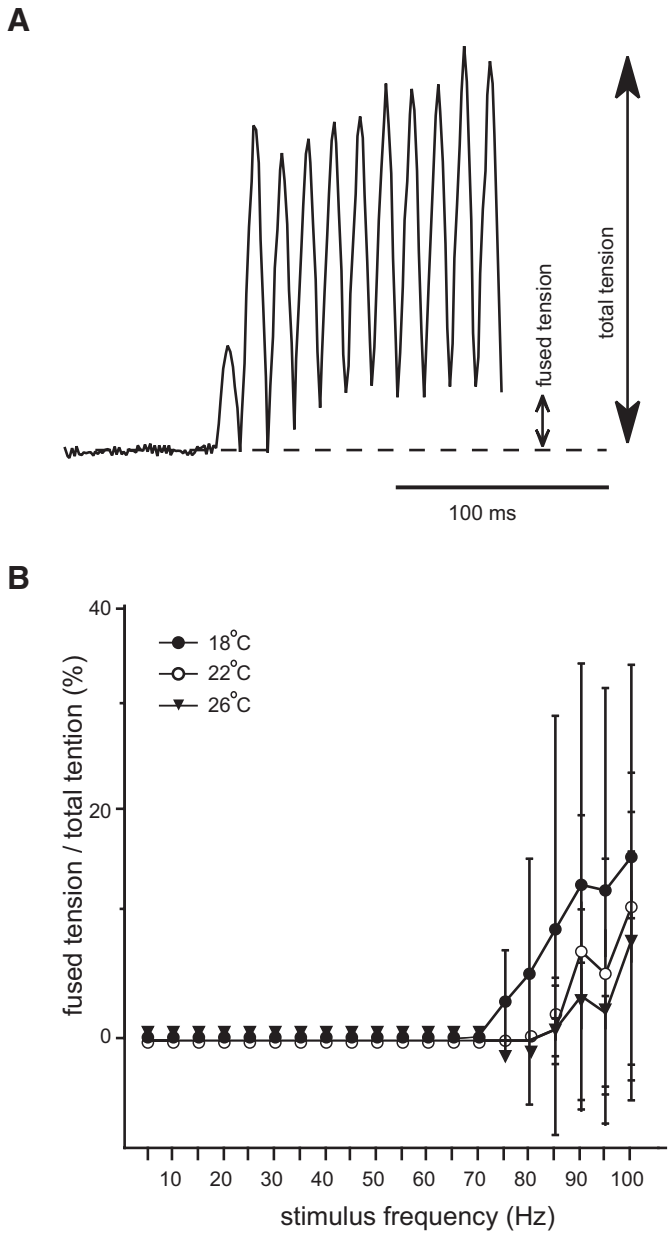

FIG. 3. Fused tension of laryngeal muscles in response to repeated stimulation at 3 different temperatures. *A*: example trace of muscle tension of a male larynx in response to repeated stimulation at 80 Hz. *B*: ratio of fused tension relative to total tension as a function of stimulus frequency, recorded at 3 different temperatures ($n = 6$ males). The fused tension ratios were similar at all temperatures. ▼, ○, ●, mean values; error bars, SD.

ature T_1 and higher temperature T_2, respectively. All statistical analyses were performed using StatView software (SAS Institute, Cary, NC).

RESULTS

Click rates of advertisement calls varied depending on the temperature

Audio recordings showed that click rates of both fast and slow trill portions of male advertisement calls varied depending on the water temperature. Comparison of twenty instantaneous click rates before and after the transition from fast to slow trills revealed that click rates of *X. laevis* became faster with an increase in temperature (Fig. 1, *B* and *C*). To analyze the relationship between temperature and click rate, we carried out a regression analysis for fast and slow trills recorded at 18, 22, and 26°C. The results showed that temperature significantly explained the variability in click rates for both fast ($F = 117.88$, $P < 0.0001$) and slow trills ($F = 98.31$, $P < 0.0001$), as shown in Fig. 1*D*. Temperature coefficients, Q_{10}, for slow and fast trills were 1.68 and 1.51, respectively, over a range from 18 to 26°C. Based on the regression model, we estimate the click rates for slow and fast trills to increase by 2.44 ± 0.23 and 1.82 ± 0.18 (SE) Hz, respectively, for every degree of temperature increase. We conclude that the ambient temperature determines the overall rates of the clicks throughout the advertisement call and that click rates for fast and slow trills increase linearly with the ambient temperature.

Contractile properties of the laryngeal muscles are not temperature-dependent across the vocal frequency range

Temperature-dependent modification in click rates likely originates in the central vocal pathways, but peripheral muscles may also show temperature sensitivity. To determine if twitch properties of laryngeal muscles of male *X. laevis* are modified by temperature, we examined contractile properties at three temperatures. In particular, we examined the ability of laryngeal muscles to contract and relax without tetanizing when stimulated at various frequencies; fused tension caused by tetanized muscle results in failure to produce clicks by the larynx (Tobias and Kelley 1987). When isolated laryngeal muscles were stimulated at frequencies ≤70 Hz (the operating frequency for advertisement calls), the muscle did not show significant tetanus (i.e., transient tension = total tension) at any temperature (Fig. 3*B*). At higher frequencies (≤100 Hz), the larynx developed tetanus, but the degree of tetanized tension developed did not differ at the three different temperatures [repeated-measure ANOVA, $F(2,15) = 1.54$, $P = 0.25$]. Thus cooler temperatures do not limit the ability of laryngeal muscles to contract at rates necessary to produce advertisement calls. Temperature-dependent modification in click rates is entirely determined by the sensitivity of the vocal CPG to the temperature.

CAP rates and duration of in vitro fictive vocalizations depend on the temperature

We next tested whether the vocal CPG is susceptible to temperature and thus potentially is the source of the temperature-dependent modification of advertisement calls in male *X. laevis*. To test this possibility, we induced fictive advertisement calls from isolated male brains by applying 30 μM 5-HT while holding the recording chamber at three different temperatures. CAPs in the laryngeal nerve, which have the same temporal pattern as vocal clicks produced in vivo (Yamaguchi and Kelley 2000), were recorded using a suction electrode. Fictive advertisement calls were induced at 18, 22, and 26°C from 13, 12, and 6 animals, respectively (Fig. 4*A*). The CAP rates of fictive advertisement calls increased with temperature (Fig. 4*B*) as click rates did in vivo. Regression analyses showed that temperature significantly accounted for the variability of CAP rates in slow and fast trills (regression ANOVA, $F = 65.50$, 62.49, $P < 0.0001$, 0.0001 for fast and slow trills, respectively, Fig. 4*D*). Q_{10} for slow and fast trills was 1.70 and 1.39,

respectively. For every degree of temperature increase, we estimate the slow and fast trill CAP rates to increase by 1.71 ± 0.22 and 1.87 ± 0.23 Hz, respectively. These data strongly suggest that the temperature-dependent modifications of advertisement calls observed in behaving frogs originate in the vocal CPG. Reducing temperature causes the vocal CPG to drive laryngeal muscles at slower rates.

In most neurons, the duration of action potentials increases as temperature decreases because of the reduced activation and inactivation rate of Na^+ and K^+ currents (Klee et al. 1974). If the action potentials generated by laryngeal motoneurons are affected by temperature in a similar manner, we predicted that the duration of fast trill CAPs (fCAPs) should also be elongated under colder temperatures for the following reason. Fast trills are thought to be generated by nearly synchronous spikes generated by the laryngeal motoneurons, whereas slow trills are considered to be the product of asynchronous motor spikes (Yamaguchi and Kelley 2000). As a consequence, the spikes underlying fast trills summate as smooth and stereotyped CAPs, whereas those underlying slow trills result in CAPs with complicated shapes with high variability due to phase cancellation (Yamaguchi and Kelley 2000) (Fig. 4*C*). If action potentials generated by individual laryngeal motoneurons are elongated, we predicted that the duration of fCAPs should also be affected. To test this prediction, we measured the half-width of fCAPs and found that it increased as temperature decreased, with a mean Q_{10} of 0.54. Regression ANOVA showed that temperature significantly explained the variability in fCAP half-width ($F = 52.79$, $P < 0.0001$, Fig. 4*E*). Thus we conclude that increased fCAP duration at the lower temperature likely reflects an increase in the duration of individual action potentials generated by laryngeal motoneurons. fCAP duration can be used as an indicator of the ambient temperature around the laryngeal motor nucleus and the motor nerve.

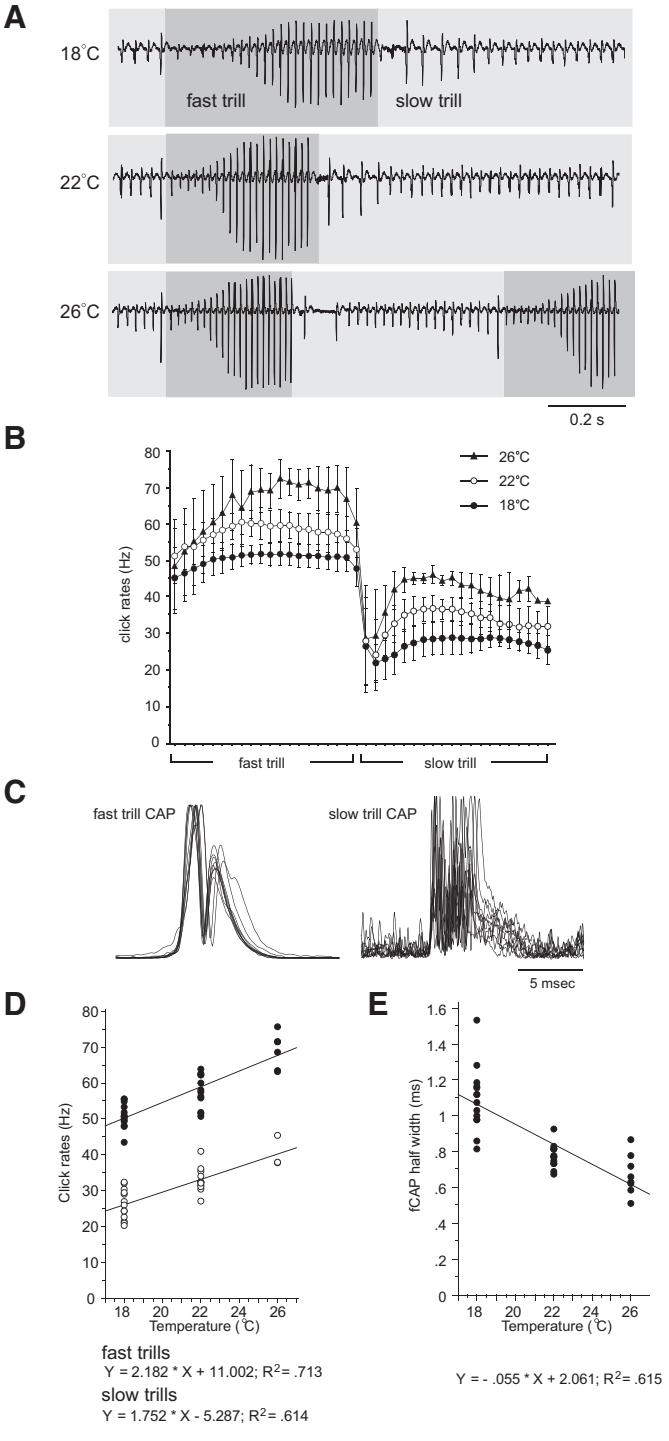

FIG. 4. Temperature-dependent change in fictive advertisement calls of male *Xenopus laevis*. *A*: example of laryngeal nerve recordings representing fictive advertisement calls induced at 3 different temperatures. Dark and light gray boxes surrounding each trace indicate fast and slow trills, respectively. Compound action potentials (CAPs) are produced at higher rates as the temperature of the recording bath increases. *B*: mean instantaneous CAP rates of 21 consecutive clicks preceding and following the transition between fast and slow trills recorded at 3 different temperatures ($n = 13$, 12, and 6 for 18, 22, and 26°C, respectively). ▲, ○, ●, mean values; error bars, SDs. *C*: overlay of 10 fast trill and 10 slow trill CAP traces obtained from a male to demonstrate that the shape of fast trill CAPs are stereotyped whereas the shape of slow trill CAPs are variable. Nerve recording traces are rectified for this figure. Each trace is normalized to its maximum amplitude. *D*: regression plots of instantaneous CAP rates for fast and slow trills as a function of temperature. ● and ○ mean fast and slow trill rates (average of all 20 intervals before and after the trill transition), respectively, for each individual at each temperature. *E*: regression plots of fast trill CAP half-width as a function of temperature.

Locus of temperature dependence

A previous study suggested that the fast trill rhythm is generated by a pool of neurons in DTAM because electrical stimuli delivered to DTAM evoked fast but not slow trills (Rhodes et al. 2007). To determine if DTAM is the source of fast trill rhythm, we inserted the cryoprobe to DTAM bilaterally. Under control conditions, the entire bath including DTAM was held at 22°C. Under experimental conditions, DTAM was cooled while the bath temperature was maintained at 22°C. If DTAM contains the fast trill rhythm generator, we predicted that selective cooling of DTAM would result in the reduction of fictive fast trill rates without affecting the slow trills.

Fictive advertisement calls were induced under control and experimental condition from all six male brains. Fictive advertisement calls obtained under control conditions were virtually identical to those obtained at 22°C in the previous experiment (Fig. 5*A*), indicating that the bilateral insertion of cryoprobes by itself does not change the vocal rhythms. When DTAM was bilaterally cooled, however, CAP rates of fictive fast trills were decreased compared with those recorded under the control condition in all six brains, and the

difference was statistically significant (Wilcoxon signed-rank test, $Z = -2.20$, $P = 0.028$, Fig. 5, *B* and *C*). To our surprise, CAP rates of slow trills were also significantly decreased by selective cooling of DTAM in all six brains ($Z = -2.20$, $P = 0.028$, Fig. 5, *A* and *B*).

Although the extent of the cooling effect across the tissue was not monitored, the temperature at the level of the laryngeal motor nucleus was confirmed to be 22°C in all six animals during the experiments. This point was further confirmed by the duration of the fCAP. When the half-width of fCAPs

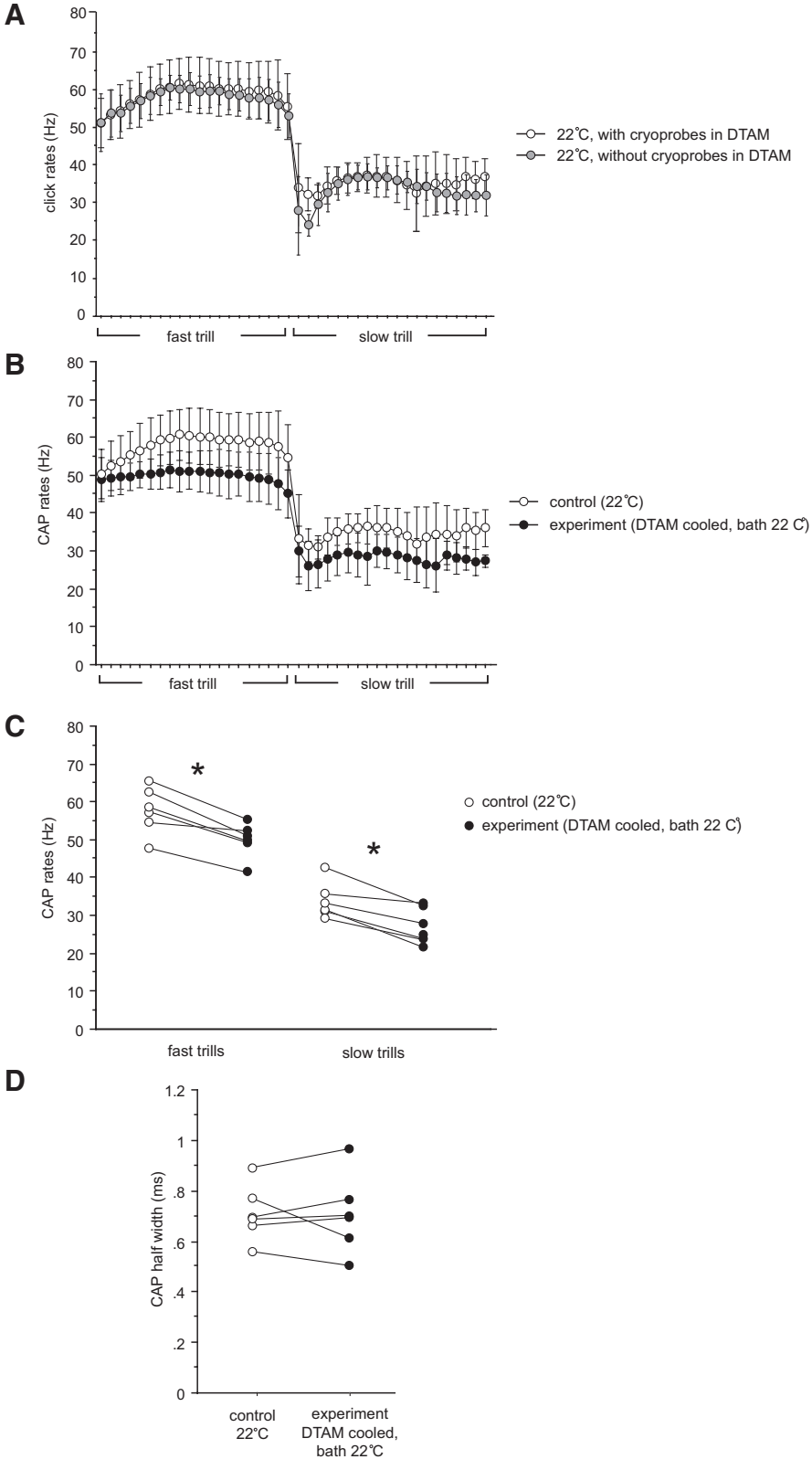

FIG. 5. Locally cooling dorsal tegmental area of the medulla (DTAM) reduces fictive fast and slow trill rates. *A*: mean instantaneous CAP rates of fictive advertisement calls recorded at 22°C with (○, same as data used in Fig. 5*B*) and without (◎, same as data used in Fig. 4*B*) the cryoprobes in DTAM. CAP rates recorded under these 2 conditions are virtually identical, indicating bilateral insertion of cryoprobes itself does not modify the CAP rates. *B*: mean instantaneous CAP rates of 21 consecutive CAPs preceding and following the transition between fast and slow trills. Recordings were made under 2 conditions from each brain ($n = 6$): at a bath temperature of 22°C (control), and while locally cooling DTAM while holding the bath temperature at 22°C (experimental). *C*: mean slow and fast trill rates for 6 animals under control and experimental conditions. When DTAM was locally cooled, both fast and slow trills were slower in all 6 males, and these differences were statistically significant (marked with *). ● and ○, mean CAP rates under experimental and controlled condition of each preparation, respectively. *D*: mean half-width of fast CAPs recorded under control and experimental conditions in each of 6 animals. The CAP half-widths recorded during local DTAM cooling were similar to those recorded at control condition. The result is consistent with the idea that the laryngeal motor nucleus was held at 22°C during DTAM was cooled.

during the DTAM cooling experiments were compared with those obtained during control condition, there was no significant difference (Wilcoxon signed-rank test, $Z = -0.31$, $P = 0.75$, Fig. 5*D*).

Thus selectively cooling DTAM alters both fast and slow trill CAP rates. This result indicates that DTAM neurons play an important role in generating both fast and slow trill rhythms.

DISCUSSION

We have demonstrated that advertisement calls of male *X. laevis* are temperature sensitive. Click rates of both slow and fast trills increase linearly as temperature increases within an ethologically relevant temperature range. A similar linear relation between temperature and CAP rates was found in serotonin-induced fictive advertisement calls in vitro, whereas temperature did not affect the contractile properties of laryngeal muscles when stimulated within the frequency range of vocalizations. We thus conclude that the temperature sensitivity of vocal behavior originates in the CPG. Finally, selective cooling of DTAM led to a deceleration of fast and slow trills, suggesting the importance of DTAM in generating both trill rhythms.

Temperature-dependent modification of advertisement calls

In its natural habitat, *X. laevis* is known to breed when water temperature ranges from 13 to 25°C (Kalk 1960). The temperature sensitivity of vocalizations shown in this study indicates that in their natural habitat, the temporal morphology of advertisement calls of male *X. laevis* will fluctuate as the water temperature changes in response to environmental factors such as air temperature, drought, and rain fall during the breeding season. In other species of ectotherms in which courtship signals of males are modified by temperature, the preference of females is also modified to match the male signals [tree frogs (Gerhardt 1978), field crickets (Pires and Hoy 1992a)]. Such a phenomenon, termed "temperature coupling" (Gerhardt 1978), has not been examined in *X. laevis*. Temperature coupling may have evolved to enhance species recognition in species the acoustic signal of which overlaps with those of sympatric heterospecifics (Gerhardt 1978). Such selective pressure may not exist in *X. laevis* because its advertisement call seems to be acoustically distinct from those of sympatric species (M. Tobias, personal communication). However, temperature coupling might still be found in *X. laevis*, not as an adaptation for species recognition, but as a byproduct of the temperature-dependent modification of the auditory neural pathways (Brenowitz et al. 1985).

Temperature independence of laryngeal muscle contraction rate

In general, the twitch properties of muscles (evoked by a single stimulus) are temperature sensitive; the rate of twitch tension development and of muscle relaxation both increase as temperature increases (reviewed by Bennett 1984). It is thus possible that when stimulated repeatedly, the laryngeal muscles of male *X. laevis* reach tetanus at lower stimulus frequency as temperature is decreased. However, within the frequency range of normally function (<70 Hz), laryngeal muscles did not develop significant tetanus at any temperature tested. Thus the deceleration of the neuronal output in decreasing temperature is not accompanied by a reduced twitch rate of laryngeal muscles. What was not tested in this study, however, was the endurance of laryngeal muscles. Male *X. laevis* can easily repeat ~40 bouts of advertisement calls (>40 s long) at room temperature without any resting interval. It is possible that temperature-dependent limitations at the level of the laryngeal muscle might have emerged in response to such long-lasting patterns of stimulation.

Temperature-dependent modulation of CPGs

X. laevis advertisement calls are generated by CPGs that can be activated in vitro by serotonin (Rhodes et al. 2007). Although fictive advertisement calls in vitro were consistently 3–8 Hz slower than actual advertisement calls of awake males at a given temperature (common in fictive motor behavior disconnected from all afferents) (Wilson 1961), the temperature-click/CAP rate relation was remarkably consistent in vivo and in vitro (Q_{10s} for in vivo and in vitro vocalizations were 1.51 and 1.39 for fast trills and 1.68 and 1.70 for slow trills, respectively). We conclude that the rhythmic motor output of the CPG is dictated by the ambient temperature of the brain and that such change is directly translated into vocal rhythms produced by the animal.

We have previously localized the *X. laevis* vocal CPG to the brain stem. Serotonin induced normal fictive vocalizations when the brain stem was isolated from the rest of the brain (Rhodes et al. 2007; Rhodes and Zornik unpublished observation). Within the brain stem, DTAM seems to be an essential component of the vocal CPG because transections that removed DTAM from the rest of the brain stem abolished 5-HT-induced fictive vocalizations entirely (Rhodes et al. 2007). In this study, we hypothesized that the fast trill rhythm is generated by neurons in DTAM because electrical stimulation delivered to DTAM evoked fictive fast trills but not slow trills (Rhodes et al. 2007). Here we confirmed that temperature of DTAM affected fast trill rates, indicating that the fast trill rhythm is generated by a pool of neurons in DTAM. However, we also found that the rate of slow trills decreased when DTAM was chilled, indicating that DTAM contains neurons that regulate the rhythm of both fast and slow trills in male *X. laevis*. Electrical stimulation in our previous experiments may have only activated a proportion of DTAM neurons responsible for fast trills or the patterns of stimuli delivered was effective only in activating fast but not slow trills.

Are these two types of trills controlled by two separate networks, each with dedicated neuronal pools (i.e., dedicated networks), or is there a single network that generates both trill types (i.e., multifunctional network)? Although the vocal motor units are shared by the two trill types (Zornik, personal communication), the present results did not allow us to answer this question because of the insufficient spatial resolution. DTAM could contain two separate pools of interneurons, each of which is a part of a dedicated network, or a single pool of interneurons that forms a network that generates both rhythms.

One insight about the architecture of the vocal networks comes from phylogeny of the genus *Xenopus*. Evolutionarily, biphasic trills seem to be derived characteristics within the genus: the advertisement calls of most other *Xenopus* species are composed of monophasic clicks repeated at rates similar to either slow or fast trills of *X. laevis* (M. Tobias personal communication). Thus the biphasic trills seemed

to have evolved more recently in a small number of species including *X. laevis*. In systems where two rhythmic motor patterns of different evolutionary history are examined, it has been argued that the newer motor patterns may utilize a pool of interneurons of the existing neural network that generate the older motor patterns (Briggman and Kristan 2006). In *Xenopus* genus, it is not clear which of the trill type (either fast or slow) is ancestral. However, if the pattern of network architecture holds true in *X. laevis*, then the derived trill network may have evolved from the preexisting ancestral trill network, and we would expect to see an overlapping, multifunctional vocal network in this species. Functional analyses of the vocal neurons offer a potential to reveal the mechanisms for generating biphasic rhythms.

DTAM as a sole rhythm generator?

Although the present results emphasize the importance of DTAM as a rhythm generator, it is possible that vocal rhythm generation also relies on interaction between DTAM and other brain stem nuclei. In leopard frogs (*Rana pipiens*), reciprocal connection between DTAM and interneurons in the N.IX-X (the laryngeal motor nucleus) are required for vocal pattern generation (Schmidt 1992). Extensive reciprocal connections between DTAM and N.IX-X are also found in *X. laevis* (Zornik and Kelley 2007), suggesting that DTAM may be necessary, but not sufficient for vocal rhythm generation. If these two nuclei interact on a cycle-by-cycle basis, then the deceleration of spike generation and synaptic transmission induced in one of the nuclei by selective cooling may result in overall elongation of the cycle period. This two-part CPG hypothesis predicts that cooling of N.IX-X should also decrease the vocal rhythms. Unfortunately, we could not test this hypothesis in the present study because the tissue surrounding N.IX-X seems to be sensitive to mechanical disturbances introduced by insertion of cryoprobes (data not shown). Nevertheless our data represent strong evidence that DTAM neurons are critically involved in rhythm generation. Future work will focus on the characterization of the types of neurons in DTAM and N.IX-X and determine how they work together as a network.

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