

5-HT_{2C}-Like Receptors in the Brain of *Xenopus laevis* Initiate Sex-Typical Fictive Vocalizations

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Yu HJ, Yamaguchi A. 5-HT_{2C}-like receptors in the brain of *Xenopus laevis* initiate sex-typical fictive vocalizations. *J Neurophysiol* 102: 752–765, 2009. First published May 27, 2009; doi:10.1152/jn.90469.2008. Vocalizations of male and female African clawed frogs (*Xenopus laevis*) are generated by brain stem central pattern generators. Serotonin (5-HT) is likely important for vocal initiation because, when applied in vitro, sex-typical fictive vocalizations are evoked from isolated brains. To explore the mechanisms underlying vocal initiation, we identified the types of serotonin receptors mediating vocal activation pharmacologically using a whole brain, fictive preparation. The results showed that 5-HT_{2C}-like receptors are important for activation of fictive vocalizations in the sexes. 5-HT_{2C} receptor agonists elicited fictive vocalizations, and 5-HT_{2C} receptor antagonists blocked 5-HT-induced fictive vocalizations, whereas agonists and antagonists of 5-HT_{2A} and 5-HT_{2B} receptors failed to initiate or block 5-HT-induced fictive vocalizations in the sexes. The results indicate that serotonin initiates fictive vocalizations by binding to 5-HT_{2C}-like receptors located either within or upstream of the vocal central pattern generator in both sexes. We conclude that the basic mechanism of vocal initiation is shared by the sexes despite the differences in the actual vocalizations between males and females. Sex-typical vocalizations, therefore, most likely arise from activation of different populations of 5-HT_{2C} receptor-expressing cells or from differential activation of downstream pattern-generating neurons.

INTRODUCTION

Rhythmic behaviors are typically generated by central pattern generators (CPGs), networks of neurons that generate motor output without sensory feedback (Grillner et al. 1998; Marder and Bucher 2001). Some rhythmic behaviors, such as locomotion and scratching, are episodic and expressed only when necessary. For episodic behaviors, neural control of the initiation and termination are as important as maintenance of that behavior. For initiation of motor patterns, tonic inputs to CPGs along with neuromodulators including monoamines, acetylcholine, and excitatory amino acids are important (Chapman and Sillar 2007; Johnson et al. 2005; Quinlan et al. 2004). Although several transmitters likely act together to initiate motor patterns in vivo (Jordan et al. 2008 review), serotonin (5-HT) is particularly important in many systems. For example, 5-HT released from the parapyramidal region of the medulla mediates the locomotion initiated by the activation of the mesencephalic locomotor region in rodents (Liu and Jordan 2005), and activation of serotonergic neurons in the raphe nucleus initiates whisking behavior in rats (Hattox et al. 2003). Identification of the types and location of 5-HT receptors involved can enhance our understanding of motor pattern initiation.

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Vocalizations are rhythmic episodic behaviors used for social communication in a wide range of species. The timing of vocal production is often determined by external sensory cues (such as olfactory and visual cues emitted from potential mates) and by the internal environment (i.e., endocrine state of the organism). Little is known, however, about the neural mechanism of vocal initiation. Given its significance in activating episodic motor patterns in other systems, we predicted that serotonin is also important in vocal motor systems. We examine this issue using African clawed frogs (*Xenopus laevis*) that produce several call types during social interactions (e.g., Fig. 1A; see Tobias et al. 2004).

Vocalizations of *X. laevis* are ideal for this analysis, because the importance of serotonergic systems has already been implicated (Brahic and Kelley 2003; Rhodes et al. 2007), and fictive vocalizations can be studied in vitro, unlike most other vocal behaviors. Anatomically, the rostral raphe nucleus (rRpd) sends serotonergic projections to premotor [dorsal tegmental area of medulla (DTAM)] and motor (n.IX-X) nuclei of the central vocal pathways in both sexes (Rhodes et al. 2007). Physiologically, application of serotonin elicits sex-typical fictive vocalizations from isolated brains of both sexes (Rhodes et al. 2007). Thus 5-HT is available to the *Xenopus* vocal pathways in vivo, and its functional importance for initiating fictive vocalizations is apparent. Furthermore, male and female *Xenopus* produce sex-typical vocalizations, providing us with the opportunity to examine whether vocal initiation mechanisms differ between the sexes.

Using a pharmacological approach, we identified the 5-HT receptor types that elicit fictive vocalizations in male and female brains. We focused on 5-HT₂ receptors because of their roles in rhythmic neural circuits in other animal models (Hattox et al. 2003; Pearlstein et al. 2005; Tryba et al. 2006; Xiang et al. 2005). Our results showed that the sexes share a similar mechanism for initiating vocalizations.

METHODS

Animals

Sexually mature *Xenopus* males ($n = 61$; 42.2 ± 8.4 g; 7.2 ± 0.5 cm) and females ($n = 50$; 72.0 ± 10.4 g; 8.7 ± 0.6 cm) were purchased from Nasco (Fort Atkinson, WI). The animals were kept in glass aquaria on a 12:12 light:dark cycle at room temperature. All experimental procedures were approved by the Boston University Institutional Animal Care and Use Committee and performed in compliance with guidelines published by the National Institute of Health.

Whole brain fictive preparation

Frogs were anesthetized with MS-222, 0.15 mg/g body weight, injected subcutaneously (Sigma, St. Louis, MO), and brains were rapidly removed in oxygenated (99% O₂-1% CO₂) ice-cold saline composed of (in mM) 96 NaCl, 20 NaHCO₃, 2 CaCl₂, 2 KCl, 0.5 MgCl₂, 10 HEPES, and 11 glucose, with pH 7.8. Brains were transferred to a recording chamber where they were continually superfused with fresh oxygenated saline (150 ml/h) and allowed to return to room temperature (~22°C) during the following hour.

In vitro nerve recordings

Methods of recording the population activity of motor nucleus IX-X were described previously (Rhodes et al. 2007). Briefly, a suction electrode was placed on the most caudal rootlet of nerve IX-X to record compound action potentials (CAPs), population activity generated by a pool of neurons. This nerve rootlet contains the axons of the laryngeal and glottal motoneurons (Simpson et al. 1986). The recorded signal was amplified (A-M Systems differential amplifier 1700), high-pass filtered (1 Hz), digitized at 10 kHz (Digidata 1322A, Molecular Devices, Sunnyvale, CA), and recorded on a PC using AxoScope software (Axon Instruments). All the recordings were made at room temperature (~22°C). Typically, there is no vocal activity recorded from the laryngeal nerve before the application of 5-HT or its agonists (Fig. 1B).

Drugs

All drugs in this study were made fresh as stock solutions on the day of use and were kept on ice until their final dilution in oxygenated saline at room temperature. Stock solutions of serotonin hydrochloride (5-hydroxytryptamine, Sigma Aldrich), 6-methyl-1-(1-methylethyl)-ergidine-8 β -carboxylic acid (LY 53857, Sigma), α -methyl-5-hydroxytryptamine (α -Me-5-HT, Sigma), 1-[5-(2-thienylmethoxy)-1H-3-indolyl]propan-2-amine HCl (BW 723C86, Sigma), ketanserin tartrate (Sigma), 6-chloro-2-(1-piperazinyl)pyrazine hydrochloride (MK-212, Tocris Bioscience, Ellsville, MO), (α S)-6-chloro-5-fluoro- α -methyl-1H-indole-1-ethanamine fumarate (Ro 60-0175, Tocris), (\pm)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI hydrochloride, Sigma), and 6-chloro-2,3-dihydro-5-methyl-N-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1H-indole-1-carboxamide dihydrochloride (SB 242084, Tocris) were also dissolved in deionized water. Stock solutions of N-(1-methyl-5-indolyl)-N'-(3-methyl-5-isothiazolyl) urea (SB 204741, Sigma), 8-[5-(2,4-dimethoxy-5-(4-trifluoromethylphenylsulphonamido) phenyl-5-oxopentyl)-1,3,8-triazaspiro[4.5] decane-2,4-dione hydrochloride (RS 102221 hydrochloride, Tocris), 3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,3-dihydro-2-thioxo-4(1H)-quinazolinone hydrochloride (altanserin hydrochloride, Tocris), α -phenyl-1-(2-phenylethyl)-4-piperidinemethanol (MDL 11939, Tocris), and 6-chloro-5-methyl-1-5-quinolycarbonyl-indoline (SB 215505, Sigma) were dissolved in DMSO. It was confirmed that application of DMSO alone at the concentration used for each experiment had no effect on 5-HT-induced fictive vocalizations ($n = 5$ males; data not shown). In some experiments, LY 53857 was used for male brains, and altanserin was used for female brains as 5-HT₂ receptor antagonists because the former antagonist became commercially unavailable. Both drugs show similar pK values for each subtype of 5-HT₂ receptors and have little affinity for adrenergic or histaminergic receptors (Lemaire et al. 1991). All concentrations of drugs and exposure times were determined by consulting the literature. When no previous in vitro use of a drug was reported in literature (i.e., SB 242084, MK-212, and Ro 60-0175), we selected a minimum dosage in the range of 10–100 μ M that consistently blocked or activated behavior based on pilot experiments, a common method used to establish a dose of drugs for selective binding to serotonin receptors in mammalian tissues (Gunther et al. 2006; Morin et al. 1992; Tryba

et al. 2006). In cases where agonists and antagonists were previously used only in brain slice preparations (Chen et al. 2003; Perrier and Hounsgaard 2003), we selected 10 times the dose for our whole brain preparation so that the drugs penetrated the tissue efficiently. Because 5-HT₂ receptors of *X. laevis* are not fully characterized pharmacologically, we will refer to the receptors identified in this study with pharmacology similar to mammals as 5-HT₂-like.

Application of pharmacological agents

5-HT was applied by replacing one half the saline in the recording chamber (20 ml) with 60 μ M 5-HT dissolved in oxygenated saline to achieve a final concentration of 30 μ M. 5-HT application took 5–10 s, after which 5-HT remained in the recording chamber for 5 min; during this time, superfusion of saline was suspended. 5-HT receptor agonists and antagonists were also applied to the bath in the same way as 5-HT. To wash 5-HT or serotonergic agents out of the bath after the treatment period, saline superfusion was reinstated at a high rate (10–20 ml/min) for 5–10 min, which is sufficient to completely exchange the solution 2.5–10 times in the recording chamber. All brains were continually superfused with oxygenated saline (150 ml/h) for 1 h between repeated applications of 5-HT. In experiments using 5-HT receptor antagonists, 5-HT was initially applied to brains to obtain baseline control fictive recordings; 1 h later, brains were exposed to the antagonist followed by 5-HT (30 μ M, the concentration of the antagonist remained constant before and during 5-HT application). For the analyses, we included only the data from brains that recovered from the effect of the drug; a brain that did not recover from the antagonist effect was not included to rule out the possibility of confounding the real effect of drugs with deteriorating health of the tissue. Only 12 of the 89 brains treated with antagonist failed to recover from antagonist application. In experiments using agonists, 5-HT receptor agonists were applied to the naïve brain that had not been exposed to any drugs, including 5-HT, to prevent any possible priming of receptors by 5-HT.

Analysis of fictive vocalizations

In this study, we focused on the activation of advertisement calls and ticking (also known as release calls in females), the two most common and best-studied vocalizations in *X. laevis*. In vivo, advertisement calls are produced exclusively by males, and ticking is produced mostly by unreceptive females but occasionally by males (Tobias et al. 2004). Similarly, fictive advertisement calls are elicited only from male brains, whereas fictive ticking can be evoked from mostly female, but occasionally from male, brains (~30% of male brains showed 5-HT-induced fictive ticking) (Rhodes et al. 2007) in response to 5-HT applied in vitro. Male fictive ticking, defined as an isolated train of four or more CAPs whose instantaneous frequency ranged from 3 to 13 Hz, was observed in a subset of males in this pharmacological study. We report the pattern of activation whenever data are available in male brains, but the data were not used for further analyses.

Fictive advertisement calls in males are characterized by a series of CAPs repeated at fast rates with progressively larger amplitude (~55 Hz; fast trill), followed by CAPs repeated at a slow rate with relatively constant amplitude (~30 Hz; slow trill; Figs. 1A and 2A1) (Rhodes et al. 2007; Yamaguchi and Kelley 2000). Both in vivo and in vitro, slow trills can sometimes be omitted (Fig. 1A). In this study, both complete and incomplete types of advertisement calls (with and without slow trills) are included in the analyses. Fictive ticking is characterized by CAPs repeated at a very slow (<10 Hz), often monotonous rate, without any systematic AM (Rhodes et al. 2007; Yamaguchi and Kelley 2000) (Fig. 2B1).

The major goal of this study was to determine whether 5-HT receptor agonists initiate fictive vocalizations and whether 5-HT receptor antagonists block 5-HT-induced fictive vocalizations. An

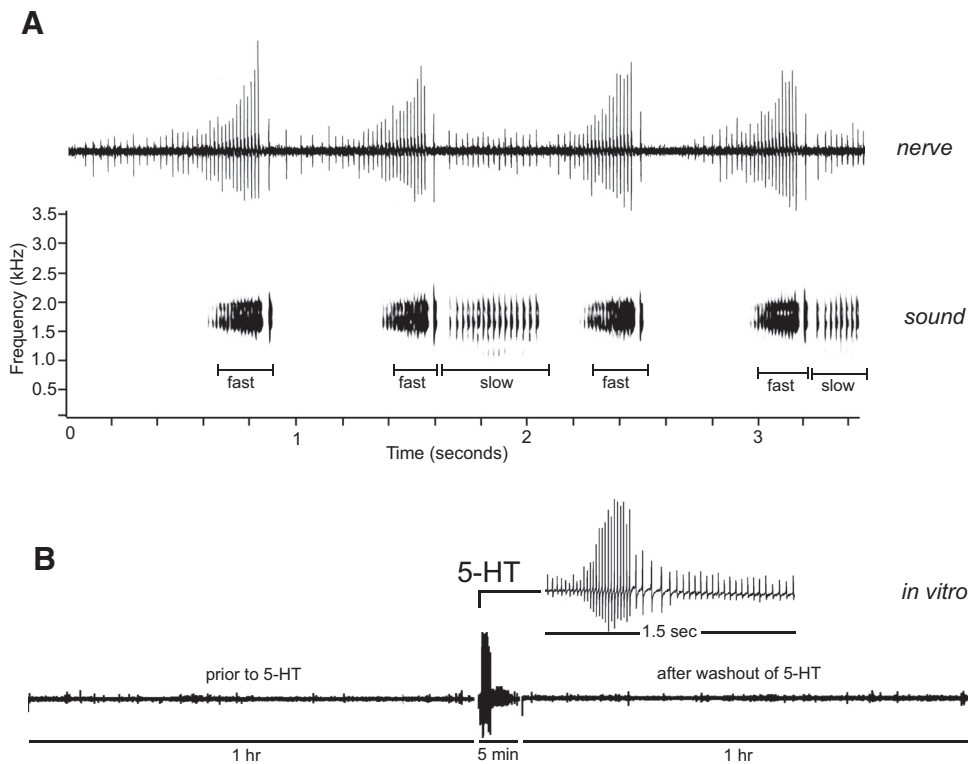


FIG. 1. Variability in vocalizations in vivo and induction of fictive vocalizations in vitro. *A*: simultaneous sound (bottom trace, sound spectrogram) and nerve (top trace) recordings obtained in vivo from a male *Xenopus* show that advertisement calls may consist of fast and slow trills (as in the 2nd and 4th bouts) or only fast trills (as in the 1st and 3rd bouts). See Yamaguchi and Kelley (2000) for the recording methods. *B*: isolated brains of *Xenopus* in vitro are typically silent (left 1-h trace) until serotonin (30 μ M) is introduced to the recording chamber (middle 5-min trace). Fictive vocal behavior continues until serotonin is washed out of the recording chamber (right 1-h trace).

additional goal of this study was to determine whether the morphology of calls induced in the presence of the pharmacological agents differs from that induced by 5-HT alone. Specifically, in cases when an agonist succeeded in eliciting vocalizations, we examined whether vocalizations induced by the agonist differ from those induced by 5-HT in the sexes. Similarly, when an antagonist failed to block 5-HT-induced vocalizations, we examined whether the vocalizations produced in the presence of antagonist differ from those induced by 5-HT alone. To this end, we characterized the temporal morphology of fictive vocalizations quantitatively, and, in the case of males, estimated overall vocal activity by counting the number of call bouts produced. The number of bouts was obtained only from males because female call bouts are variable in length. The temporal morphology of fictive vocalizations was characterized using mean CAP rates for fast and slow trill and maximum sustained CAP rates (defined as the fastest rate at which 10 consecutive inter-CAP intervals are produced) for 10 randomly selected consecutive advertisement calls from each male brain; mean CAP rates and maximum sustained CAP rates for the entire 5 min for ticking from each female brain.

Up to 10 bouts of advertisement calls within the 5 min of serotonin exposure were randomly sampled from each male brain. Female brains either tick in long continuous trains, or in bouts (\sim 10 ticks) interrupted by periods of silence ($>$ 1 s). Because of this variability and difficulty in defining a bout, female vocal behavior was analyzed as one continuous 5-min bout of ticking, and all CAPs produced during 5 min of 5-HT exposure were sampled for each female brain for analysis. Pauses longer than 1 s between bouts of female ticking were eliminated from the data.

All traces were rectified and low-pass filtered at 2 kHz with Clampfit 10.0 software (Molecular Devices). CAPs corresponding to laryngeal motor neuron activity were identified in Clampfit using a threshold search (threshold set at 3σ of background noise, minimum event duration = 0.4 ms). The instantaneous CAP rates were calculated, and the frequency distributions of instantaneous CAP rates (with bin size of 1 Hz) were plotted (Fig. 2, *A* and *B*). The frequency histogram for each animal showed either a bimodal (male) or unimodal (female) distribution with one exception: the frequency histogram

from one male brain showed a unimodal distribution with mean rates corresponding to fast trills (i.e., advertisement calls without slow trills). These histograms were well fit with two (except 1 male) or one (1 male and all females) Gaussian curves ($R^2 > 0.9$), with means of μ_1 , μ_2 , (2 Gaussian) or μ (1 Gaussian) (Fig. 2, *A* and *B*). μ_1 and μ_2 were used as estimates of mean slow and fast trill rates, and μ was used as an estimate of mean ticking rates. In the case of one male with a unimodal distribution, μ was used as an estimate of fast trill rates (i.e., the equivalent of μ_2). Maximum sustained CAP rates for males and females were calculated using a sliding window (i.e., by averaging 10 consecutive instantaneous CAP rates and taking the maximum). Total number of advertisement bouts in male brains was also compared across conditions. A bout of advertisement call was defined as a vocalization that consisted of CAPs produced at fast trill rates ($>$ 48 Hz) that is amplitude modulated. μ_1 , μ_2 , μ , maximum sustained CAP rates, and number of advertisement bouts were used for further statistical analyses to determine whether fictive vocalizations elicited in the presence of 5-HT₂ receptor agonists and antagonists differ quantitatively from those induced by 5-HT.

Statistical analyses

All statistical analyses were done using StatView software (SAS Institute, Cary, NC). For experiments in which the antagonist did not block vocal behavior, we examined whether there was any quantitative change in the fictive vocalizations in the presence of the antagonist compared with those induced in the absence of the antagonist. To this end, we compared each μ , maximum sustained CAP rate, and number of advertisement bouts (males only) obtained in the presence of antagonist to those parameters obtained in response to 5-HT alone using a Wilcoxon signed rank test. For experiments in which the agonist elicited vocal behavior, we compared fictive calls elicited by the agonist in experimental brains to those elicited by 5-HT in a set of control brains ($n = 5$ males and 5 females) using a Mann-Whitney *U* test comparing across treatments. A Kruskal-Wallis test was also used to examine if the temporal structure of fictive vocalizations initiated in the

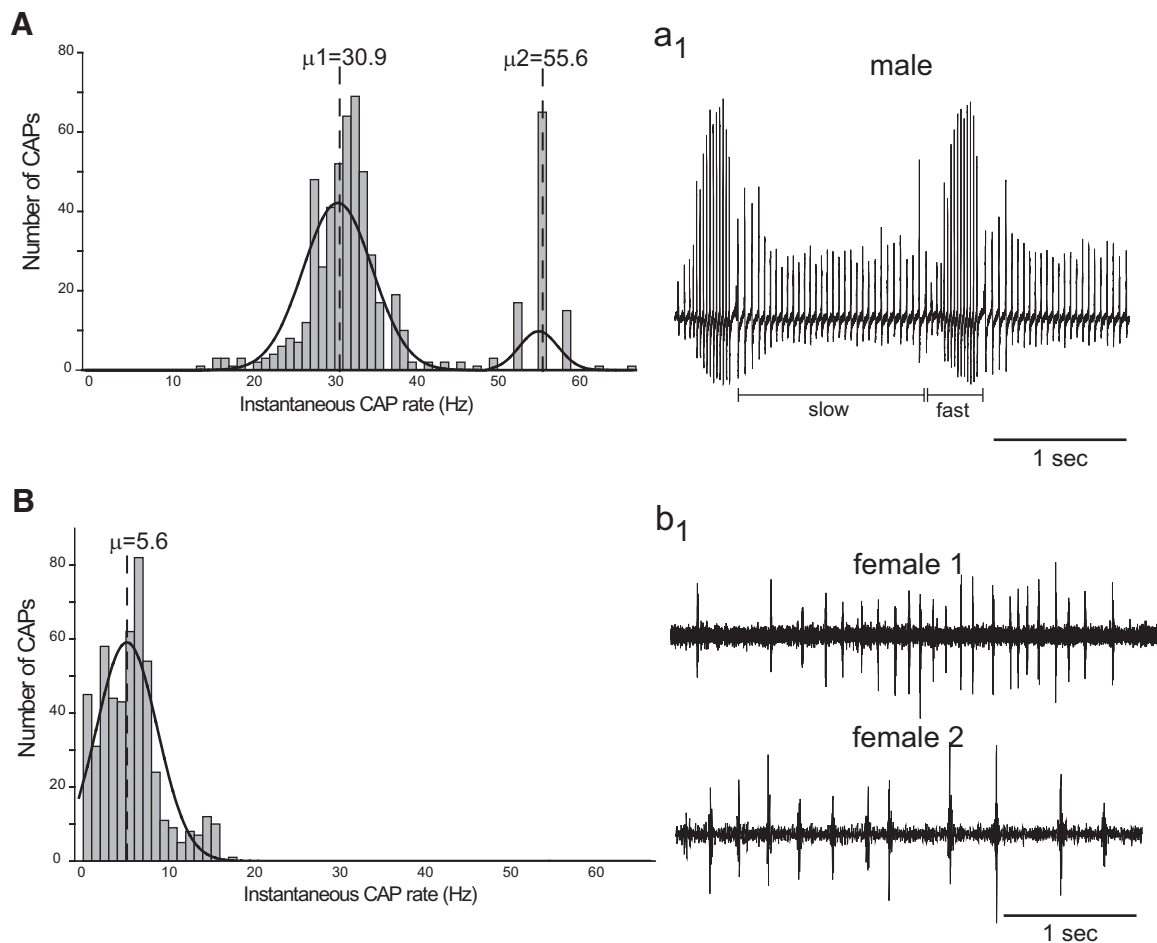


FIG. 2. Quantitative characterization of fictive vocalizations of *Xenopus*. *A*: an example frequency histogram (bin size 1 Hz) of instantaneous compound action potential (CAP) rate calculated from 10 bouts of fictive advertisement calls of a male brain. The histogram was fit with bimodal Gaussian curves. Higher and lower peaks (μ_2 and μ_1) are used as estimates of mean fast and slow trill rates. *A1*: example trace of fictive advertisement call from which the histogram in *A* was plotted. Slow and fast trills are labeled. *B*: an example frequency histogram (bin size 1 Hz) of instantaneous CAP rates calculated from fictive ticking obtained from 10 females. A large unimodal peak (μ) is used as an estimate for mean ticking rate. *B1*: 2 traces each from 2 different females, showing inter- and intraindividual variation in the rate of CAPs.

presence of agonists or antagonists systematically differs from those initiated by 5-HT.

RESULTS

In this section, we first address whether agonists and antagonists to 5-HT receptors activated or blocked fictive vocalizations. We next address whether the temporal morphology of fictive vocalizations produced in the presence of agonists or antagonists differ from that induced by 5-HT alone.

5-HT₂-like receptors are important for initiating fictive vocal behavior in both male and female brains

We first examined how blocking 5-HT₂-like receptors affect 5-HT-induced fictive vocalizations in both sexes. Bath application of a 5-HT₂ receptor antagonist (3 males: LY 53857, 100 μ M; 3 females: altanserin, 100 μ M, ≤ 10 min) to the brain in vitro blocked 5-HT-induced fictive vocalizations in both sexes. In males, 5-HT-induced fictive advertisement calls were blocked in the presence of antagonist in all brains (Fig. 3*A*, middle). 5-HT-induced fictive ticking, which was observed in one of the male brains, was also blocked by the antagonist

(data not shown). After 5-HT₂ receptor antagonist was washed out of the recording chamber, 5-HT once again elicited fictive vocalizations (including ticking in the one male brain) from all brains (Fig. 3*A*, right). Similarly, in female brains, 5-HT-induced fictive ticking was also abolished after the application of the antagonist and recovered after washout (Fig. 3*B*). We conclude that 5-HT₂-like receptors are important for 5-HT-induced fictive vocalizations in both sexes.

We next examined whether the activation of 5-HT₂-like receptors can activate fictive vocalizations in both sexes. Before agonist treatment, all brains were silent (Fig. 3, *C* and *D*, left traces). However, bath application of a 5-HT₂ receptor agonist (α -Me-5-HT, 30 μ M, ≤ 10 min) initiated fictive advertisement calls and ticking (Fig. 3, *C* and *D*, right traces) from all male and female brains, respectively ($n = 5$ and 5, respectively). Fictive ticking was also elicited by the agonist in one male brain (data not shown). The total number of advertisement call bouts initiated by the agonist was similar to those initiated by 5-HT (Tables 1 and 2). Thus the effect of the 5-HT₂ receptor agonist in initiating fictive vocalizations was no different from the effect of 5-HT. These results suggest that 5-HT

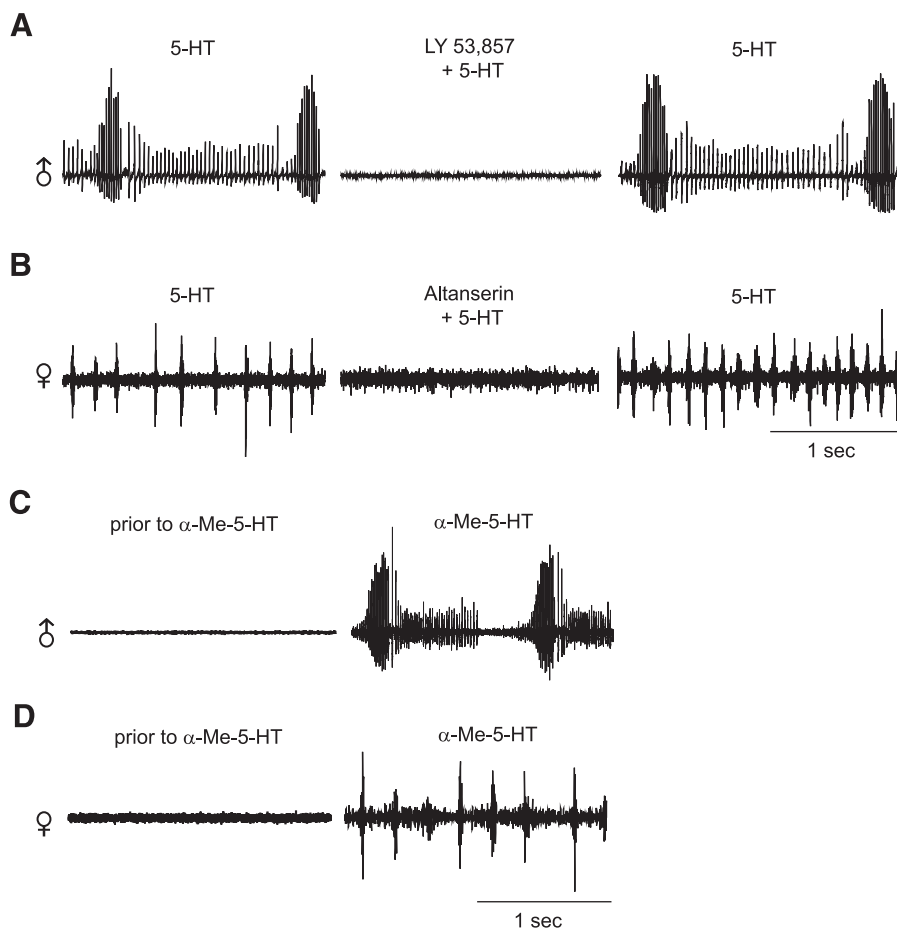


FIG. 3. 5-HT₂-like receptors are important for the initiation of fictive vocalizations in males and females. *A*: 5-HT induces fictive advertisement call before (*left*) and after washout (*right*) but not during (*middle*) 5-HT₂ receptor antagonist (LY 53857; 100 μ M, 10 min) application in a male brain. *B*: 5-HT induces fictive ticking before (*left*) and after washout (*right*) but not during (*middle*) 5-HT₂ receptor antagonist (altanserin; 100 μ M, 10 min) application in a female brain. *C*: fictive advertisement calls were recorded from a male brain only when a 5-HT₂ receptor agonist (α -Me-5-HT; 30 μ M; 5 min) was applied (*left*) and not before the treatment (*right*). *D*: before treatment with agonist, brains do not produce fictive ticking (*left*). Application of a 5-HT₂ receptor agonist (α -Me-5-HT; 30 μ M; 5 min) initiates fictive ticking in a female brain (*right*).

initiates fictive vocalizations by binding to 5-HT₂-like receptors in both sexes. In the case of male brains, activation of 5-HT₂ receptors may mediate the activation of ticking in addition to advertisement call.

Which 5-HT₂ receptors play a role in activating fictive vocalizations?

There are three known subtypes of 5-HT₂ receptors. To determine which subtype(s) are involved in vocal initiation and

whether the sexes use different subtypes, we carried out further pharmacological experiments using more selective agents.

5-HT_{2A} receptors

5-HT_{2A} receptor antagonists failed to block 5-HT-induced fictive vocalizations in both sexes. When 5-HT was applied to male and female brains ($n = 5$ and 5, respectively) that were preincubated with the 5-HT_{2A} receptor antagonist, ketanserin (40 μ M, 5 min), fictive advertisement calls and ticking were

TABLE 1. Temporal structure and the vocal activity of calls induced under experimental and control conditions in males

Receptor	Drug	μ_1		μ_2		Max Sust. CAP Rate		No. Bouts		<i>n</i>
		Control	Experiment	Control	Experiment	Control	Experiment	Control	Experiment	
5-HT ₂	α -Me-5-HT	30.85 \pm 9.69	34.66 \pm 7.02	52.89 \pm 3.44	54.61 \pm 3.34	57.34 \pm 3.78	56.94 \pm 2.26	15.98 \pm 2.02	11.38 \pm 2.94	5
5-HT _{2A}	Ketanserin	26.12 \pm 8.16	30.66 \pm 8.44	50.94 \pm 0.75	52.64 \pm 3.80	54.63 \pm 2.38	61.22 \pm 10.64	13.89 \pm 1.87	13.16 \pm 2.11	5
5-HT _{2A}	MDL	22.77 \pm 7.75	28.04 \pm 6.06	56.17 \pm 6.81	54.13 \pm 2.86	58.64 \pm 8.07	60.45 \pm 4.64	8.65 \pm 3.73	9.67 \pm 2.05	5
5-HT _{2B}	SB 204,741	25.19 \pm 5.96	33.39 \pm 10.83	54.05 \pm 3.56	55.62 \pm 5.53	54.92 \pm 3.94	57.68 \pm 5.32	38.76 \pm 1.78	14.88 \pm 2.13	5
5-HT _{2B}	SB 215505	32.19 \pm 5.77	24.53 \pm 13.46	55.59 \pm 3.08	52.83 \pm 2.92	57.49 \pm 3.56	56.22 \pm 2.51	23.97 \pm 3.86	4.20 \pm 3.11	5
5-HT _{2C}	Ro 60-0175	30.85 \pm 9.69	21.92 \pm 5.19	52.89 \pm 3.44	52.47 \pm 3.31	57.34 \pm 3.78	54.60 \pm 3.21	15.98 \pm 2.02	3.77 \pm 2.2	7
5-HT _{2C}	MK-212	30.85 \pm 9.69	23.17 \pm 5.82	52.89 \pm 3.44	54.58 \pm 6.98	57.53 \pm 3.49	58.01 \pm 7.43	15.98 \pm 2.02	8.58 \pm 5.11	5
5-HT _{2A/C}	DOI	31.30 \pm 9.69	23.20 \pm 6.58	53.79 \pm 3.44	53.29 \pm 4.67	57.45 \pm 3.78	56.28 \pm 4.90	15.98 \pm 2.02	8.19 \pm 3.70	6
5-HT _{2C}	DOI/Ket	23.02 \pm 8.38	34.20 \pm 9.32	56.76 \pm 6.72	58.86 \pm 0.16	59.67 \pm 7.53	62.29 \pm 2.18	15.98 \pm 2.02	12.79 \pm 1.41	3

Mean and SD of instantaneous CAP rate for slow trill (μ_1) and fast trill (μ_2), maximum sustained CAP rate, and the number of bouts for each condition. For experiments in which 5-HT_{2A} and 5-HT_{2B} receptor antagonists are used, measurements are made between calls induced in response to 5-HT alone (*control*) and in response to 5-HT in the presence of the antagonists (*experiment*) from the same set of preparations with sample size indicated in the table. For experiments in which agonists are used, measurements are made between calls induced in response to agonists by the experimental preparations (sample size indicated in the table) and in response to 5-HT by control preparations ($n = 5$). CAP, compound action potentials.

TABLE 2. Comparisons of call structure and the number of calls induced under different experimental conditions in males

Receptor	Drug	μ_1		μ_2		Max Sust CAP Rate		No. Bouts		<i>n</i>
		Test Statistic	<i>P</i>	Test Statistic	<i>P</i>	Test Statistic	<i>P</i>	Test Statistic	<i>P</i>	
5-HT ₂	α -Me-5-HT	U = -1.15	0.25	U = 0.73	0.46	U = 0.00	1.00	U = -0.31	0.75	5
5-HT _{2A}	Ketanserin	Z = -1.46	0.14	Z = -0.67	0.50	Z = -1.48	0.14	Z = -0.14	0.89	5
5-HT _{2A}	MDL	Z = -1.21	0.22	Z = -0.14	0.89	Z = -0.67	0.50	Z = -0.41	0.69	5
5-HT _{2B}	SB 204,741	Z = -0.94	0.35	Z = -0.94	0.35	Z = -1.75	0.08	Z = -2.02	0.04*	5
5-HT _{2B}	SB 215505	Z = -1.46	0.14	Z = -1.21	0.22	Z = -0.67	0.50	Z = -1.10	0.27	5
5-HT _{2C}	Ro 60-0175	U = -1.89	0.06	U = -0.08	0.94	U = -1.22	0.22	U = -2.52	0.01*	7
5-HT _{2C}	MK-212	U = -1.15	0.25	U = -0.73	0.46	U = -0.52	0.60	U = -0.52	0.60	5
5-HT _{2A/C}	DOI	U = -0.73	0.46	U = -0.31	0.75	U = -0.10	0.92	U = -0.82	0.41	6
5-HT _{2C}	DOI/Ket	U = -1.34	0.18	U = -1.34	0.18	U = -1.34	0.18	U = -0.75	0.46	3

For experiments in which 5-HT_{2A} and 5-HT_{2B} receptor antagonists are used, comparisons are made between calls induced in response to 5-HT alone (*control*) and in response to 5-HT in the presence of the antagonists (*experiment*) by the same preparations using a Wilcoxon signed-rank test. For experiments in which agonists are used, comparisons are made between calls induced in response to agonists by the experimental preparations (sample size indicated in each row) and in response to 5-HT by control preparations (*n* = 5) using Mann-Whitney *U*-tests. *Z*-values are listed for the test statistic for Wilcoxon signed-rank test and *U*-values are listed for the test statistic for Mann-Whitney *U*-test. Asterisks by the *P* value indicate significant differences.

readily induced in all males and females (Fig. 4, *A* and *B*, respectively, right traces). Fictive ticking was also observed in three males before and after treatment with ketanserin (data not shown), indicating that 5-HT_{2A}-like receptors are likely not required for initiation of male fictive ticking. To rule out the possibility that the dose of the antagonist was too low or the duration of exposure was too brief, we repeated the experiment with a higher dose (ketanserin, 100 μ M, 5 min, 1 male), and for a longer duration (ketanserin, 50 μ M, 10 min, 1 male). Both treatments still failed to block 5-HT-induced fictive advertisement calls. When a different 5-HT_{2A} receptor antagonist (MDL, 50 μ M, 10 min) was applied to a different set of male and female brains (*n* = 5 and 5, respectively), 5-HT-induced fictive advertisement calls and ticking persisted in all males and females (traces not shown). The overall vocal activity in male brains (total number of bouts) was unaffected by 5-HT_{2A} receptor antagonists (Tables 1 and 2). Because two types of 5-HT_{2A} receptor antagonist failed to block 5-HT-induced fictive vocalizations and had no effect on the total amount of vocalizations in males, we conclude that 5-HT_{2A}-like receptors are likely not important for 5-HT-induced fictive vocalizations in either sex.

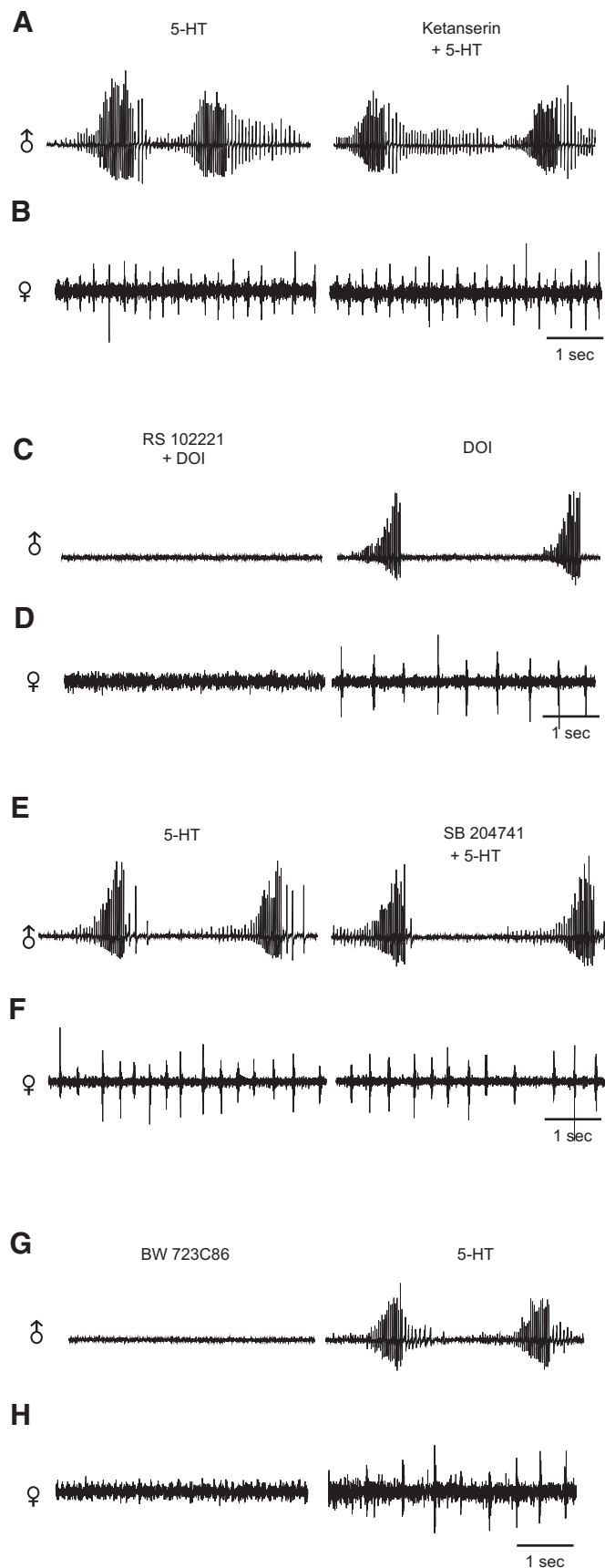
We next tested if activation of 5-HT_{2A}-like receptors can initiate fictive vocalizations in the sexes. Because there is no 5-HT_{2A}-selective agonist commercially available we used a combination of the 5-HT_{2A/C} receptor agonist DOI and the 5-HT_{2C} receptor antagonist RS 102221 (Krebs-Thomson et al. 1998). Combined application of agonists and antagonists has been performed routinely to dissect mixed actions of agonists in other behavioral models (Bishop et al. 2004; Krebs-Thomson et al. 1998; Wolf et al. 1999). Application of DOI (50 μ M, 10 min) to male (*n* = 2) and female (*n* = 3) brains preincubated with RS 102221 (50 μ M, 15 min) failed to activate fictive advertisement calls or ticking (Fig. 4C and D, left traces), whereas DOI (50 μ M, 10 min) alone readily activated fictive vocalizations in both sexes (Fig. 4C, D, right traces). In a third male brain, application of α -Me-5-HT (30 μ M, 10 min), a broad-spectrum 5-HT₂ receptor agonist that readily activates fictive vocalizations when applied alone (see above), failed to initiate fictive advertisement call when it was preincubated with the 5-HT_{2C} receptor selective antagonist, RS 102221 (50 μ M, 15 min) and the 5-HT_{2B} receptor selective antagonist, SB 215505 (50 μ M, 15 min) verifying our results that activation of

5-HT_{2A} receptors is not sufficient for initiation of fictive vocal behavior. Thus we conclude that 5-HT_{2A}-like receptors are likely not critical for vocal initiation in the sexes.

5-HT_{2B} receptors

5-HT_{2B} receptor antagonists were mostly ineffective in blocking 5-HT-induced fictive vocalizations in both sexes. When 5-HT was applied to male (*n* = 5) and female (*n* = 5) brains that were preincubated with a 5-HT_{2B} receptor antagonist (SB 204741, 100 μ M, 20 min), fictive advertisement calls and ticking were initiated in all male and female brains, respectively (Fig. 4, *E* and *F*, right traces). However, males produced significantly fewer bouts of advertisement call in the presence of the antagonist compared with controls (5-HT alone; Tables 1 and 2). To explore the role of the 5-HT_{2B}-like receptor further, we used another type of 5-HT_{2B} antagonist on a different set of brains. When SB 215505 (50 μ M, 15 min) was applied to male (*n* = 6) and female (*n* = 5) brains, the antagonist failed to block fictive advertisement calls in five of six male brains and all five female brains (traces not shown). The antagonist also failed to block 5-HT-induced fictive ticking that was produced by one of the male brains (data not shown). The overall vocal activity was not reduced by SB 215505 (Tables 1 and 2). Although it is not clear why SB 215505 blocked 5-HT-induced fictive vocalizations in one brain but not in the other five brains, our results showed that two different types of 5-HT_{2B} antagonist failed to block 5-HT-induced vocalizations in a majority of the brains (91% male, 100% female brains). The decreased number of bouts obtained in the presence of SB 204741 may indicate that the antagonists may reduce the overall excitability of the vocal circuit without blocking the vocal initiation mechanism. Taken together, the results are consistent with the idea that 5-HT_{2B}-like receptors are not critical for initiating fictive vocalizations in the sexes.

We next tested the effect of activating 5-HT_{2B}-like receptors on vocal production in the sexes. Application of a 5-HT_{2B} receptor agonist (BW 723C86, 100 μ M, \leq 20 min) to male (*n* = 5) and female (*n* = 5) brains yielded no fictive advertisement call or ticking (Fig. 4, *G* and *H*, left traces), even though subsequent application of 5-HT resulted in fictive vocalizations in all brains (Fig. 4, *G* and *H*, right traces). Furthermore, we tested the role of activating 5-HT_{2B}-like



receptors by using a combination of drugs that effectively acts as a 5-HT_{2B} receptor agonist: a broad spectrum 5-HT₂ receptor agonist combined with antagonists to 5-HT_{2A} receptors and 5-HT_{2C} receptors ($n = 5$ males, 5 females). This combination of drugs was used because there is no other selective 5-HT_{2B} receptor agonist available. Although all brains responded initially to 5-HT₂ receptor agonist alone, as shown earlier (Fig. 3, C and D), the combination of α -Me-5-HT (5-HT₂ agonist 30 μ M) + ketanserin (5-HT_{2A} antagonist, 40 μ M) + RS 102221 (5-HT_{2C} antagonist, 50 μ M, all applied for 15 min) failed to elicit any fictive advertisement calls or ticking from all male and female brains, respectively. All brains subsequently recovered from the effects of the antagonists (traces not shown). Thus the activation of 5-HT_{2B}-like receptors does not result in vocal initiation in either sex. Taken together, we concluded that 5-HT_{2B}-like receptors are not critical for vocal initiation in the sexes because the agonists failed to initiate vocalizations and the antagonists exhibited very minor effects, if any, on the fictive vocalizations. It is possible, however, that the activation of 5-HT_{2B}-like receptors may play a supportive role in vocal initiation and production in males.

5-HT_{2C} receptors

In contrast to 5-HT_{2A} and 5-HT_{2B} receptors, we discovered that 5-HT_{2C}-like receptors are important for 5-HT-evoked fictive vocalizations in both sexes. 5-HT_{2C} receptor selective antagonists blocked fictive vocalizations in both sexes. When 5-HT was applied to male ($n = 5$) and female ($n = 5$) brains preincubated with the 5-HT_{2C} receptor antagonist (RS 102221; 50 μ M, 15 min), fictive vocalizations were not initiated from any of the brains (Fig. 5, A and B, middle). The antagonist also blocked 5-HT-induced fictive ticking that we observed in two of the male brains before the antagonist application (data not shown). In one brain, this experiment was repeated twice after the recovery from the first application of antagonist: the 5-HT_{2C} receptor antagonist blocked vocalizations both times. These results cannot be ascribed to ill health of the tissue because all males and females recovered from the effect of the antagonist and later responded to 5-HT with fictive vocalizations (Fig. 5, A and B, right). In the two male brains that initially produced both advertisement call and ticking, both call types recovered on washout. To further confirm the importance of 5-HT_{2C}-like receptors, we used an additional 5-HT_{2C} re-

FIG. 4. Vocal initiation is not governed by the activation of 5-HT_{2A}-like and 5-HT_{2B}-like receptors. A: 5-HT induces male fictive advertisement call before (left) and during (right) 5-HT_{2A} receptor antagonist (ketanserin, 40 μ M, 5 min) administration. B: 5-HT induces fictive ticking before (left) and during (right) 5-HT_{2A} receptor antagonist administration. C: 5-HT_{2A/C} receptor agonist (DOI, 50 μ M, 10 min) evokes fictive advertisement call (right) but not in the presence of a 5-HT_{2C} receptor antagonist (RS102221, 50 μ M, 15 min; left). D: 5-HT_{2A/C} receptor agonist (DOI, 50 μ M, 10 min) evokes fictive ticking (right) but not in the presence of a 5-HT_{2C} receptor antagonist (RS102221, 50 μ M, 15 min). E: 5-HT induces male fictive advertisement call before (left) and during (right) the application of 5-HT_{2B} receptor antagonist (SB 204741, 100 μ M, 20 min). F: 5-HT induces female fictive ticking before (left) and during (right) the application of 5-HT_{2B} receptor antagonist (SB 204741, 100 μ M, 20 min). G: 5-HT_{2B} receptor agonists (BW 723C86, 100 μ M, 20 min) do not elicit fictive advertisement call from male brains (left), yet subsequent application of 5-HT to the same brains initiates fictive advertisement calls (right). H: 5-HT_{2B} receptor agonists do not activate fictive ticking from female brains (left), although subsequent application of 5-HT to the same brains initiates fictive ticking (right). Scale = 1 s for all traces.

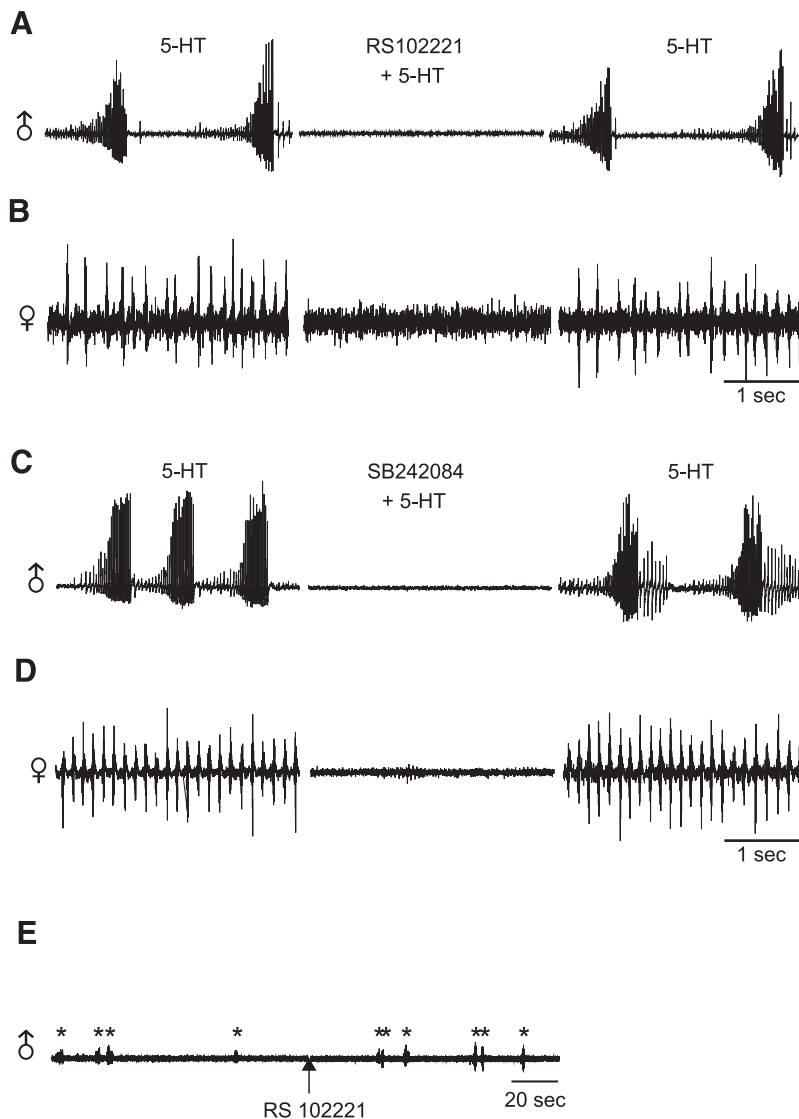


FIG. 5. Blocking 5-HT_{2C}-like receptors prevents 5-HT-induced fictive vocal behavior in both males and females but does not block respiration. *A*: 5-HT initiates male fictive advertisement calls before (*left*) and after the wash out (*right*) but not during 5-HT_{2C} receptor antagonist (RS 102221, 50 μ M, 10 min) administration. *B*: 5-HT initiates female fictive ticking before (*left*) and after the wash out (*right*) but not during (middle) 5-HT_{2C} receptor antagonist (RS 102221, 50 μ M, 10 min) administration. *C*: 5-HT induces male fictive advertisement calls before (*left*) and after the wash out (*right*) but not during an alternative 5-HT_{2C} receptor antagonist (SB242084, 100 μ M, 10 min) administration. *D*: 5-HT induces female fictive ticking before (*left*) and after the wash out (*right*) but not during (middle) administration of an alternative 5-HT_{2C} receptor antagonist (SB242084, 100 μ M, 10 min). *E*: 5-HT_{2C} receptor antagonists do not block fictive breathing. An example trace from a male brain showing episodes of fictive breathing before (*left* trace) and during (*right* trace) application of a 5-HT_{2C} receptor antagonist (RS 102221; 50 μ M, 15 min). The trace is truncated at the time of antagonist application indicated by an arrow. Asterisks indicate episodes of fictive breathing.

ceptor antagonist (SB 242084; 100 μ M, 15 min) on five new males and five new females. This antagonist also blocked fictive vocalizations in all male and female brains (Fig. 5, *C* and *D*, middle traces). Subsequently, these brains also recovered from the effect of the antagonist after washout (Fig. 5, *C* and *D*, right traces). From these experiments, we concluded that 5-HT initiates both types of fictive calls (advertisement calls and ticking) by activating 5-HT_{2C}-like receptors in *Xenopus* brains.

Interestingly, the activation of 5-HT_{2C}-like receptors influenced vocalizations without affecting the respiratory rhythm. The respiratory activity in addition to vocal activity can be monitored via the laryngeal nerve recordings (Rhodes et al. 2007; Zornik and Kelley 2008), because the fourth rootlet of nerve IX–X contains axons of both glottal and laryngeal motoneurons (Simpson et al. 1986). Unlike vocalizations, the respiratory activity occurs spontaneously in this whole brain preparation. Although the 5-HT_{2C} receptor antagonists blocked 5-HT-induced fictive vocalizations, they failed to block respiratory activity in either sex (Fig. 5*E*, male example shown with RS 102221). Likewise, the 5-HT₂ receptor antagonists used in this study did not affect fictive respiratory activity either (data

not shown). Thus selective blockade of 5-HT_{2C}-like receptors eliminates vocal activity without affecting the other motor systems.

Finally, to test if the activation of 5-HT_{2C}-like receptors can initiate fictive vocalizations in both sexes, we used three types of 5-HT_{2C} receptor agonists: MK-212, Ro 60–0175, and a combination of DOI, the 5-HT_{2A/C} receptor agonist, and ketanserin, the 5-HT_{2A} receptor antagonist (Barnes and Sharp 1999). Application of MK-212 to silent brains (Fig. 6, *A* and *B*, left traces) elicited fictive vocalizations (Fig. 6, *A* and *B*, right traces) in all brains (5 male and 5 female brains). The number of call bouts induced by MK-212 in male brains was similar to those induced by 5-HT (Tables 1 and 2).

Application of another 5-HT_{2C} receptor agonist, Ro 60–0175 (50 μ M, \leq 10 min) to male ($n = 7$) and female ($n = 5$) brains led to mixed results. In males, Ro 60–0175 applied to silent brains (Fig. 6*C*, left trace) induced fictive advertisement calls in all seven brains (Fig. 6*C*, right trace) and fictive ticking from four of these brains (data not shown). However, the number of bouts initiated by the agonist was significantly less than that initiated by 5-HT (Tables 1 and 2). Thus Ro 60–0175 was able to initiate fictive advertisement calls in male brains,

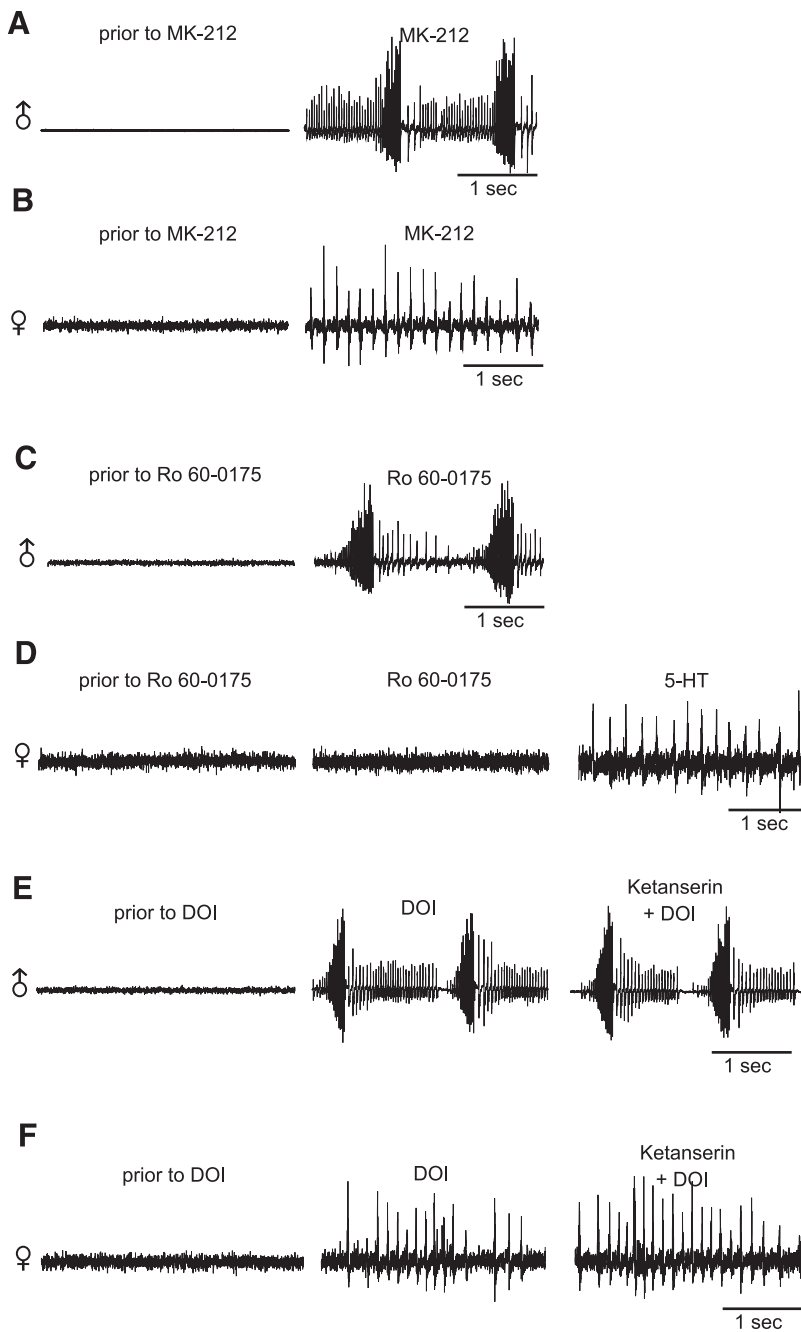


FIG. 6. 5-HT_{2C} receptor agonists initiate fictive vocalizations. *A* and *B*: a 5-HT_{2C} receptor agonist (MK-212, 50 μ M, 10 min) initiates fictive advertisement calls from male brains and fictive ticking from female brains (right traces), whereas before application of the agonist the brains were silent (left traces). *C*: an alternative 5-HT_{2C} receptor agonist, Ro 60-0175 (50 μ M, 10 min) initiates fictive advertisement calls in male brains (right), whereas before application of the agonist the brains were silent (left). *D*: the same 5-HT_{2C} receptor agonist, Ro 60-0175 (50 μ M, 10 min) fails to initiate fictive ticking in female brains (middle). This was not caused by ill health of the brains because all brains were capable of ticking in response to 5-HT (right). *E*: the application of 5-HT_{2A/C} receptor agonist (DOI; 50 μ M, 10 min) to male brains preincubated with 5-HT_{2A} receptor antagonist (ketanserin, 40 μ M, 5 min, $n = 3$) initiates fictive advertisement calls (right), as in application of DOI alone (middle). Before treatment, all brains were silent (left). *F*: the application of 5-HT_{2A/C} receptor agonist (DOI; 50 μ M, 10 min) to female brains preincubated with 5-HT_{2A} receptor antagonist (ketanserin, 40 μ M, 5 min, $n = 3$) initiates fictive ticking (right), as in application of DOI alone (middle). Before treatment, all brains were silent (left).

although it is not as potent as 5-HT in maintaining the vocal activity. In female brains, in contrast, Ro 60-0175 (50 μ M, ≤ 10 min) was largely ineffective in initiating fictive vocalizations; fictive ticking was induced in only one female (data not shown), and not in other four (Fig. 6*D*, middle). These results were not caused by the ill health of the tissue, because all five of the female brains responded to subsequent 5-HT application with fictive ticking (Fig. 6*D*, right). Thus Ro 60-0175 initiated fictive vocalizations in males but not in females.

The results obtained using the final agonist, a combination of DOI, the 5-HT_{2A/C} receptor agonist (50 μ M, 10 min) co-applied with the 5-HT_{2A} receptor antagonist (ketanserin, 40 μ M, 5 min), were more consistent with the results obtained using MK-212. The application of the combination agonist

readily activated fictive vocalizations in all female ($n = 3$) and male ($n = 3$) brains (Fig. 6, *E* and *F*, right traces). The overall vocal activity in male brains was no different from that induced by 5-HT in control brains (Tables 1 and 2). As described above (Fig. 4*D*, left trace), DOI-induced fictive vocalizations can be blocked by the 5-HT_{2C} receptor antagonist (RS 102221, 50 μ M, 15 min) in all female and male brains tested, indicating that DOI initiates fictive vocalizations by acting on 5-HT_{2C}-like receptors. Thus we used DOI alone as a 5-HT_{2C} receptor agonist in seven additional female and three additional male brains and confirmed that fictive vocalizations were induced from all brains.

In summary, all three types of 5-HT_{2C} receptor agonists initiated fictive vocalizations from all male brains and two out of three types of 5-HT_{2C} receptor agonists served a virtually

identical function as 5-HT in females. Although it is not clear why Ro 60–0175 was less effective than other agonists in initiating vocalizations in females and less potent in maintaining the vocal activity in males, we conclude that the 5-HT_{2C}-like receptor is by far the most important of all three types of 5-HT₂ receptors for the activation of fictive vocalizations in both males and females; blockade of 5-HT_{2C}-like receptors prevents 5-HT-initiated fictive vocalizations, and activation of 5-HT_{2C}-like receptors initiates fictive vocalizations in both sexes. Thus 5-HT most likely binds to 5-HT_{2C}-like receptors to initiate fictive vocalizations in both sexes.

Temporal morphology of fictive vocalizations in the presence of pharmacological agents is no different from that induced by 5-HT alone

Finally, we examined whether the temporal morphology of fictive vocalizations induced by 5-HT_{2C} agonists and that induced by 5-HT in the presence of 5-HT_{2A} and 5-HT_{2B}

antagonists differs from the temporal morphology of fictive vocalizations induced by 5-HT alone. Although we showed that the activation of 5-HT_{2C}-like receptors mediates vocal initiation (as evident in the presence or absence of fictive vocalizations in response to the agonists and antagonists), it is possible that other types of 5-HT receptors are important for generation and maintenance of vocal patterns. To this end, we compared the temporal morphology of fictive vocalizations. Mean instantaneous CAP rates [estimates of slow and fast trill rates (μ_1 and μ_2) in males, and ticking rates (μ) in females, see METHODS] and maximum sustained CAP rates obtained under all experimental and control conditions (Fig. 7) were examined using a Kruskal-Wallis test. The results showed that the application of any of the agonists or antagonists used in this study did not account for variability in the temporal parameters of the fictive vocalizations (Kruskal-Wallis test-males μ_1 : $H = 12.86$, $P = 0.23$; μ_2 : $H = 2.99$, $P = 0.93$; maximum sustained CAP rate: $H = 9.18$, $P = 0.33$; number of calls: $H = 8.76$, $P = 0.36$;

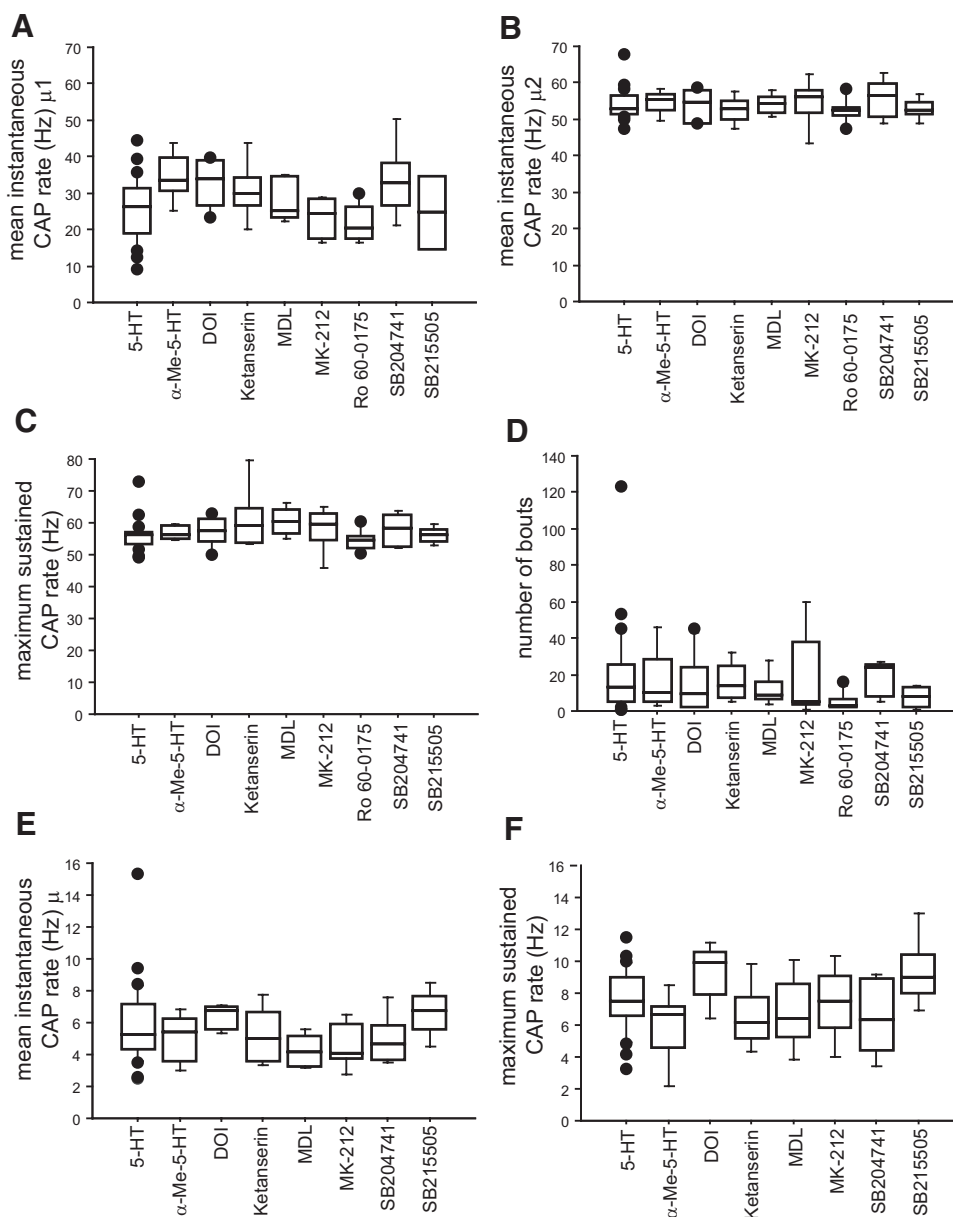


FIG. 7. Vocalizations initiated in the presence of agonists and antagonists are similar to those induced by 5-HT alone. *A*: box plots of mean instantaneous CAP rate for slow trills (μ_1) in males divided by treatment; $H = 12.86$, $P = 0.07$. *B*: box plots of mean instantaneous CAP rate for fast trills (μ_2) in males by treatment; $H = 2.99$, $P = 0.93$. *C*: box plots of maximum sustained CAP rate for male advertisement call across treatment; $H = 9.18$, $P = 0.33$. *D*: box plots of number of bouts of advertisement call across treatment; $H = 8.76$, $P = 0.36$. *E*: box plots of mean instantaneous CAP rate for ticking (μ) in females by treatment; $H = 9.27$, $P = 0.23$. *F*: box plots of maximum sustained CAP rate for female ticking by treatment; $H = 10.74$, $P = 0.15$. All comparisons were done using the Kruskal-Wallis test. Lines within boxes indicate median, box bounds are 25th and 75th percentiles, whiskers are 10th and 90th percentiles, and dots indicate data <10th percentile or >90th percentile.

females μ : $H = 9.27$, $P = 0.23$; maximum sustained CAP rate: $H = 10.74$, $P = 0.15$).

Furthermore, in case there were some subtle differences that were not detected in the Kruskal-Wallis test, we carried out pairwise comparisons. To test the effect of 5-HT_{2A} and 5-HT_{2B} antagonists, we compared the morphology of 5-HT-induced calls in the presence and absence of the antagonists obtained from the same set of brains using a Wilcoxon signed-rank test (i.e., within-individual comparison). The results showed that none of the antagonists modified the morphology of fictive vocalizations in the sexes (males: Tables 1 and 2; females: Tables 3 and 4). To test the effect of 5-HT₂ and 5-HT_{2C} agonists, we compared the agonist-induced fictive vocalizations to 5-HT-induced vocalizations obtained from five control male and female brains using a Mann-Whitney U test, because 5-HT was not applied to the brain tested with the agonists (see METHODS). The results showed that fictive songs induced by the agonists were also similar to those induced by 5-HT (males: Tables 1 and 2; females: Tables 3 and 4).

Vocalizations of *Xenopus* are variable both in vivo (Fig. 1A) and in vitro (Fig. 7). The results showed that fictive vocalizations initiated by the activation of 5-HT_{2C}-like receptors fall within the normal range of variability found in 5-HT-induced fictive vocalizations. We conclude that 5-HT initiates fictive vocalizations by binding to 5-HT_{2C}-like receptors in both sexes.

DISCUSSION

Previous studies have shown that *Xenopus* serotonergic neurons project to the brain stem vocal nuclei, and application of exogenous 5-HT evokes fictive vocalizations from isolated brains (Rhodes et al. 2007). These results establish a role for 5-HT in vocal initiation in male and female *Xenopus*. In this study, we set out to identify the type of 5-HT receptors that mediate vocal activation and asked whether they differ between the sexes.

To determine the types of 5-HT receptors that are of functional importance to the vocal pathways, pharmacological experiments were conducted using isolated brain preparations of male and female *X. laevis*. Of the seven identified types of 5-HT receptors, we suspected that the 5-HT₂-like receptor may be the key component for the vocal pathways of *Xenopus*, both because of its involvement in activation and modulation of

rhythmic motor patterns in other systems (Hattox et al. 2003; Pearlstein et al. 2005; Tryba et al. 2006; Xiang et al. 2005) and because of its role in the reproductive behavior of mammals (Bancila et al. 1999; Heisler et al. 2007; Millan et al. 1997; Stafford et al. 2006; Wada et al. 2006; Wolf et al. 1999). Based on these pharmacological studies, we conclude that 5-HT_{2C}-like receptors mediate vocal initiation in *X. laevis*.

Pharmacology in an amphibian model system

There are several challenges associated with pharmacological identification of the receptor types that mediate vocal activation in *X. laevis*. The first challenge is that the pharmacological agents used in this study were originally developed for mammalian species. However, we consider the evolutionary distance of the species to be less of an issue because these pharmacological agents have been previously used routinely and effectively in amphibian species including *X. laevis* (Holohean and Hackman 2004; Holohean et al. 1990; Scrymgeour-Wedderburn et al. 1997). The second challenge is "cross-talk" exhibited by most drugs on some level. For example, the 5-HT₂ receptor antagonist, altanserin, is known to activate adrenergic receptors in addition to 5-HT₂ receptors (Megens et al. 1986). Our strategy to tackle this problem was to use multiple drugs for a given receptor, to rule out the possibility that "cross-talk" with other receptors caused the observed effects. To this end, we used at least two drugs, and sometimes three, to reach a conclusion. In the case of altanserin, we used two additional antagonists, RS 102221 and SB 242084, each with high selectivity for the 5-HT_{2C} receptor and no affinity for adrenergic receptors (Bonhaus et al. 1997; Kennett et al. 1997) and discovered that they both blocked fictive vocalizations as altanserin did. Similarly, we ruled out the possibility that activation of receptors other than 5-HT_{2C} receptors induced fictive vocalizations because at least two agonists (a selective agonist, MK-212 and a combination agonist/antagonist, DOI/ketanserin), each with different binding affinities for other receptors (Knight et al. 2004; Porter et al. 1999), were effective in eliciting fictive vocalizations. The third challenge is determining the concentration of the drugs to be used. We believe the concentration of drugs used in this study was appropriate for our in vitro whole brain preparation compared with other studies. For example, although ketanserin is reported to block 5-HT_{2C} receptors in addition to 5-HT_{2A} receptors (although the

TABLE 3. Temporal structure of calls induced under experimental and control conditions in females

Receptor		μ		Max Sust. CAP Rate		<i>n</i>
		Control	Experiment	Control	Experiment	
5-HT2	α -Me-5-HT	5.40 \pm 1.99	5.03 \pm 4.59	5.62 \pm 2.67	5.90 \pm 2.37	5
5-HT2A	Ketanserin	6.58 \pm 5.11	5.20 \pm 1.86	7.49 \pm 1.94	6.54 \pm 2.10	5
5-HT2A	MDL	4.37 \pm 1.67	4.26 \pm 1.06	6.19 \pm 2.54	6.82 \pm 2.39	5
5-HT2B	SB 204,741	5.47 \pm 1.15	1.63 \pm 1.63	7.12 \pm 1.31	6.50 \pm 2.52	5
5-HT2B	SB 215505	7.02 \pm 1.47	6.61 \pm 1.49	8.92 \pm 1.44	9.37 \pm 2.28	5
5-HT2C	MK-212	5.40 \pm 1.99	4.63 \pm 1.49	5.19 \pm 2.04	7.40 \pm 2.38	5
5-HT2A/C	DOI	5.40 \pm 1.99	6.37 \pm 0.81	7.99 \pm 3.92	9.27 \pm 1.88	7
5-HT2C	DOI/Ket	5.40 \pm 1.99	4.75 \pm 2.67	7.99 \pm 3.92	5.19 \pm 2.94	3

Mean and SD of instantaneous CAP rate (μ) and maximum sustained CAP rate for each condition. For experiments in which 5-HT_{2A} and 5-HT_{2B} receptor antagonists are used, measurements are made between calls induced in response to 5-HT alone (control) and in response to 5-HT in the presence of the antagonists (experiment) from the same set of preparations. For experiments in which agonists are used, measurements are made between calls induced in response to agonists by the experimental preparations and in response to 5-HT by control preparations ($n = 5$). See Table 1 for abbreviation.

TABLE 4. Comparisons of call structure of fictive calls induced under different experimental conditions in females

Receptor	Drug	μ		Max Sust. CAP Rate		<i>n</i>
		Test Statistic	<i>P</i>	Test Statistic	<i>P</i>	
5-HT ₂	α -Me-5-HT	U = -0.52	0.60	U = -0.10	0.92	5
5-HT _{2A}	Ketanserin	Z = -0.41	0.69	Z = -1.21	0.22	5
5-HT _{2A}	MDL	Z = -0.67	0.50	Z = -0.67	0.50	5
5-HT _{2B}	SB 204,741	Z = -1.75	0.80	Z = -0.67	0.50	5
5-HT _{2B}	SB 215505	Z = -0.41	0.69	Z = -0.14	0.89	5
5-HT _{2C}	MK-212	U = -0.52	0.60	U = -1.57	0.12	5
5-HT _{2A/C}	DOI	U = -0.52	0.60	U = -0.94	0.35	7
5-HT _{2C}	DOI/Ket	U = -0.75	0.46	U = -1.94	0.05	3

For experiments in which 5-HT_{2A} and 5-HT_{2B} receptor antagonists are used, comparisons are made between calls induced in response to 5-HT alone (*control*) and in response to 5-HT in the presence of the antagonists (*experiment*) by the same preparations using Wilcoxon signed-rank test. For experiments in which agonists are used, comparisons are made between calls induced in response to agonists by the experimental preparations and in response to 5-HT by control preparations using Mann-Whitney *U*-test. Sample size listed in each row represents the number of experimental preparations used for the analyses. *Z*-values are listed for the test statistic for Wilcoxon signed-rank test and *U*-values are listed for the test statistic for Mann-Whitney *U*-test. See Table 1 for abbreviation.

selectivity is 2 orders of magnitude lower for the 5-HT_{2C} receptor than for the 5-HT_{2A} receptor; Barnes and Sharp 1999; Baxter et al. 1995), it is unlikely that the 5-HT_{2C} receptors were blocked by the dose of ketanserin used in our study, because when a similar dose (10 μ M) was applied to a brain slice preparation (a preparation into which a drug penetrates more readily), it blocked the 5-HT_{2A} receptor-mediated persistent sodium currents without blocking the 5-HT_{2C} receptor-mediated persistent sodium currents in spinal motoneurons of a rat (Harvey et al. 2006). Similarly, a concentration of DOI similar to that used in this study was used to activate 5-HT₂ receptors in a mammalian slice preparation (Chen et al. 2003). Thus we believe the conclusion we reached after using a battery of the pharmacological agents at carefully determined doses in this study is appropriate—the 5-HT-induced initiation of fictive vocalization depends on, and results from, activation of 5-HT_{2C}-like receptors in both sexes.

How do 5-HT_{2C}-like receptors initiate vocal behavior in *Xenopus*?

Where are the 5-HT_{2C}-like receptors distributed within the brain, and how do they overlap with the central vocal pathways? Although the location of 5-HT_{2C}-like receptors within the brain was not identified in this study, the *Xenopus* brain stem contains the central pattern generator, a network of neurons that generates vocalizations without sensory feedback (Rhodes et al. 2007). The vocal nuclei in the *Xenopus* brain stem include laryngeal motor nucleus (n.IX–X), the raphe nucleus, and the dorsal tegmental area of medulla (DTAM), a major premotor nucleus. In particular, the importance of DTAM in generating fictive vocalization is apparent in *Xenopus*. Bilateral removal of DTAM abolishes 5-HT-induced fictive vocalizations in both sexes, electrical stimulation delivered to the male DTAM results in fictive fast trills in the absence of exogenous 5-HT (Rhodes et al. 2007), and selective cooling of male DTAM results in reduced CAP rates of advertisement calls (Yamaguchi et al. 2009). At the cellular level, 5-HT_{2C} receptors are known to depolarize the membrane potential of a neuron and enhance its excitability by opening voltage-gated calcium channels via phosphoinositide hydrolysis mediated by phospholipase C (Di Giovanni et al. 2006; Roth and Chuang 1987). Thus we predict that binding of 5-HT to 5-HT_{2C}-like receptors in the brain stem of *Xenopus* initiates

vocalizations by activating DTAM either directly or indirectly. It is possible, however, that other 5-HT receptor types are involved in the generation and maintenance of vocal behavior. For example, once the vocal circuits are turned on, the activation of 5-HT_{2B}-like receptors may be important in maintaining their excitability. Such supportive roles of 5-HT_{2B}-like receptors may account for the reasons why one of the 5-HT_{2B} receptor antagonists reduced the overall vocal activity and the other blocked the 5-HT-induced fictive vocalizations in a small proportion of males.

How can one receptor mediate two distinct vocalizations?

How can a single neurotransmitter elicit distinct vocal patterns (advertisement call and ticking) from male and female brains using the same receptor subtypes expressed in the brain? It is possible that male and female vocal pathways are wired differently so that the postsynaptic targets of neurons expressing 5-HT_{2C}-like receptors differ between the sexes. Another possibility is that the 5-HT_{2C}-like receptors of males and females may be qualitatively different so that sexually distinct cellular responses may be elicited in response to 5-HT. Interestingly, results from this study showed that male brains were sensitive to three types of 5-HT_{2C} receptor agonist (DOI/ketanserin, MK-212, and Ro 60-0175), whereas female brains were sensitive to only two types (DOI/ketanserin, and MK-212, but not to Ro 60-0175). This may indicate that the 5-HT_{2C}-like receptors of the sexes show differential receptor-effector coupling, perhaps because of RNA editing of the 5-HT_{2C} receptor (Berg et al. 2001), or that the 5-HT_{2C}-like receptors are expressed in different amounts in males and females and that the effect of each individual agonist depends on the number of receptors saturated in the vocal circuit.

These explanations, however, cannot account for how 5-HT elicits two types of fictive vocalizations from a male brain (advertisement calls and ticking). One possibility that accounts for the activation of two motor outputs is that the differential excitation of neurons that express 5-HT_{2C}-like receptors could in turn lead to differential recruitment of downstream CPG interneurons and may provide the basis for two distinct vocalizations in male brains. This hypothesis is plausible in light of the findings by Li et al. (2007), who suggested that different motor programs (swimming vs. struggling) in *Xenopus tad-*

poles can be elicited by the same CPG depending on the amount of recruitment of interneurons within the CPG.

Role of 5-HT_{2C} receptor-mediated vocal behavior in vivo

Does activation of 5-HT_{2C}-like receptors also initiate *Xenopus* vocalizations in vivo? We have previously shown that dense serotonergic fibers are found within the raphe nucleus, DTAM, laryngeal motor nucleus IX–X, and inferior reticular formation (Rhodes et al. 2007). In this study, we showed that 5-HT_{2C}-like receptors in the brain are a likely part of the 5-HT-induced activation system for vocal behavior. Thus endogenous serotonin is available throughout the brain where functionally important 5-HT_{2C}-like receptors reside. If 5-HT_{2C}-like receptors indeed mediate vocal initiation in the two sexes, we predict that application of 5-HT_{2C} receptor antagonists in vivo should impair vocal activation in male and female *Xenopus*.

Although we did not show that 5-HT is the only substance that can initiate vocalizations in *Xenopus*, based on our work in this study, 5-HT likely acts at 5-HT_{2C} receptors to initiate vocalizations, and 5-HT is a likely part of the normal activation system in vivo. Further work needs to be done to assess if other transmitter systems such as the dopaminergic and adrenergic systems can also activate vocal behavior, and furthermore, if blocking 5-HT_{2C}-like receptors can block vocal activation by these other transmitters.

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