5-HT$_{2C}$-like receptors in the brain of *Xenopus laevis* initiate sex-typical fictive vocalizations

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Abstract

Vocalizations of male and female African clawed frogs (*Xenopus laevis*) are generated by brainstem 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, while 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 (Quinlan et al., 2004; Johnson et al., 2005; Chapman and Sillar, 2007).

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 (Hatton et al., 2003). Identification of the types and location of 5-HT receptors involved can enhance our understanding of motor pattern initiation.

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; Tobias et al., 2004)

Vocalizations of Xenopus laevis are ideal for this analysis, because the importance of serotonergic systems has already been implicated (Rhodes et al, 2007; Brahic and
Kelley, 2003), 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; Xiang et al., 2005; Pearlstein et al., 2005; Tryba et al., 2006). Our results showed that the sexes share a similar mechanism for initiating vocalizations.

Methods

Animals

Sexually mature Xenopus males (n = 61; 42.2 g +/-8.4; 7.2 cm +/-0.5) and females (n = 50; 72.0 g +/-10.4; 8.7 cm +/-0.6) 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 anaesthetized 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, 11 glucose with pH 7.8. Brains were then transferred to a recording chamber where they were continually superfused with fresh oxygenated saline (150 ml/hr) 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, Inc.). All the recordings were made at room temperature (~22° C). Typically, there is no vocal activity recorded from the laryngeal nerve prior to 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, St. Louis, MO), 6-methyl-1(1-methylethyl)-ergdine-8β-carboxylic acid (LY 53,857; 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), (αS)-6-Chloro-5-fluoro-α-methyl-1H-indole-1-ethanamine fumarate (Ro 60-0175; Tocris), (±)-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-carboxyamide 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-triaspiro[4.5] decane-2,4-dione hydrochloride (RS 102221 hydrochloride; Tocris), 3-[2-[4-(4-Fluorobenzoyl)-1-piperidinyl]ethyl]-2,3-dihy dro-2-thioxo-4(1H)-quinazolinone hydrochloride (Altanserin hydrochloride; Tocris), a-Phenyl-1-(2-phenylethyl)-4-piperidinemethanol (MDL 11,939; Tocris), and 6-Chloro-5-methyl-1-5-quinolycarbamoyl-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 53,857 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-HT$_2$ receptors and have little affinity for adrenergic or histaminergic receptors (Lemaire, 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, Ro 60-0175), we selected a minimum dosage in the range of 10 to 100 μM that consistently blocked or activated behavior based on pilot experiments, a common method employed to establish a dose of drugs for selective binding to serotonin receptors in mammalian tissues (Gunther et al., 2006; Tryba et al., 2006; Morin et al., 1994). In cases where agonists and antagonists were previously used only in brain slice preparations (Perrier and Hounsgaard, 2003; Chen et al., 2003), we selected ten times the dose for our whole brain preparation lest the drugs not penetrate the tissue efficiently. Since 5-HT$_2$ receptors of *Xenopus laevis* are not fully characterized pharmacologically, we will refer to the receptors identified in this study with pharmacology similar to mammals as 5-HT$_2$-like.

Application of pharmacological agents

5-HT was applied by replacing half the saline in the recording chamber (20 ml) with 60 μM 5-HT dissolved in oxygenated saline to achieve a final concentration of 30 μM. 5-HT application took 5-10 seconds, after which 5-HT remained in the recording chamber for 5 minutes; 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 minutes, which is sufficient to completely exchange the solution 2.5 to 10 times in the recording chamber.
All brains were continually superfused with oxygenated saline (150 ml/hr) for 1 hour 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; one hour later brains were exposed to the antagonist followed by 5-HT (30 μM, 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 out 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 Xenopus 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 – 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; Fig.
1A, 2a; Yamaguchi and Kelley, 2000; Rhodes et al., 2007). 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 amplitude modulation (Yamaguchi and
Kelley, 2000; Rhodes et al., 2007; Figure 2b).

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 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 (see below). 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 ten 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 minutes for ticking from each female brain.

Up to ten bouts of advertisement calls within the 5 minutes of serotonin exposure were randomly sampled from each male brain. Female brains either tick in long continuous trains, or in bouts (~10 ticks) interrupted by periods of silence (> 1 sec). Because of this variability and difficulty in defining a bout, female vocal behavior was analyzed as one continuous five minute bout of ticking, and all CAPs produced during 5 minutes of 5-HT exposure were sampled for each female brain for analysis. Pauses longer than 1 second 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 $3\sigma$ of background noise, minimum event duration $= 0.4$ ms). The instantaneous CAP rates were then calculated, and the frequency distributions of instantaneous CAP rates (with bin size of 1 Hz) were plotted (Fig. 2A, 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 one male) or one (one male and all females) Gaussian curves ($R^2 > 0.9$) with means of $\mu_1$, $\mu_2$ (2 Gaussian) or $\mu$ (1 Gaussian); (Fig. 2A, B). $\mu_1$ and $\mu_2$ were used as estimates of mean slow and fast trill rates, and $\mu$ was used as an estimate of mean ticking rates. In the case of one male with a unimodal distribution, $\mu$ was used as an estimate of fast trill rates (i.e. the equivalent of $\mu_2$). Maximum sustained CAP rates for males and females were calculated using a sliding window (i.e. by averaging ten 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. $\mu_1$, $\mu_2$, $\mu$, maximum sustained CAP rates, and number of advertisement bouts were used for further statistical analyses to determine if fictive vocalizations elicited in the presence of 5-HT$_2$ 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 if there was any quantitative change in the fictive vocalizations in the presence of the antagonist compared to those induced in the absence of the antagonist. To this end, we compared each $\mu$, 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
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 53,857, 100 μM; 3 females: Altanserin, 100 μM, up to 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. 3A center). 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. 3A right). Similarly in female brains, 5-HT-induced fictive ticking was also abolished after the application of the
antagonist and recovered after washout (Fig. 3B). We conclude that 5-HT$_2$-like receptors are important for 5-HT-induced fictive vocalizations in both sexes.

We next examined whether the activation of 5-HT$_2$-like receptors can activate fictive vocalizations in both sexes. Prior to agonist treatment all brains were silent (Fig. 3C, D left traces). However, bath application of a 5-HT$_2$ receptor agonist (α-Me-5-HT, 30 μM, up to 10 min) initiated fictive advertisement calls and ticking (Fig. 3C, D right traces) from all male and female brains, respectively (n = 5, 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-HT$_2$ receptor agonist in initiating fictive vocalizations was no different from the effect of 5-HT. These results suggest that 5-HT initiates fictive vocalizations by binding to 5-HT$_2$-like receptors in both sexes. In the case of male brains, activation of 5-HT$_2$ receptors may mediate the activation of ticking in addition to advertisement call.

Which 5-HT$_2$ receptors play a role in activating fictive vocalizations?

There are three known subtypes of 5-HT$_2$ receptors. To determine which subtype(s) are involved in vocal initiation, and whether the sexes utilize 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, 5 respectively) that were pre-incubated with the 5-HT$_{2A}$ receptor antagonist, Ketanserin (40 μM, 5 min), fictive advertisement calls and ticking were readily induced in all males and females (Fig. 4A, 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 μM, 5 min, 1 male), and for a longer duration (Ketanserin, 50 μM, 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 μM, 10 min), was applied to a different set of male and female brains (n = 5, 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 (Krebs-Thomson et al., 1998; Wolf et al., 1999;
Bishop et al., 2004). Application of DOI (50 μM, 10 min) to male (n = 2) and female (n =
3) brains pre-incubated with RS 102221 (50 μM, 15 min) failed to activate fictive
advertisement calls or ticking (Fig. 4C and D, left traces), whereas DOI (50 μM, 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 μM, 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-
HT2C receptor selective antagonist, RS 102221 (50 μM, 15 min) and the 5-HT2B receptor
selective antagonist, SB 215505 (50 μM, 15 min) verifying our results that activation of
5-HT2A receptors is not sufficient for initiation of fictive vocal behavior. Thus, we
conclude that 5-HT2A-like receptors are likely not critical for vocal initiation in the sexes.

5-HT2B receptors

5-HT2B 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 pre-incubated with a 5-HT2B receptor antagonist (SB 204741, 100
μM, 20 min), fictive advertisement calls and ticking were initiated in all male and female
brains, respectively (Fig. 4E, F right traces). However, males produced significantly
fewer bouts of advertisement call in the presence of the antagonist compared to controls
(5-HT alone; Tables 1 and 2). To explore the role of the 5-HT2B-like receptor further, we
used another type of 5-HT2B antagonist on a different set of brains. When SB 215505 (50
μM, 15 min) was applied to male (n = 6) and female (n = 5) brains, the antagonist failed
to block fictive advertisement calls in 5 out of 6 male brains and all 5 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-HT2B 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-
HT2B-like receptors are not critical for initiating fictive vocalizations in the sexes.

We next tested the effect of activating 5-HT2B-like receptors on vocal production
in the sexes. Application of a 5-HT2B receptor agonist (BW 723C86, 100 μM, up to 20
min) to male (n = 5) and female (n = 5) brains yielded no fictive advertisement call or
ticking (Fig. 4G, H, left traces), even though subsequent application of 5-HT resulted in
fictive vocalizations in all brains (Fig. 4G, H, right traces). Further, we tested the role of
activating 5-HT2B-like receptors by using a combination of drugs that effectively acts as a
5-HT2B receptor agonist, a broad spectrum 5-HT2 receptor agonist combined with
antagonists to 5-HT2A receptors and 5-HT2C receptors (n=5 males, 5 females). This
combination of drugs was used because there is no other selective 5-HT2B receptor
agonist available. Although all brains responded initially to 5-HT2 receptor agonist alone,
as demonstrated earlier (Fig. 3C and D), the combination of α-Me-5-HT (5-HT2 agonist
30 μM) + Ketanserin (5-HT$_{2A}$ antagonist, 40 μM) + RS 102221 (5-HT$_{2C}$ antagonist, 50 μM, 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 since 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.

$5HT_{2C}$ 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 pre-incubated with the 5-HT$_{2C}$ receptor antagonist (RS 102221; 50 μM, 15 min), fictive vocalizations were not initiated from any of the brains (Fig. 5A, B, center). The antagonist also blocked 5-HT-induced fictive ticking that we observed in two of the male brains prior to 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. 5A, B, right). In the two male brains
that initially produced both advertisement call and ticking, both call types recovered upon washout. To further confirm the importance of 5-HT$_{2C}$-like receptors, we used an additional 5-HT$_{2C}$ receptor antagonist (SB 242084; 100 μM, 15 min) on 5 new males and 5 new females. This antagonist also blocked fictive vocalizations in all male and female brains (Fig. 5C, D, center traces). Subsequently, these brains also recovered from the effect of the antagonist after washout (Fig. 5C, 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 (Zornik and Kelley, 2008; Rhodes et al., 2007), 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. 5E, male example shown with RS 102221). Likewise, the 5-HT$_2$ receptor antagonists used in this study did not affect fictive respiratory activity either (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. 6A, B, left traces) elicited fictive vocalizations (Fig. 6A, 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, 2).

Application of another 5-HT$_{2C}$ receptor agonist, Ro 60-0175 (50 μM, up to 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. 6C, left trace) induced fictive advertisement calls in all seven brains (Fig. 6C, 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, 2). Thus, Ro 60-0175 was able to initiate fictive advertisement calls in male brains although it is not as potent as 5-HT in maintaining the vocal activity. In female brains, in contrast, Ro 60-0175 (50 μM, up to 10 min) was largely ineffective in initiating fictive vocalizations; fictive ticking was induced in only one female (not shown), and not in other four (Fig. 6D, middle). These results were not due to the ill-health of the tissue, because all five of the female brains responded to subsequent 5-HT application with fictive ticking (Fig. 6D, 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 μM, 10 min) co-applied with the 5-HT$_{2A}$ receptor antagonist (Ketanserin, 40 μM, 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. 6E 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. 4D, left trace), DOI-induced fictive
vocalizations can be blocked by the 5-HT$_{2C}$ receptor antagonist (RS 102221, 50 μM, 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 7 additional female and 3 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. While it is not clear why Ro 60-0175 was less effective as 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-HT$_2$ 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 have demonstrated 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 (µ1 and µ2) 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 µ1: H = 12.86, p = 0.23; µ2: 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; 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 pair-wise comparisons. To test the effect of 5-HT2A and 5-HT2B 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, 2, females: Tables 3, 4). To test the effect of 5-HT2 and 5-HT2C 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, 2, females: Tables 3, 4).
Vocalizations of *Xenopus* are variable both *in vivo* (Fig. 1A) and *in vitro* (Fig. 7). The results demonstrate that fictive vocalizations initiated by the activation of 5-HT$_2$C-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$_2$C-like receptors in both sexes.

**Discussion**

Previous studies have shown that *Xenopus* serotonergic neurons project to the brainstem 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 if they differ between the sexes. To determine the types of 5-HT receptor that are of functional importance to the vocal pathways, pharmacological experiments were conducted using isolated brain preparations of male and female *Xenopus laevis*. Of the seven identified types of 5-HT receptors, we suspected that the 5-HT$_2$-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; Xiang et al., 2005; Pearlstein et al., 2005; Tryba et al., 2006), and because of its role in the reproductive behavior of mammals (Millan et al., 1997; Wolf et al., 1999; Bancila et al., 1999; Stafford et al., 2006; Wada et al., 2006; Heisler et al., 2007). Based on these pharmacological studies, we conclude that 5-HT$_2$C-like receptors mediate vocal initiation in *Xenopus laevis*.
Pharmacology in an amphibian model system

There are several challenges associated with pharmacological identification of the receptor types that mediate vocal activation in *Xenopus laevis*. The first challenge is that the pharmacological agents used in the present 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 *Xenopus laevis* (Holohean et al., 1990; Scrymgeour-Wedderburn et al., 1997; Holohean and Hackman, 2004). 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-HT₂C 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-HT₂C 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 when compared to other studies. For example, although Ketanserin is reported to block 5-HT$_{2C}$ receptors in addition to 5-HT$_{2A}$ receptors (although the selectivity is two 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 μM) 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-HT$_2$ 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* brainstem 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* brainstem
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., 2008). 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 (Roth and Chuang, 1987; Di Giovanni et al., 2006). Thus, we predict that binding of 5-HT to 5-HT$_{2C}$-like receptors in the brainstem 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 post-synaptic targets of neurons expressing 5-HT\textsubscript{2C}-like receptors differ between the sexes. Another possibility is that the 5-HT\textsubscript{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 the current study showed that male brains were sensitive to three types of 5-HT\textsubscript{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\textsubscript{2C}-like receptors of the sexes show differential receptor-effector coupling, perhaps due to RNA-editing of the 5-HT\textsubscript{2C} receptor (Berg et al., 2001), or that the 5-HT\textsubscript{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\textsubscript{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 suggest that different motor programs (swimming versus struggling) in *Xenopus* tadpoles can be elicited by the same CPG depending on the amount of recruitment of interneurons within the CPG.

*The role of 5-HT\textsubscript{2C} receptor-mediated vocal behavior in vivo*
Does activation of 5-HT$_{2C}$-like receptors also initiate *Xenopus* vocalizations *in vivo*? We have previously demonstrated 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 the present study, we have demonstrated 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 have not demonstrated 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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References


Figure captions

Figure 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 demonstrate that advertisement calls may consist of fast and slow trills (as in the second and the fourth bouts) or only fast trills (as in the first and the third bouts). See Yamaguchi and Kelley (2000) for the recording methods. B, Isolated brains of Xenopus in vitro are typically silent (left 1 hour trace) until serotonin (30 μM) 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 hour trace).

Figure 2. Quantitative characterization of fictive vocalizations of Xenopus. A, An example frequency histogram (bin size 1 Hz) of instantaneous CAP (compound action potential) 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 (μ2 and μ1) 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, Two traces each from two different females, showing inter- and intra-individual variation in the rate of CAPs.
Figure 3. 5-HT$_2$-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-HT$_2$ receptor antagonist (*LY 53,857; 100 μM, 10 min*) application in a male brain. **B**, 5-HT induces fictive ticking before (*left*), and after washout (*right*), but not during (*middle*) 5-HT$_2$ receptor antagonist (*Altanserin; 100 μM, 10 min*) application in a female brain. **C**, Fictive advertisement calls was recorded from a male brain only when a 5-HT$_2$ receptor agonist (*α-Me-5-HT; 30 μM; 5 min*) was applied (*left*), not before the treatment (*right*). **D**, Before treatment with agonist, brains do not produce fictive ticking (*left*). Application of a 5-HT$_2$ receptor agonist (*α-Me-5-HT; 30 μM; 5 min*) initiates fictive ticking in a female brain (*right*).

Figure 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 μM, 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 μM, 10 min*) evokes fictive advertisement call (*right*), but not in the presence of a 5-HT$_{2C}$ receptor antagonist (*RS102221, 50 μM, 15 min; left*). **D**, 5-HT$_{2A/C}$ receptor agonist (*DOI, 50 μM, 10 min*) evokes fictive ticking (*right*), but not in the presence of a 5-HT$_{2C}$ receptor antagonist (*RS102221, 50 μM, 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 μM, 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 μM, 20 min*). **G**, 5-HT$_{2B}$
receptor agonists, \((BW\ 723C86,\ 100\ \mu M,\ 20\ min)\) 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 sec for all traces.

**Figure 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 \((RS\ 102221,\ 50\ \mu M,\ 10\ min)\) 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 \((RS\ 102221,\ 50\ \mu M,\ 10\ min)\) 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 \((SB242084,\ 100\ \mu M,\ 10\ min)\) 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 \((SB242084,\ 100\ \mu M,\ 10\ min)\). \(E\), 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 \((RS\ 102221\; 50\ \mu M,\ 15\ min)\). The trace is truncated at the time of antagonist application indicated by arrow. Asterisks indicate episodes of fictive breathing.
Figure 6. 5-HT$_{2C}$ receptor agonists initiate fictive vocalizations. **A** and **B**, A 5-HT$_{2C}$ receptor agonist (MK-212, 50 μM, 10 min) initiates fictive advertisement calls from male brains and fictive ticking from female brains (right traces) whereas prior to application of the agonist the brains were silent (left traces). **C**, An alternative 5-HT$_{2C}$ receptor agonist, Ro 60-0175 (50 μM, 10 min) initiates fictive advertisement calls in male brains (right) whereas prior to application of the agonist the brains were silent (left). **D**, The same 5-HT$_{2C}$ receptor agonist, Ro 60-0175 (50 μM, 10 min) fails to initiate fictive ticking in female brains (middle). This was not due to 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 μM, 10 min) to male brains pre-incubated with 5-HT$_{2A}$ receptor antagonist (Ketanserin, 40 μM, 5 min, n = 3) initiates fictive advertisement calls (right), as in application of DOI alone (middle). Prior to treatment, all brains were silent (left). **F**, The application of 5-HT$_{2A/C}$ receptor agonist (DOI; 50 μM, 10 min) to female brains pre-incubated with 5-HT$_{2A}$ receptor antagonist (Ketanserin, 40 μM, 5 min, n = 3) initiates fictive ticking (right), as in application of DOI alone (middle). Prior to treatment, all brains were silent (left).

Figure 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; $H = 9.18, p = 0.33$. **D**, Box plots of number of bouts of advertisement call across treatment; $H = 8.76, p$
Box plots of mean instantaneous CAP rate for ticking (μ) in females by treatment; H = 9.27, p = 0.23. 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.

Table 1. Temporal structure and the vocal activity of calls induced under experimental and control conditions in males. Mean and standard deviations of instantaneous CAP rate for slow trill (μ₁) and fast trill (μ₂), maximum sustained CAP rate, and the number of bouts for each condition. For experiments in which 5-HT₂A and 5-HT₂B 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).

Table 2. Comparisons of call structure and the number of calls induced under different experimental conditions in males. For experiments in which 5-HT₂A and 5-HT₂B 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.

**Table 3.** Temporal structure of calls induced under experimental and control conditions in females. Mean and standard deviation of instantaneous CAP rate (μ) and maximum sustained CAP rate for each condition. For experiments in which 5-HT2A and 5-HT2B 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).

**Table 4.** Comparisons of call structure of fictive calls induced under different experimental conditions in females. For experiments in which 5-HT2A and 5-HT2B 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.
A

(nerve)

(sound)

B

(in vitro)

prior to 5-HT

after washout of 5-HT

1 hr

5 min

1 hr

5-HT
A

Number of CAPs

Instantaneous CAP rate (Hz)

μ1 = 30.9
μ2 = 55.6

male

slow

fast

0 sec

1 sec

B

Number of CAPs

Instantaneous CAP rate (Hz)

μ = 5.6

female 1

female 2

0 sec

1 sec
A

B

C

D

prior to \( \alpha\)-Me-5-HT

prior to \( \alpha\)-Me-5-HT

\( 5\text{-HT} \) + \( 5\text{-HT} \)

\( \text{Altanserin} + 5\text{-HT} \)

\( \alpha\)-Me-5-HT

\( \alpha\)-Me-5-HT

1 sec

1 sec
Prior to MK-212

prior to Ro 60-0175

prior to DOI

Prior to DOI
A  
B

mean instantaneous CAP rate (Hz) μ

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

mean instantaneous CAP rate (Hz) μ²

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

maximum sustained CAP rate (Hz)

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

number of bouts

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

maximum sustained CAP rate (Hz)

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

mean instantaneous CAP rate (Hz) μ

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

maximum sustained CAP rate (Hz)

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

number of bouts

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

maximum sustained CAP rate (Hz)

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

mean instantaneous CAP rate (Hz) μ

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

maximum sustained CAP rate (Hz)

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

number of bouts

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

maximum sustained CAP rate (Hz)

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

mean instantaneous CAP rate (Hz) μ

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

maximum sustained CAP rate (Hz)

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

number of bouts

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

maximum sustained CAP rate (Hz)

5-HT α-Me-5-HT DOI Ketanserin MDL Ro 60-0175 SB204741 SB215505

mean instantaneous CAP rate (Hz) μ
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<th>μ2 control</th>
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