Melatonin action in a midbrain vocal-acoustic network

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Short title: Melatonin regulation of vocal pathways
Key words: Melatonin, vocalization, periaqueductal gray, midshipman fish

SUMMARY

Melatonin is a well-documented time-keeping hormone that can entrain an individual’s physiology and behavior to the day-night cycle, though surprisingly little is known about its influence on the neural basis of social behavior, including vocalization. Male midshipman fish (Porichthys notatus) produce several call types distinguishable by duration and by daily and seasonal cycles in their production. We investigated melatonin’s influence on the known nocturnal- and breeding season-dependent increase in excitability of the midshipman’s vocal network (VN) that directly patterns natural calls. VN output is readily recorded from the vocal nerve as a “fictive call.” Five days of constant light significantly increased stimulus threshold levels for calls electrically evoked from vocally active sites in the medial midbrain, supporting previous findings that light suppresses VN excitability, while 2-iodomelatonin (2-IMel; a melatonin analogue) implantation decreased threshold. 2-IMel also increased fictive call duration evoked from medial sites as well as lateral midbrain sites that produced several-fold longer calls irrespective of photoregime or drug treatment. When stimulus intensity was incrementally increased, 2-IMel increased duration only at lateral sites, suggesting melatonin action is stronger in the lateral midbrain. For animals receiving five days of constant darkness, known to increase VN excitability, systemic injections of either of two mammalian melatonin receptor antagonists increased threshold and decreased duration for calls evoked from medial sites. Our results demonstrate melatonin modulation of vocal network excitability and suggest that social context-dependent call types differing in duration may be determined by neuro-hormonal action within specific regions of a midbrain vocal-acoustic network.

INTRODUCTION

Conserved features of vertebrate vocal-acoustic communication include the production of context-dependent vocal call types, occurrence over predictable daily and seasonal cycles, and the ability of neuro-hormones to modulate vocal motor output by acting upon dedicated neural networks (Bass and Remage-Healey, 2008; Goodson and Bass, 2001; Tramontin and Brenowitz,
Most studies to date on fish circadian rhythms have investigated the effects of photoperiod and the time-keeping pineal hormone, melatonin, on locomotor or feeding activity, while little attention has been paid to melatonin’s action on courtship behaviors such as vocalization, or more generally on underlying neural circuitry (Azpeleta et al., 2010; López-Olmeda et al., 2006; Piccinetti et al., 2010; Pinillos et al., 2001; Zhdanova et al., 2001). In songbirds, a melatonin-sensitive circadian rhythm in song and call behaviors has only recently been shown (Wang et al., 2012), even though melatonin influence on song nuclei volume and expression of song nuclei-specific melatonin receptors has been well-documented (Bentley, 2003; Bentley and Ball, 2000; Bentley et al., 1999; Bentley et al., 2013; Cassone et al., 1995; Cassone et al., 2008; Gahr and Kosar, 1996; Jansen et al., 2005; Whitfield-Rucker and Cassone, 1996). Additionally, melatonin inhibited the spontaneous firing rate of a vocal premotor nucleus in the zebra finch, suggesting that it can act directly on vocal circuits to influence vocal patterning (Jansen et al., 2005). Here, we use a fish model to investigate melatonin influence on the temporal patterning of a brainstem neural circuit dedicated to sound production.

Male plainfin midshipman (Porichthys notatus) contract sonic swim bladder muscles at ~100Hz to produce several call types distinguishable mainly by their duration and the social context under which they are produced (Brantley and Bass, 1994; Bass et al., 1999). Nest-guarding males produce very long duration (mins-h) advertisement/courtship “hums” or short duration agonistic “grunts” (~50-200 ms) (e.g., Fig. 1A) (Bass et al., 1999; Bass and McKibben, 2003; Brantley and Bass, 1994). Well-defined forebrain, midbrain, and hindbrain nuclei form a vocal-acoustic network (Bass et al., 1994; Goodson and Bass, 2002), the output of which is readily recorded in-vivo from paired vocal occipital nerves that innervate the sonic muscles and are considered homologues of hypoglossal nerve roots (Fig. 1B) (Bass et al., 2008). The spike-like vocal nerve motor volley is referred to as a fictive call in the absence of muscle activation because each nerve spike directly translates into a single muscle contraction and, in turn, one sound pulse (Fig. 1A) (Bass and Baker, 1990; Cohen and Winn, 1967). Each spike reflects the synchronous firing of vocal motor neurons whose activity is patterned by hindbrain premotor nuclei (Bass and Baker, 1990; Chagnaud et al., 2011; Chagnaud et al., 2012). Hence, fictive calls are a reliable proxy for assessing hormonal influences mediated by specific receptors on a discrete vocal network that directly determines natural call properties (Forlano et al, 2005; Forlano et al., 2010; Goodson and Bass, 2000a; Goodson and Bass, 2000b; Goodson et al., 2003; Fergus and Bass, 2013; Remage-Healey and Bass, 2004; Remage-Healey and Bass, 2007). Given the importance of day length on regulating reproductive physiology, melatonin’s role as the main time-keeping hormone among vertebrates, and evidence of its interaction with the hypothalamo-
pituitary-gonadal axis (Azpeleta et al., 2010; Bhattacharya et al., 2007; Falcón et al., 2007; Falcón et al., 2010), the midshipman presents a tractable model for investigating potential melatonin action on the excitability of neural networks regulating courtship behaviors.

Midshipman courtship vocalization follows dramatic daily and seasonal rhythms, occurring at night during the summer breeding season (Brantley and Bass, 1994; Ibara et al., 1983). Field and captive studies of fish vocal behavior report robust daily periodicity, with activity peaking during nighttime in most species identified, including closely related toadfish (Locascio and Mann, 2008; Rice and Bass, 2009). Directly complementing behavioral studies, in-vivo neurophysiology in midshipman demonstrates a nocturnal increase in vocal network excitability, measured in increased duration and decreased stimulation threshold of midbrain evoked fictive calls (Rubow and Bass, 2009). Constant light conditions abolish the nocturnal rise in excitability, while constant dark substantially increases excitability (Rubow and Bass, 2009).

Although these studies support the existence of either daily or circadian rhythms in fish vocal behavior and neural circuit plasticity, potential control mechanisms remain unknown.

Given the ability of constant light to abolish pineal melatonin production and constant dark to increase baseline melatonin in many species of fish (Bayarri et al., 2002; Bhattacharya et al., 2007; Falcón et al., 2010), we tested the hypothesis that the stimulatory effects of constant darkness on vocal excitability were due, in part, to an increase in melatonin action in discrete vocal nuclei. Within the midshipman vocal network, the midbrain periaqueductal gray (PAG) and surrounding midbrain tegmentum play a crucial role in vocalization initiation, consistent with other vertebrates (Kittelberger and Bass, 2013). We report that, compared to medial stimulation sites, lateral midbrain sites produce longer duration calls comparable to natural advertisement/mate calls that are more sensitive to the stimulatory effects of melatonin than brief calls evoked from medial sites. We propose the existence of a neuroendocrine center located laterally within the previously described midbrain vocal-acoustic network that contributes to the generation of social context-dependent calls. To our knowledge, this study is the first demonstration of melatonin effects on the excitability of a neural network underlying vocal behavior in fishes, and one of few such studies in all of vertebrates (Jansen et al., 2005).

RESULTS

We used two electrical microstimulation regimes in the midbrain to probe vocal network excitability. The first regime evoked fictive responses at ten time points over 120 min, following Rubow and Bass (2009). The second stimulation regime, referred throughout as a stimulus-
response curve (SRC), assessed fictive call responses to increasing stimulus current levels. Prior
to each time point in 120 min sessions and each SRC, we measured the minimum stimulus
current required to elicit responses (threshold). Figure 1C, D provide schematics of photoperiod,
drug treatment, and neurophysiology stimulation regimes used in this study.

Although earlier studies were suggestive that more robust vocal stimulation sites could be
found in lateral portions of the midbrain PAG and portions of the surrounding tegmentum in
comparison to medial sites (Goodson and Bass, 2000b), no study systematically compared vocal
output between midbrain regions or examined their neuroendocrine control. In addition to
eliciting vocal output from medial sites in the medial PAG and nearby tegmentum (Kittelberger
and Bass, 2013), we also stimulated sites in the lateral midbrain that could reliably elicit up to a
magnitude longer duration output. We first provide evidence of photoregime effects and
melatonin modulation in medial and lateral midbrain regions followed by detailed comparisons of
their excitability.

Effects of photoperiod on vocal excitability inferred from control animals

Fish were held under three different photoregimes: one group was maintained on a 15:9h L:D
cycle (LD), consistent with their breeding season photoperiod during late spring-summer.
Another group was moved from LD to 24h of light for five days (5LL). Constant light conditions
have been equated to “functional pinealectomy”, which in fish, as in mammals, abolishes the
nocturnal increase in melatonin secretion from the pineal (Bayarri et al., 2002; Bhattacharya et
al., 2007; Carter et al., 1982; Porter et al., 1999). A third group was moved from LD to 24h of
darkness for five days (5DD). In constant darkness, the pineal of most teleosts exhibits
endogenous cycling of melatonin production albeit at higher daytime levels than under normal
light-dark cycles, with the exception of some that show constant up-regulation of melatonin
production within the duration of darkness (Ekström and Meissl, 1997; Oliveira et al., 2009).
Either 5LL or 5DD was chosen because these regimes exaggerate the observed natural decrease
or increase in vocal excitability associated with daytime and nighttime, respectively (Rubow and
Bass, 2009).

We first compared vocal output in control animals from each of the photoperiod groups
(see above and Results sections below) to examine effects of photoperiod manipulation alone and
to validate previous findings that 5LL decreased and 5DD increased vocal excitability,
respectively (Rubow and Bass, 2009). For 120 min sessions and medial site SRC comparisons
(Fig. 2A-C), data were from animals tested at medial stimulation sites, including 5LL implant
controls for testing effects of a melatonin agonist on excitability (n=10, Fig. 3), 5DD vehicle-
injected controls for testing effects of melatonin antagonists on excitability (n=6, Fig. 4), and LD animals for comparing vocal motor output from medial vs. lateral midbrain sites (n=5, Fig. 5). For lateral SRCs (Fig. 2D), data were from the same 5LL and 5DD control animals as above, as well as LD animals that first received lateral stimulation over 120 min sessions (n=5, Fig. 5). We present the following results with the caveat that although all were control animals, differences in treatment methods (no treatment, implant, injection) could have contributed to variation in vocal excitability.

For the medial midbrain, we observed a strong trend of photoregime effect on call duration recorded over 120 min, with 5DD animals having the longest fictive calls (Fig. 2A) (P=0.06). There was a significant effect of photoregime on stimulus threshold over the 120 min session (Fig. 2B) (F(2,18.0)=6.02, P=0.01) and a significant photoregime*time interaction (F(18,161)=2.68, P=0.0005), with 5LL animals having the highest thresholds (Tukey-HSD: 5LL vs 5DD: P=0.018; 5LL vs LD: P=0.043). These results for medial sites suggested that 5DD increased and 5LL suppressed vocal excitability, as best revealed by threshold levels.

There was a similar strong trend for a photoregime effect on medial site SRC burst duration (P=0.066), with 5DD animals having the highest duration (Fig. 2C). For lateral site SRC (Fig. 2D), we detected no significant photoregime effect (P=0.73). There was no photoregime*stimulus-intensity interaction for either medial (P=0.12) or lateral (P=0.17) SRCs.

Finally, we compared medial vs. lateral site excitability by examining SRC thresholds in control animals (Fig. 2E). When we examined the effects of photoregime, stimulus site, and their interaction on SRC thresholds, we found a near-significant photoregime effect (P=0.061), significant stimulus site effect (F(1,1)=12.48, P=0.001), and photoregime*stimulus site interaction (F(2,2)=6.41, P=0.004). Post-hoc Tukey-HSD showed that the lateral SRC threshold of LD animals who did not receive prior medial stimulation was significantly higher than all others who received medial stimulation first (P<0.042) (Fig. 2E). These results imply that stimulation at medial sites first with a 120 min session disinhibits lateral sites by decreasing threshold, but stimulation at lateral sites first does not.

We concluded that photoregime had a significant influence on vocal excitability. This was most strikingly revealed by threshold comparisons (Fig. 2B), consistent with the results of Rubow and Bass (2009) and our hypothesis that constant light inhibits vocal excitability.

2-Iodomelatonin treatment in 5LL animals

To test the prediction that melatonin replacement can rescue the decreased vocal motor excitability seen in 5LL animals (Rubow and Bass, 2009), fish held in 5LL received an implant
for five days of either a vehicle or 2-iodomelatonin (2-IMel), a high affinity melatonin analogue
that acts on both MT1/Mel1a and MT2/Mel1b receptor subtypes in mammals (Dubocovich et al.,
2010; Boutin et al., 2005; Stankov et al., 1993).

For 120 min recording sessions at medial sites, 2-IMel significantly increased fictive call
duration in a time-dependent manner (Fig. 3A). There was a significant effect of time
\( F_{(10,200)} = 2.9, P = 0.002 \) and time*treatment interaction \( F_{(10,200)} = 2.0; P = 0.038 \), but no treatment
effect alone \( P = 0.09 \). Additionally, 2-IMel significantly decreased overall stimulus threshold in
5LL males across 120 min sessions (Fig. 3B) \( F_{(1,20)} = 4.3, P = 0.0514 \) and there was a significant
treatment*time interaction \( F_{(9,180)} = 2.17, P = 0.026 \). In sum, 120 recording sessions revealed that
2-IMel increased vocal excitability at medial stimulation sites in 5LL fish.

For SRCs, 2-IMel exerted a site-dependent effect on duration with a significant
stimulatory effect at lateral, but not medial, stimulation sites (Fig. 3C,D). At lateral sites, 2-IMel
treatment significantly increased duration \( F_{(1,20)} = 4.8, P = 0.041 \), with a significant
treatment*stimulus-intensity interaction \( F_{(4,80)} = 5.6, P = 0.0055 \). At medial sites, we observed no
treatment \( P = 0.59 \) or treatment*stimulus-intensity interactions \( P = 0.91 \). We also compared the
threshold stimulus levels for SRCs (Fig. 3E). There was a significant treatment effect of 2I-Mel
for decreasing threshold \( F_{(1,40)} = 5.28, P = 0.027 \), but no stimulus-site effect \( P = 0.44 \) or stimulus-
site*treatment interaction \( P = 0.33 \).

Taken together, the results indicated that 2-IMel increased excitability at vocal midbrain
sites, irrespective of stimulating electrode location. However, the SRC paradigm revealed a
particularly robust stimulatory effect of melatonin on call duration specific to lateral midbrain
sites.

**Melatonin receptor antagonist treatment in 5DD animals**

We set out to test the hypothesis that melatonin action on specific receptor subtypes could explain
the increased vocal excitability observed in 5DD males. Since our comparisons of control groups
showed that 5DD decreased threshold levels at medial sites (Fig. 2B), we predicted that treatment
with melatonin receptor antagonists would reverse this effect. Fish held in 5DD received daily
intramuscular injections for five days of a receptor antagonist or vehicle (Fig. 1C). The
antagonists used were luzindole, a general Mel1a/b antagonist, or 4-phenyl-2-
propionamidotetralin (4P-PDOT), a Mel1b specific antagonist (Dubocovich et al., 2010).

For 120 min sessions, although luzindole values for duration were generally lower at all
time points, there was no significant effect \( P = 0.16 \) or treatment*time interaction \( P = 0.58 \) when
compared to animals injected with vehicle control. No treatment \( P = 1.00 \) or treatment*time
interaction (P=0.76) on duration were observed between vehicle and 4P-PDOT (Fig. 4A). For vehicle to luzindole stimulus threshold comparisons, there was no effect of treatment (P=0.84) or treatment*time interaction (P=0.74). For vehicle to 4P-PDOT threshold comparisons, we found no effect of treatment (P=0.18) alone, but there was a significant treatment*time interaction (Fig. 4B) (F(9,90)=4.34, P=0.001). In sum, 4P-PDOT increased threshold in a time-dependent manner, suggesting that disrupting Mel1b-mediated melatonin action impacts vocal excitability at medial sites in 5DD animals.

For SRCs evoked at medial sites, luzindole significantly decreased fictive call duration (F(1,10)=6.42, P=0.03) with a significant treatment*stimulus-intensity interaction (F(4,40)=4.23, P=0.006); 4P-PDOT had no effect (P=0.32) (Fig. 4C). In lateral SRCs, treatment with either luzindole (P=0.21) or 4P-PDOT (P=0.25) had no effect on duration (Fig. 4D). Thus, luzindole, like 4P-PDOT, inhibited vocal excitability at medial sites in 5DD animals. Finally, there was no significant treatment effect on SRC threshold levels (luzindole: P=0.56; 4P-PDOT: P=0.11) or treatment*stimulus-site interaction (Fig. 4E) (luzindole: P=0.75; 4P-PDOT: P=0.59), but there was a stimulus-site effect of lateral sites having significantly higher thresholds (Fig. 4E) (luzindole: F(1,9.4)=14.5; P=0.004; 4P-PDOT: F(1,10)=5.0; P=0.048).

In summary, for calls evoked from medial sites, antagonizing Mel1b receptors (4P-PDOT) increased threshold in 120 min sessions, while antagonizing Mel1a/b receptors (luzindole) decreased duration in SRCs. This was consistent with our predication that the increased vocal excitability seen in 5DD control animals was due to melatonin action on specific melatonin receptors.

Medial vs. lateral midbrain vocal sites

We wanted to ensure that the several-fold longer duration calls consistently elicited from stimulation sites in the lateral compared to the medial midbrain (Figs. 2C,D; 3C,D; 4C,D) were not due to a priming effect of stimulating for 120 min at a medial site first. We tested non-treated LD males at either (1) only a medial or only a lateral site for a 120 min session delivering only 40 stimuli at the 120 min trial, followed by one SRC at the same site (Fig. 1D) or (2) a lateral site for a 120 min session and SRC followed by a second SRC at a medial site (Fig. S1).

Stimulation at lateral midbrain sites often led to several-fold longer duration fictive calls than those elicited from medial sites (e.g., Fig. 5Ai,ii; Bi,ii). Examination of fictive call frequency, measured in inter-pulse intervals (IPIs), of all fictive calls taken from one pair of representative medial and lateral SRCs revealed no significant site-dependent differences (P=0.14) (e.g. Fig. 5Aiii, Biii). For 120 min trials, lateral-evoked calls had a significantly longer
duration (Fig. 5C) \( (F_{1,7.9}=217.6, P<0.0001) \), higher threshold (Fig. 5D) \( (F_{1,7.9}=14.0, P=0.006) \),
and longer latency (Fig. 5E) \( (F_{1,8.0}=316.8, P<0.0001) \). Medial site stimulation successfully
evoked fictive calls for 98.8±1.0% (mean±s.e.m, 120 min trial means) of the 40 stimuli delivered
in each trial, whereas lateral stimulation was less reliable at 65.0±2.2% (data not shown).

Similar to the results for 120 min trials (Fig. 5C), lateral site SRCs had significantly
longer calls (Fig. 5F) \( (F_{1,8.0}=68.4, P<0.0001) \). In a pilot study \( (n=3) \), we found the same response
patterns when first stimulating in a lateral site for a 120 min session and SRC and then moving to
a medial site for a second SRC (Fig. S1) \( (F_{1,18}=138.9, P<0.0001) \).

Taken together, although calls elicited from lateral sites were longer duration than those
from medial sites, they required more stimulus current to initiate and exhibited longer latencies.

Electrolytic lesions in a subset of animals, including those from drug treatment
experiments, localized medial \( (n=11) \) and lateral \( (n=10) \) midbrain stimulation sites. Fictive call
duration was highly dependent upon the site of stimulation segregated along the medial-lateral
midbrain axis (Fig. S2). Medial sites were located in the midbrain tegmentum and the medial
PAG. Lateral sites were in the paratoral tegmentum (PTT), deep layer of the torus semicircularis
(TSd), and just below and within the ventral aspect of the paralemniscal tegmentum (PL).

**DISCUSSION**

Our results support the hypothesis that nocturnal melatonin action contributes to increased vocal
excitability during the midshipman breeding season. The SRC stimulation paradigm highlighted
the lateral midbrain vocal-acoustic network as a potential neuroendocrine node that contributes to
the production of distinct social context-dependent vocal outputs. We propose that these findings
apply to other lineages of vocal vertebrates given wide occurrence of daily and seasonal cyclicity
in vocal behaviors and conserved vocal network organization.

**Melatonin regulation of vocal excitability**

We found that in control animals, constant light (5LL) inhibited vocal excitability by increasing
stimulus threshold at the medial site compared to fish held in constant dark (5DD) and normal
light-dark cycles, consistent with previous findings (Rubow and Bass, 2009). This inhibitory
effect was rescued by melatonin agonist (2-IMel) implants in fish held under 5LL (Fig. 3B). 2-
IMel implant also led to increased call duration across 120 min sessions in a time-dependent
manner. Interestingly, 2IMel increased duration in SRCs only at lateral, but not medial, midbrain
stimulation sites (Fig. 3C-E). For 5DD fish, daily injections of 4P-PDOT, a Mel1b-specific
receptor antagonist, increased thresholds across 120 min sessions in a time dependent manner; luzindole, a general Mel1a/b antagonist, resulted in consistently low call durations in both 120 min sessions and SRCs, but these effects were only significant in SRCs (Fig. 4). These mammalian-specific antagonists may be less effective in fish (Dubocovich et al., 2010), though some fish studies have effectively antagonized melatonin effects on locomotor and feeding activity via peripheral delivery of luzindole (Pinillos et al., 2001; Zhdanova et al., 2001).

Together, our results support the hypothesis that a nocturnal melatonin action increases vocal network excitability in male midshipman fish during the breeding season.

Our experimental design was inspired by a previous study where 5DD and 5LL manipulations either significantly increased or decreased vocal network excitability, respectively (Rubow and Bass, 2009). Surprisingly at first, we found that medial midbrain stimulation in 5DD fish did not readily evoke the grunt-hums observed in the prior study that are structurally comparable to natural amplitude-modulated growls (see Rubow and Bass, 2009). Rather, we consistently elicited long duration fictive calls reminiscent of non-amplitude-modulated hum advertisement calls (see Fig. 1A), but only from the lateral midbrain. Our comparisons of control groups showed that although a 5DD effect on duration was apparent, it was only near significant (Fig. 2A). Two prominent methodological differences could explain this inconsistency. First, Rubow and Bass (2009) noted that an increase in stimulus intensity in conjunction with the 100 stimulus presentation at 120 min was needed to evoke the grunt-hums, whereas in the current study stimulus intensity was kept low and consistent across the 120 min sessions. Second, the long duration fictive calls reported by Rubow and Bass (2009) were only readily evoked in males that were held in captivity for less than one month (Rubow, 2010; A. Bass, personal communication). Most fish used in the current study were first tested after being held captive for at least one month and did not produce long calls either at baseline or with repeated stimulation at the medial site, consistent with our earlier studies.

Results from control animal comparisons (Fig. 2) may shed light on the apparent conundrum that midshipman are both long-day and nocturnal breeders. The short duration of nocturnal melatonin experienced under long-days would lead to the prediction that melatonin is inhibitory to courtship behaviors, while being nocturnally active during peak diel levels of melatonin would lead to the inverse prediction. In support for an inhibitory role of melatonin in vocalization of diurnally active species, melatonin treatment in songbirds mimics the effect of short days by decreasing song nuclei volumes (Bentley et al., 1999; Cassone et al., 2008). Furthermore, daily melatonin treatment in pinealectomized zebra finches kept in constant dim light entrained song and call activity to occur during periods without melatonin (Wang et al.,
Our results in nocturnally active midshipman showed that fictive call duration was increased in 5LL+2-Imel animals, especially in the lateral midbrain (Fig. 3D), but not for vehicle-injected 5DD animals (Fig. 2D). One feasible explanation is that the combination of melatonin treatment and long durations of light experienced by 5LL+2-Imel animals increases vocal excitability by increasing melatonin sensitivity in the lateral midbrain, perhaps in the form of a higher density of melatonin receptors. On the other hand, in fish that have experienced long durations of darkness (5DD), sensitivity to melatonin could have been diminished at the lateral midbrain, leading to no change in vocal excitability in response to constant darkness when melatonin levels are putatively high. However we did observe significantly lower threshold levels at medial midbrain sites in 5DD animals (Fig. 2B), suggesting 5DD does increase aspects of vocal excitability. These are testable hypotheses to be investigated in future studies on melatonin levels in 5DD animals, as well as photoperiod regulation of receptor density and localization.

Alternatively, increases in midshipman vocal excitability at night may rely on an increase in peak nocturnal melatonin levels during the summer breeding season. In some fish species, it has been found that although the duration of a nocturnal rise in circulating melatonin levels is shortened in response to long-day lengths, the amplitude of the rise is higher during the spring/summer breeding season and is positively correlated with water temperature (García-Allegue et al., 2001; Iigo and Aida, 1995; Vera et al., 2007). If this is also true for the midshipman, as males migrate from wintering in deeper, colder waters to shallow, warmer intertidal zones for breeding (see Bass, 1996), a higher nocturnal peak in melatonin levels could lead to increased vocal excitability at night.

Melatonin has been shown to interact with neuropeptide and steroid pathways in many vertebrates, including teleost fishes (Falcón et al., 2010; Maitra and Chattoraj, 2006), frogs (Lutterschmidt and Wilczynski, 2012), and birds (Chowdhury et al., 2010; Ubuka et al., 2005). Unlike nonapeptides and steroid hormones (Goodson and Bass, 2000a; Remage-Healey and Bass, 2004), pilot studies showed that acute melatonin injections did not rapidly (5-120 min) change the output of the midshipman vocal system under our testing conditions (N. Feng, personal observations). Hence, the effects of melatonin on vocal excitability documented here were likely via slower, transcriptionally dependent events. Melatonin may interact with multiple neurotransmitter and neuromodulatory systems synergistically to stimulate the full expression of nocturnal behavior. In other words, melatonin could increase baseline vocal network excitability at night so that in the presence of other activating factors such as neuro-hormones and social cues, the vocal system is capable of responding by producing long duration calls.
A potential mechanism for melatonin to influence network excitability is through the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Acute melatonin application to mammalian brain slices increases or decreases GABAergic currents via Mel1a or Mel1b receptors, respectively (Wan et al., 1999). Supporting a stimulatory role for Mel1b-mediated action, antagonizing Mel1b receptors in zebra finches (which are specifically expressed in song control nuclei) at night decreased song duration the following day (Jansen et al., 2005). Song nucleus-specific Mel1b mRNA expression is also positively correlated with immediate early gene expression (Bentley et al., 2013), suggesting melatonin action contributes to increased neural activity in vocal centers. Our result of 4P-PDOT (mammalian Mel1b antagonist) increasing threshold during the 120 min session is consistent with Mel1b-mediated events as stimulatory on the vocal system. Given GABA’s essential role in midshipman vocal network function (Chagnaud et al., 2011; Chagnaud et al., 2012), melatonin might alter vocal network excitability via GABA.

### Medial versus lateral midbrain vocal excitability

How the brain generates social context-dependent calls, distinguishable by vocal attributes such as duration, is still largely unresolved. Together with evidence from some mammalian species (Bandler and Carrive, 1988; Fenzl and Schuller, 2007), our study supports the possibility that vocal-acoustic centers in the midbrain contribute to modulation of different call types. We showed that activation of medial and lateral midbrain regions led to either short or long duration calls, respectively. The vocally active sites identified here are inclusive of those anatomically mapped in prior studies of midshipman (Goodson and Bass, 2002; Kittelberger et al., 2006; Kittelberger and Bass, 2013). Whether medial and lateral sites represent two parallel subdivisions of the descending vocal system or the lateral midbrain feeds into the medial to activate the hindbrain vocal central pattern generator (CPG) is beyond the scope of this study and requires further investigation of midbrain microcircuitry. We propose, for vertebrates in general, that the neural control of vocal attributes is sculpted by differential activation of midbrain populations given that (1) call duration is a salient trait distinguishing natural call types in midshipman (Bass and McKibben, 2003), and vertebrates in general (Bradbury and Vehrencamp, 2011), and (2) midbrain vocal sites are present in tetrapods (see Kittelberger et al., 2006).

Specifically, we showed that the duration of fictive calls elicited from lateral midbrain sites was up to an order of magnitude longer than those elicited from medial sites, regardless of stimulus order. In some experiments, medial stimulation may have directly activated the MLF that also evokes brief calls (Kittelberger et al., 2006). Calls elicited from lateral sites also
exhibited higher stimulus thresholds and longer latencies than medial-elicited calls, suggesting that the lateral site is overall less excitable and/or is part of a multi-synaptic pathway that eventually leads to activation of the hindbrain vocal CPG (see below). Strikingly, the latency of lateral midbrain-evoked fictive calls are comparable to the latency of the hum component in medial-midbrain evoked “grunt-hums”/growls (Rubow and Bass, 2009), suggesting that this component could have been produced by a disinhibited lateral site. Importantly, glutamate injection into the lateral midbrain elicits long duration responses like those we report here (Weeg et al., 2005), suggesting stimulation of local neuronal populations. A more complete investigation using, for example, focal glutamate injection can more precisely map vocally active sites in the midbrain. We propose that divergent medial versus lateral midbrain responses is reliant on differential patterns of vocal-acoustic connectivity and/or neuroendocrine modulation. In midshipman, the midbrain PAG is a crucial node in vocalization initiation and highly interconnected with auditory centers, consistent with its role in sensorimotor integration and vocal initiation in birds and mammals (Jürgens, 2009; Holstege 1989; Kingsbury et al., 2011; Kittelberger and Bass, 2013). Recent studies reveal that the midshipman lateral PAG is more extensively connected than medial PAG to auditory-recipient nuclei including the TSd (Kittelberger and Bass, 2013), consistent with our finding that TSd stimulation can elicit longer calls (Fig. S2). The lateral PAG also shows greater connectivity to other brainstem vocal sites, as well as the anterior, ventral tuberal (vT) hypothalamus that is directly connected to and activates the PAG (Goodson and Bass, 2000b; Goodson and Bass, 2002; Kittelberger et al., 2006; Kittelberger and Bass, 2013). The latency (Fig. 5E) and percentage of successfully evoked fictive calls from lateral midbrain sites recorded here (~65%, see Results) closely resemble values for calls evoked from vT, providing a neurophysiological complement to anatomical evidence showing stronger vT input to lateral than medial PAG (Kittelberger et al., 2006; Kittelberger and Bass, 2013). However, our lateral sites evoked notably longer calls than those reported for vT stimulation (Kittelberger et al., 2006), suggesting that while we stimulated the vT-PAG pathway, we were also activating a more extensive network. Furthermore, lateral PAG has stronger connections with the anterior tuberal hypothalamus (AT), a stimulation site that elicits calls similar to those from the lateral midbrain and PL (see Goodson and Bass, 2000b Fig. 4), so both AT and vT inputs into the lateral midbrain could be important for generating long calls. Rostromedial PAG, by contrast, has stronger connections to downstream hindbrain auditory-recipient nuclei that are absent from lateral PAG output (Kittelberger and Bass, 2013). Together, these results support a functional separation of the medial and lateral PAG/midbrain tegmentum...
in eliciting context-dependent call types, e.g., brief agonistic grunts vs. longer advertisement
calls, dependent on sensory inputs and local neuroendocrine influences.

Vocal and acoustic structures in the midbrain such as the PAG, torus semicircularis,
parataoral tegmentum (PTT), and paralemniscal tegmentum (PL) are highly interconnected and
have been described together as the midbrain vocal-acoustic complex (mVAC) (Bass et al., 2000;
Goodson and Bass, 2002). Anatomical studies of neuropeptide input and steroid receptor
distribution in the midshipman midbrain support potential medial versus lateral nodes of
neuroendocrine action. Isotocin expressing fibers and terminals originating from the POA show
dense expression in the mVAC, including the PTT that receives dense input from the lateral PAG
and is a stimulation site resulting in long calls (Fig. S2D,E) (Goodson et al., 2003; Weeg et al.,
2005; Kittelberger and Bass, 2013). Sparse labeling of isotocin was also found in the TSd,
another site for eliciting long fictive calls (Fig. S2D). A dorsal bundle of AVT expressing fiber
and terminals, also originating from the POA, can be found in the PTT. Unlike the diffuse
expression of isotocin throughout the caudal midbrain, AVT immunoreactivity was concentrated
in a dense band within the midbrain paralemniscal tegmentum (PL) (Goodson and Bass, 2000b;
Goodson et al., 2003). Although Goodson and Bass (2000b) report no effects of AVT on fictive
call duration following AVT injections in the PL (unlike in POA, Goodson and Bass, 2000a), the
one illustration of a stimulation site (their Fig. 5A, comparable to our Fig. S2F) suggests that
injections were at sites caudal and medial to the majority identified in our study (Fig. S2E).

Previous in-vivo physiology experiments are suggestive of steroid action at midbrain
levels, where sustained (>30 min) stimulatory effects of steroids on fictive call duration are
dependent on descending midbrain input into the hindbrain CPG (Remage-Healey and Bass,
2004). Subsequent studies show androgen and estrogen receptor subtypes in the PAG (Forlano et
al., 2010; Fergus and Bass, 2013), and high concentrations of the estrogen-synthesizing enzyme
aromatase in the medial PAG (see Forlano et al., 2005). Presumably, endogenous steroids could
act upon midbrain specific receptors to increase vocal network excitability. However, since these
studies used systemic steroid injections, medial vs. lateral midbrain specific effects cannot be
parsed out.

Taken together, the available evidence strongly suggests that further investigation is
warranted to assess differential peptidergic and steroid control of specific midbrain vocal-acoustic
regions.

Concluding remarks
Vertebrates occupy divergent temporal niches (Challet, 2007; Helfman, 1993; Reebs, 2002; Steiger et al., 2013) despite sharing a highly conserved and predictable pattern of melatonin secretion from the pineal at night (but see Wikelski et al., 2005). Thus, species-typical patterns of melatonin-dependent circadian and diel behaviors must rely on divergent downstream molecular and physiological events to interpret the melatonin signal accordingly at a particular time of day and year for a given species. Evidence for such plasticity is found in the varying distribution and abundance of melatonin receptors within neural pathways of songbirds across species, sex, development, and seasons (Bentley et al., 1999; Bentley et al., 2013; Cassone et al., 1995; Gahr and Kosar, 1996; Whitfield-Rucker and Cassone, 1996), the pro- or anti-gonadal effects of exogenous melatonin treatment in fishes (Maitra and Chattoraj, 2006), and the differential effects of melatonin on locomotor activity in nocturnal vs. diurnal fishes (López-Olmeda et al., 2006).

Results presented here suggest that differential melatonin sensitivity exists within subregions of a central vocal motor network to regulate the production of seasonal and nocturnal-dependent advertisement calling. Comparative approaches studying behaviors with different daily and seasonal expression patterns at the level of specific neural pathways, such as the vocal network controlling midshipman nocturnal vocalization, will contribute to a more predictive mechanistic model for melatonin regulation of behavior.

**MATERIALS AND METHODS**

**Animals**

Midshipman fish have two male reproductive morphs: type I males that acoustically court females and are the focus of this study, and type II males that sneak spawn (Brantley and Bass, 1994). Nesting type I males (standard length 15.1±0.24cm) were hand collected from rocky intertidal zones in northern California between May 2011 and August 2012. Fish were temporarily (1-7 d) held in large outdoor tanks with running seawater at the University of California Bodega Marine Laboratory before shipping overnight to Cornell University where they were housed in individual tanks under 15 h light (L) : 9 h dark (D) for at least two weeks before experimentation. To better simulate sunrise and sunset, a small lamp turned on at 02:00 EST, half an hour before room lights turned on, which turned off half an hour before the small lamp at 17:00 EST. Visual inspection of the gonads and swim bladder muscles at the time of sacrifice confirmed type I male status (Bass, 1996). All methods were approved by the Institutional Animal Care and Use Committee at Cornell.
Drug treatment

Fish held in 5LL received either a control implant (coconut oil vehicle) or a 50 mM 2-IMel implant (Santa Cruz Biotechnology Inc, CA, USA) for five days (Fig. 1C). 2-IMel was dissolved to 2 μg/μl in heat-liquefied coconut oil, sonicated to homogeneity, pipetted into 10 mm plastic tubing with 0.5 mm inner diameter (BD Infusion Therapy Systems Inc., Sandy, UT, USA) and stored at -20°C in parafilm sealed Eppendorf tubes until use. After normalization by body weight (bw) of implanted males (n=12), implant dosages were 0.09±0.01 μg/g bw, lower than reported doses used in fishes and tetrapods that range from 0.1 μg/g bw to 300 μg/g bw (Aarseth et al., 2010; Alvariño et al., 2001; Amano et al 2000; Dubocovich et al., 1998; Handeland et al., 2013; López-Olmeda et al., 2006; Pinillos et al., 2001; Porter et al., 1998; Rubio et al., 2004; Stankov et al., 1993). In European sea bass, 0.1 μg/g bw melatonin treatment raised day time plasma levels to physiological nocturnal levels (Rubio et al., 2004). Thus, we are confident that our dosage was in a physiological range. Following general anesthesia in 0.025% benzocaine (Sigma, St. Louis, MO, USA), implants were inserted into the abdominal cavity of males through a small incision, which was closed by suture and Vetbond Tissue Adhesive (3M Animal Care Products, St. Paul, MN, USA). Fish were observed to fully recover within approximately 15 min after surgical procedure.

Fish held in 5DD received daily intramuscular injections for five days via 27 gauge butterfly needles (BD Infusion Therapy Systems Inc.) of 2 μg/g bw of a receptor antagonist or only the vehicle (dimethyl sulfoxide, DMSO; J.T. Baker, Phillipsburg, NJ, USA) (Fig. 1C).

Stocks of 50 mM luzindole and 4P-PDOT were dissolved in DMSO and stored at -20°C in parafilm-sealed Eppendorf tubes until use. On the first day of injection, fish were anesthetized under 0.025% benzocaine and weighed in order to calculate the appropriate volume of luzindole, 4P-PDOT, or DMSO to reach 2 μg/g bw, to be added to 5 μl/g bw teleost saline solution or water as an injection carrier. Subsequently, daily injections were done within 1 h before subjective lights off, between 16:00-17:00 EST except for the day of neurophysiology (Fig. 1C). We chose to use 2 μg/g bw for luzindole and 4P-PDOT because this dose was within the range used in studies looking at locomotion or feeding (Dubocovich et al., 1998; Pinillos et al., 2004). Treatment groups were staggered to ensure that fish were tested under similar conditions, such as total time spent in captivity.

In-vivo neurophysiology

The in-vivo fictive call (defined in Introduction) preparation used here follows Rubow and Bass (2009). Briefly, a dorsal craniotomy exposed the brain, rostral spinal cord, and vocal/occipital
nerve after general anesthesia with 0.025% benzocaine and local injection of 0.25% Bupivacaine (Hospira, Inc., Lake Forest, IL, USA), a long-lasting local anesthetic. After surgery, fish were immobilized with an intramuscular injection of pancuronium bromide (0.5 mg/kg; MP Biomedicals, LLC, Solon, OH, USA) and stabilized on a platform within a Plexiglas tank with chilled saltwater (15 -17°C) perfused through the mouth. After 1 h of acclimation, an insulated tungsten electrode (125 μm diameter, 8° tip angle, 5 MΩ impedance, 20 μm exposed tips; A-M Systems, Sequim, WA, USA) was used to evoke fictive calls from midbrain sites using well-documented surface landmarks and depth measurements as guidance for electrode placement (Goodson and Bass, 2002; Kittelberger et al., 2006). Fictive calls were recorded from vocal nerve roots unilaterally (reflects synchronous bilateral activity; Bass and Baker, 1990) with an extracellular Teflon coated silver electrode with an exposed ball tip (50-100 μm diameter) and digitized using MATLAB software designed by Dr. Bruce R. Land (School of Electrical and Computer Engineering, Cornell).

In 120 min sessions, each stimulation trial consisted of 40 brief stimulus trains delivered to midbrain sites. Baseline (0 min) and subsequent recording trials were performed at 5, 15, 30, 45, 60, 75, 90, 105, and 120 min (Fig. 1C,D). For 5LL and 5DD animals (Fig. 1C), we delivered 100 stimulus trains instead of 40 at the time of the 120 min trial because Rubow and Bass (2009) found that the additional 60 stimuli evoked longer duration responses resembling natural calls in 5DD and 14:10 h L:D males. We generated a SRC at the end of the 120 min recording session to measure the relationship between stimulus intensity and fictive call duration (Fig. 1C,D). At 10 min after the 120 min recording session and without moving the stimulating electrode, we recorded 10 fictive calls evoked by 100%, 125%, 150%, 175% and 200% of threshold stimulus, with each recording separated by 5 min. For some animals (see Fig. 1C,D) the stimulating electrode was immediately moved to a lateral midbrain site and another SRC was recorded. Each stimulus train consisted of 5 square pulses (0.1 ms pulse width, 200 Hz), with inter-train intervals of 1 s were delivered via a stimulus isolation unit (SIU; Model A350D-A, World Precision Instruments, Sarasota, FL, USA). SIU current output (nA) was interpolated from a linear relationship between SIU dial level and current output derived using the Ohms Law by measuring voltage across a resistor.

All animals were tested 1 h post surgery starting between 17:00 EST to 18:00 EST under darkness with experimenter wearing a red head lamp (except for 5LL animals who were tested under light). 5DD fish experienced approximately 15 min of exposure to low-level scope light during craniotomy.
Medial vs. lateral midbrain lesions

At the end of the experiments in a subset of animals including fish from drug treatment experiments that received stimulation at medial and/or lateral sites, electrolytic lesions were made by passing 10 μA of current with 50% duty cycle for 4–15 s through the stimulus electrode to mark the stimulation site. Fish were then deeply anesthetized under 0.025% benzocaine and perfused transcardially with teleost Ringer followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.2). Prior to sectioning, brains were cryoprotected in 30% sucrose solution overnight, frozen in Cryo-M-Bed (Hacker Instruments, Huntington, UK), sectioned at 30 μm in the transverse plane on a cryostat, and mounted onto Superfrost Plus slides (Erie Scientific, Portsmouth, NH, USA). Photomicrographs were taken of selected sections using the Spot FLEX imaging system (Diagnostics Instruments, Inc, Sterling Heights, MI, USA) on a Nikon Eclipse E800 compound microscope.

Neurophysiological and statistical analysis

Measurements of fictive call duration, latency (delay from end of stimulus to first fictive pulse), and IPI were performed by a customized MATLAB program designed by Dr. Bruce Land, Cornell University. In some cases where more than one fictive burst was elicited from the lateral midbrain, we combined the total duration of all bursts for analysis.

All statistical analyses were performed in JMP 9 (SAS Institute Inc., Cary, NC, USA) using means obtained from individual recording trials and in consultation with the Cornell University Statistical Consulting Unit. Log or square root transformations were performed whenever assumptions of normality were violated. We used a linear mixed model with appropriate fixed effects (details below) and fish nested within treatment as a random effect.

In order to examine effects of photoperiod manipulation, we first compared data taken from 5LL and 5DD control animals (oil-implanted or vehicle-injected) and LD non-treated animals used in medial vs. lateral comparisons. For 120 min trial and SRC measurements, our fixed effects were photoregime, time, and photoregime*time interaction. Three-way ANOVA followed by Tukey-HSD post-hoc comparison was performed on SRC stimulus thresholds.

For 5LL and 5DD 120 min sessions, we looked for effects of treatment, time, and treatment*time interaction on fictive call duration and threshold. Since luzindole and 4P-PDOT act on melatonin receptors with different affinities (Dubocovich et al., 2010), we performed separate analyses for each. The 100 stimuli delivered on the 120 min trial in 5LL and 5DD animals were split into the first 40 and last 60 calls for analyses. We combined data from 2011...
and 2012 5LL animals since year was not a significant factor (120 min session duration: \( P=0.34 \); 120 min session threshold: \( P=0.27 \); medial SRC duration: \( P=0.80 \); lateral SRC duration: \( P=0.76 \)).

For SRC duration comparisons, fixed effects were treatment, stimulus-intensity, and treatment*stimulus-intensity. To compare SRC threshold levels across treatment groups, we performed a three-way ANOVA for each antagonist.

In LD groups used for medial vs. lateral comparisons, we looked for effects of stimulus-site, time, and stimulus-site*time interaction on duration, stimulus threshold, and latency of fictive responses in 120 min sessions. The fixed effects for SRCs were stimulus-site, stimulus-intensity, and their interaction. Additionally, we performed a *Student’s t-test* on a total of 116 IPIs from medial SRC and 1149 IPIs from lateral SRC in one individual to assess potential site-dependent differences in call frequency.

**LIST OF SYMBOLS AND ABBREVIATIONS**

- 2-iodomelatonin (2-IMel)
- 4-phenyl-2-propionamidotetralin (4P-PDOT)
- 15:9h light:dark cycle (LD)
- 24h of light for five days (5LL)
- 24h of darkness for five days (5DD)
- anterior tuberal hypothalamus (AT)
- arginine vasotocin (AVT)
- body weight (bw)
- central pattern generator (CPG)
- deep layer of the torus semicircularis (TSd)
- dimethyl sulfoxide (DMSO)
- interpulse interval (IPI)
- midbrain vocal-acoustic complex (mVAC)
- paralemniscal midbrain tegmentum (PL)
- paratal midbrain tegmentum (PTT)
- periaqueductal gray (PAG)
- stimulus isolation unit (SIU)
- stimulus-response curve (SRC)
- torus semicircularis (TS)
- ventral tuberal hypothalamus (vT)
ACKNOWLEDGEMENTS

We sincerely thank Margaret Marchaterre for support with fish collection and neuroanatomy, Boris Chagnaud for help with neurophysiology and comments on the manuscript, Dan Fergus and Kevin Rohmann for statistical advice, and Bruce Land for programming support.

FUNDING

Support from NSF IOS 1120925, Cornell Research in Animal Behavior grant, Cornell Sigma Xi research grant.

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**FIGURE LEGENDS**
Fig. 1. (A) Natural (adapted from Rubow and Bass, 2009) and fictive calls of male midshipman fish. Long-duration advertisement hums are produced in the summer at nighttime. Short-duration agonistic grunt trains can be produced at any time of day or year. (B) A schematic sagittal view of the midshipman brain showing the vocal control network (adapted from Chagnaud et al., 2011). Stimulation in the midbrain vocal-acoustic complex (mVAC) evokes readily recorded fictive calls from the vocal nerve. mVAC receives input from forebrain vocal-acoustic complex (fVAC), from which fictive calls can also be evoked. mVAC drives the hindbrain vocal pattern generator, which provides a precise and synchronous code for sonic muscle contraction and consists of vocal pre-pacemaker nucleus (VPP), vocal pacemaker nucleus (VPN), and vocal motor nucleus (VMN). (C-D) Schematic of photoperiod, drug treatment, and neurophysiology stimulation regimes used in this study. (C) 5LL fish were implanted with 2-IMel or vehicle before subjective lights-off and moved to constant light (LL) for 5 days (d). Light grey boxes represent subjective night. 5DD fish were held in 5 days of constant darkness (DD) and injected daily around subjective lights-off with vehicle, luzindole, or 4PPDOT. Dark grey boxes represent subjective day. Black arrows indicate time of treatment, and gray arrow indicates time of neurophysiology for both 5LL and 5DD fish. 120 min sessions consisted of stimulation at indicated times after fish acclimated on the rig for 1 h. 40 stimuli were delivered at each time point except for 120 min when an additional 60 stimuli for a total of 100 was delivered (highlighted by *). 10 min later, a stimulus response curve (SRC) was collected without moving the electrode, where stimulus intensity was increased to indicated % of baseline threshold, recording 10 fictive calls every 5 min. The stimulus electrode was then immediately moved to a lateral site in the midbrain to collect another SRC. (D) LD fish were tested for medial and lateral midbrain stimulation comparisons and were housed in 9 h of darkness (D) and 15 h of light (L). The neurophysiology stimulation regime followed that of 5LL and 5DD animals except only 40 stimuli were delivered at the 120 min trial, and only one SRC was collected after 120 min sessions without moving the electrode.

Fig. 2. Effects of photoperiod on vocal excitability inferred from control animals. Fictive call duration (A) and threshold (B) over 120 min sessions, as well as medial (C) and lateral (D) SRCs recorded from control fish of each photoregime/treatment group: LD, 5LL, and 5DD (see legend in A). (E) Threshold levels at baseline trials of medial and lateral SRCs. For LD fish that received lateral midbrain stimulation only, lateral SRCs were recorded without prior medial SRCs (Lateral 1st). All other groups received medial stimulation first (Medial 1st). Data are presented as mean±standard error. n.s., non-significant. * indicates P<0.042.
Fig. 3. Characterization of fictive calls elicited from animals kept under five days of continuous light (5LL) and implanted with 2-iodomelatonin (2-IMel) or vehicle control. (A) 2-IMel treatment increased fictive call duration in a time-dependent manner during the 120 min stimulus trial and decreased stimulus threshold (B) (see legend in A). (C-D) 2-IMel increased fictive call duration in stimulus response curves (SRC) only when lateral midbrain was stimulated. (E) 2I-Mel decreased stimulus threshold measured at the onset of SRCs. All fish were stimulated at a medial site first for the 120 min session and SRC, followed by recording a second SRC at a lateral site. Data are presented as mean±standard error. Abbreviations: Stim, stimulus intensity; n.s., non-significant. Note for 120 min session duration, the means from the first 40 responses were separated from the last 60 fictive call responses (displaced to the right), hence the two data points at 120 min.

Fig. 4. Melatonin receptor antagonists luzindole (luz) and 4P-PDOT have mixed effects on fictive call 120 min session duration (A), 120 min session threshold (B), medial SRC duration (C), and lateral SRC duration (D) in fish kept under five days of constant dark (5DD) (see legend in A). (E) Treatments had no effect on SRC threshold levels, which were higher at lateral sites. Data are presented as mean±standard error. stim, stimulus intensity; n.s., non-significant. Note for 120 min session duration measurements the means from the first 40 responses were separated from the last 60 fictive call responses (displaced to the right), hence the two data points at 120 min.

Fig. 5. (A-B) Five representative fictive vocal responses evoked by medial (Ai) and lateral (Bi) midbrain stimulation at 200% SRC, taken from a fish that was treated with 2-IMel. Fictive vocal traces outlined in grey boxes were enlarged in (Aii) and (Bii). Grey carat signs point to stimulus artifacts. From the same fish and stimulation trial, fictive call firing frequency, measured in inter-pulse intervals (IPI), taken from 200% threshold medial SRC trial (Ai) and lateral SRC trial (Biii) showed no significant site-dependent differences. (C-F) Quantification of fictive call duration (C), stimulus threshold (D), latency (E), and stimulus response curves (F) resulting from either medial or lateral midbrain stimulation in non-treated fish held in normal light:dark (LD) cycles (n=5/group). Data are presented as mean±standard error. All stimulus-site comparisons shown in C-F are statistically significant (P<0.006).

Fig. S1. Medial sites evoked shorter fictive calls, even after lateral stimulation. Three males held in normal 15 h light:9 h dark cycle were tested at the lateral site first for a 120 min session and a
SRC at the same site, followed by recording a second SRC at the medial site. (A) Fictive call duration recorded over 120 min from the lateral site. (B) Fictive call latency recorded over 120 min from the medial site. (C) Threshold stimulus required to elicit fictive calls over 120 min sessions. (D) Fictive calls elicited during lateral SRC are significantly longer than medial SRC, demonstrating that regardless of stimulus order, lateral midbrain stimulation reliably elicits longer duration calls. P value is shown for the fixed effect of stimulus-site. Data are presented as mean±standard error.

Fig S2. Localization of lateral and medial midbrain lesion sites. Sample lateral (A) and medial (B) lesions are indicated by arrows. (C) Dorsal view of the midshipman brain, showing relative midbrain levels at which coronal sections in D-F were taken. Abbreviations: F, forebrain; M, midbrain; C, cerebellum; H, hindbrain. (D-F) The relative positions of medial (x) and lateral (o) lesion sites were mapped onto midbrain sections at three transverse levels adapted from Kittelberger and Bass (2013). Of the medial lesions, seven fell within the midbrain tegmentum adjacent to the medial longitudinal fasciculus (MLF) that connects the vocal midbrain with the hindbrain vocal pattern generator (E,F), one was on the medial edge of the PAG (E), one was within the MLF (F), and two were below the MLF (F). Of the lateral lesions, five were in the paratoral tegmentum (PTT) that lies below the lateral periaqueductal gray (PAG) (D, E), one within the deep layer of the torus semicircularis (TSd) (D), and four just below or along ventral aspect of the paralemniscal tegmentum (PL) that borders the lateral lemniscus (ll) (E).

Abbreviations: Is, isthmal nucleus; nll, nucleus of the ll; nMLF, nucleus of the MLF; nlv, nucleus lateralis valvulae; TE, midbrain tectum; TL, torus longitudinalis.