



SEASONAL AND DIEL RHYTHMS REGULATE MULTISTABILITY IN A TELEOST VOCAL PATTERN GENERATOR

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SEASONAL AND DIEL RHYTHMS REGULATE MULTISTABILITY IN A
TELEOST VOCAL PATTERN GENERATOR

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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August 2010

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SEASONAL AND DIEL RHYTHMS REGULATE MULTISTABILITY IN A
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Cornell University 2010

The central pattern generator controlling vocalizations in songbirds has been investigated for more than 30 years and produced a wealth of information about the morphological and physiological underpinnings of seasonal change in song production and stereotypy. Now departing the aerial lifestyle for an aquatic one, we find a teleost fish that presents not only an annual reproductive rhythm in vocal motor circuit function, but a daily and activity-dependent one as well. The plainfin midshipman, *Porichthys notatus*, spends winters in the deep, offshore waters of the Pacific coast from Baja to Alaska from where they migrate into the tidal zone in spring and summer to spawn. At night, the parental and highly vocal, type I male uses an acoustic beacon, the advertisement hum, to attract the females to his rocky excavation to lay her eggs, a nest also defended with several agonistic calls. These calls can be studied in a neurophysiological, or “fictive call” preparation, in which the vocal circuit is activated by microelectrical stimulation and the rhythmic output easily monitored by an extracellular electrode on the ventral root nerve that innervates the vocal muscle of the swim bladder. Since this rhythmic motor volley, “or fictive vocalization” directly predicts the temporal properties of the natural calls, it serves as a valid measure of natural plasticity in a dedicated motor circuit. The following studies present for the first time the full repertoire of midshipman fictive calls and how seasonal and diel physiological changes in vocal circuit function determine its variable output.

Furthermore, the activity-dependence of these rhythmic fictive calls and their patterning by spatially dynamic levels of GABAergic inhibition may reveal a functional partitioning in the circuit, such that rhythm and duration are controlled at one level, and the frequency shift that distinguishes a broadband grunt from a multiharmonic hum occurs at another.

BIOGRAPHICAL SKETCH

Tine Rubow was born in Denmark, raised in the Bay Area in California, received her B.S. from Northern Arizona University in 2005 and her doctorate in neurobiology from Cornell in 2010.

ACKNOWLEDGMENTS

I wish to express appreciation to Professor Andrew Bass, my thesis committee chair, for his support and patience and for sharing in the discovery of new fictive calls with me. I would like to thank my committee members, Drs Joseph Fetcho, David Deitcher and Ned Place for their thoughtful insights and practical expertise along the way.

I am also grateful to the Cornell University fellowship program and two NIH predoctoral training grants 5-T32-MH15793 and GM007469 for my financial support.

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CHAPTER 1

INTRODUCTION

The Rhythms of a Fish: A Symphony in three movements

From macroscopic seasonal change to microscopic single cell oscillations, rhythms organize life, ultimately synchronizing seasonal and daily patterns of behavior with social and environmental cues to enhance survival (reviewed in Herzog, 2007). Regulated by external cues and yet autonomous internally, ongoing neuronal oscillations interact with incoming sensory stimulation to alter perception, sensorimotor integration, memory formation, and the final patterning of motor behavior (Witham et al., 2007; Mormann et al., 2008; Buzsaki, 2005). This rhythm-supported synchrony with its optimization of neural processing and conservation of energy (Buzsaki and Chrobak, 1995) is evident in the network-binding gamma oscillations of the cortex (Buzsaki and Draguhn, 2004), in the smooth and regular gait of a tetrapod or in the undulations of a fish.

The beauty and complexity arises in the temporal and spectral overlaps, as if in a vertical stacking of pitches or chords or the horizontal weaving of melodic lines. Indeed, the molecular periodicity of metronomic “clock” gene expression appears to be the limiting factor or “tempo” for the patterning of other central network rhythms—such as vocalizations. Interpulse intervals of the *Drosophila* courtship song fluctuated rhythmically and were actually shortened in *per* mutants in conjunction with the abbreviation of their circadian locomotor rhythm, while another *per* mutation lengthened both rhythms (Kyriacou et al., 1980). They concluded that products of the *per* clock gene control a fundamental property of temporal regulation in the fruitfly, whether it is the circadian period or the more rapid oscillations of the courtship song.

While fish are not yet unanimously considered to have a hypothalamic nucleus with pacemaker functions like the mammalian suprachiasmatic nucleus (SCN), robust clock gene rhythms occur in almost all species, produced in the pineal and retina as well as in peripheral tissues of zebrafish (Cahill, 2002; Whitmore et al. 1998; Kaneko et al., 2006). Although relatively few specific behaviors have been linked to these distributed “clocks”, mounting evidence across invertebrate and vertebrate species for more omnipresent circadian pacemaker cells in the central nervous system and peripheral tissues raises questions about their effect on physiological and behavioral plasticity (Abe et al., 2002; Lyons et al., 2006a; Vansteensel et al., 2008). For example, in the rat there are multiple isolated brain regions containing damped oscillators with their own rhythmicity and kinetics, measured by *Per1* expression, including the olfactory bulb and various structures of the telencephalon (Abe et al., 2002), while in a marine mollusk, an extra-ocular circadian oscillator modulates long-term memory (Lyons et al., 2006b).

More than twenty years of studying the seasonal morphological and physiological changes in the avian vocal circuit have generated a detailed story of another rhythm with an audible output shaped by peripherally and centrally cycling hormones. The avian song control system has been a model for the periodic anatomical and functional plasticity in the adult CNS (Tramontin and Brenowitz, 2000; Meitzen et al, 2009). Although elevations of gonadal steroids strongly influence the morphological and physiological changes measured in the male vocal nuclei, there is evidence for testis-independent effects of photoperiod as well (Meitzen et al., 2007). High-affinity melatonin binding sites have been identified in several of the song nuclei of the song sparrow and zebra finch (Whitfield-Rucker and Cassone 1996; Gahr and Kosar 1996), and exogenous melatonin has been shown to decrease the size of HVC and Area X in castrated starlings (Bentley et al 1999). This suggests that photoperiod

and clock genes, which ultimately regulate the reproductive axis via circulating melatonin and steroids, may also directly, and in parallel with the hormones, govern the volumetric and physiological changes in song control nuclei. Such synergy between environmental cues, gonadal hormones, fluctuating melatonin secretion and even *local* control by circadian pace-making cells must finally be the more complex and comprehensive explanation for periodic oscillations in CNS function that control diverse behaviors, including vocalization. Thus, it is arguably as important to consider endogenous biological rhythms when investigating the neurophysiology of a behavior as one does when studying the natural behavior itself.

Different species have exemplified different biological rhythms--but there may yet be one homely fish to unite them all. The vocalizing plainfin midshipman, *Porichthys notatus*, a member of the toadfish family, Batrachoididae, offers its life history and vocal motor system as a platform for the multilevel study of biological periodicity, from the temporal properties of their social calls to the hormone-supported reproductive behaviors and the pattern-generating networks that support them. Reproductive, type I, parental males enter their most enhanced vocal state during the summer when a convergence of biological changes prime their brains and bodies for long nights of courtship humming that solicit females to their rocky nests to spawn. Some of the biological underpinnings of their seasonal behaviors have already been documented, beginning with the most fundamental rhythms of steroid secretion and the differential central expression of the testosterone-converting enzyme, aromatase (Sisneros et al., 2004; Forlano and Bass, 2005). Peripherally, cycling androgens also promote the annual hyperplasia of the sonic muscle that is attached to the lateral walls of the sound-producing swim bladder (Brantley et al., 1993), while centrally, androgens along with estrogen and corticosteroids enhance functioning of the vocal motor circuit in both midshipman and their cousin the Gulf toadfish, *Opsanus beta*

(Remage-Healey and Bass, 2004, 2005, 2006). Ingested 11kT increased the call rate of wild toadfish while systemic injections of these steroids increased the duration of the “fictive grunt.”

“Fictive calls” are the neural correlates of the natural call that can be recorded from the occipital nerve root in an *in vivo* neurophysiological preparation. The discharge frequency of the motoneurons determines the contraction rate of the vocal muscle and thus the fundamental frequency of harmonic calls and the pulse repetition rate of non-harmonic vocalizations like the broadband grunt (Bass and Baker, 1990). The rhythmic properties of the vocalizations reflect the output of a vocal pattern generator that is evolutionarily linked to comparable networks in tetrapods (Bass and Baker, 1990; Bass et al., 1994; Bass et al., 2008). The hindbrain-spinal pattern generator responsible for the calls is comprised of a rostral hindbrain pre-pacemaker nucleus (VPP), which projects to the paired columns of vocal pacemaker neurons (VPN), ventrolaterally abutting the paired vocal motor nuclei (VMN) that straddle the midline of the caudal medulla and rostral spinal cord. Vocal motoneurons can be activated by electrical microstimulation in the forebrain’s pre-optic area-anterior hypothalamus (POA-AH), the midbrain periaqueductal grey or PAG, or in VPP. The firing patterns of the vocal motor circuit determine frequency, duration and amplitude, the stereotyped and easily recorded properties of the natural calls by which the vocalizations are neurally encoded. On the receiving side, it has been shown that during the reproductive summer, estrogen tunes the sensitivity of the female auditory system to the higher harmonics of the advertisement call. The perception of the upper harmonics of the hums, as measured by the temporal encoding of frequency by eighth nerve saccular afferents, is as acute in estrogen-treated (30 d), non-gravid females, as in freshly caught, gravid females (Sisneros et al., 2004).

The natural, multiharmonic hum has a highly stable fundamental frequency around 100 Hz at ambient temperatures that does not vary across the duration of the call and shows almost no amplitude modulation. The hum contrasts sharply with the very brief (50-100 ms), higher frequency (~110 Hz) and broadband agonistic grunt produced singly by nesting parental males (type I), an alternative male morphotype (type II) that either sneaks or satellite spawns, and females. During agonistic encounters with other males, the grunt is also produced repetitively as a “grunt train” by nesting males at rates of 1.5-3 Hz for as long as several minutes (Bass et al., 1999; Brantley and Bass, 1994; Cohen and Winn, 1967; McKibben and Bass, 1998). A second agonistic call, the “growl”, is exclusive to the nest-building males and most frequently recorded at night (Bass et al., 1999). Growls are the most complex call; they overlap hums in duration (~200 ms – 5 s) and are reiterative sequences of grunt and hum-like signals, and have thus, in these studies been dubbed the “grunt-hum.”

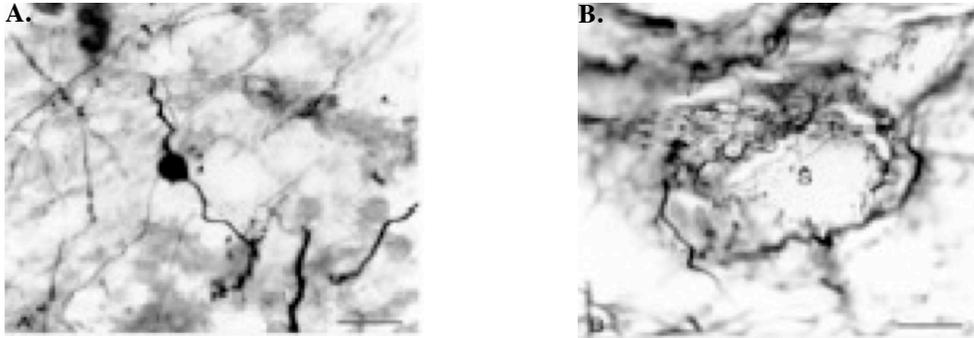
In spite of this detailed understanding of the midshipman’s seasonal vocal repertoire and auditory behavior, and their dependence in part upon seasonal fluctuations in systemic and central hormone levels, the fictive correlates of the very calls that are the signatures of their peak reproductive state: the hum, the growl or “grunt-hum” and the grunt train--were never consistently or fully evoked in a neurophysiological preparation. This is in stark contrast to the malleable *in vitro* evocation of fictive advertisement calls from male *Xenopus laevis*, at any time of day or year after systemic injection of human chorionic gonadotropin (hCG) and bath application of 5-HT to activate the circuit (Rhodes and Yamaguchi, 2007). The wild-caught midshipman has not been so cooperative, as if remaining temperamentally connected to its natural environment after captivity. This has been both a hurdle and a gift. By taciturnly mumbling only their monosyllabic grunts, midshipman compel us to find the keys that would unlock the other calls, thereby exposing a more intricate

system of hormonal and neurological mechanisms that control the cyclic tuning of their brains, the variable output of the vocal pattern generator, and their natural vocal rhythms.

Presented here, for the first time, are some of the fundamental requirements for the production of the fictive correlates of these long duration, lower frequency calls typically recorded during reproductive summer nights (Rubow and Bass, 2009) (Chapter 2). The natural and fictive hum and growl are clearly enhanced by seasonally and nocturnally dependent physiological states, while the free-running grunt train is only slightly less constrained. Furthermore, with the gradual development of a new stimulation paradigm, it became clear that the evocation of these fictive calls depended upon increased stimulation time and intensity, while paradoxically exhibiting a significant drop in call threshold. Site-specificity was also revealed: fictive growls and hums are most consistently evoked by stimulation in the most medial PAG, as opposed to the lateral, while the grunt train is triggered by stimulation in VPP or in the fibers projecting to VPN. Brief grunts are not dependent upon any of these parameters and reflect the minimal activation of the vocal motor system, as if they are indeed the simplest “phoneme” in the midshipman vocal repertoire. In a final effort to reveal mechanisms controlling the seasonal and nocturnal enhancement of fictive call production that supersedes or compliments the influence of steroids, the type I males were housed for five days in 24h darkness vs. 24h light. All of the 24D animals exhibited a dramatic potentiation of growls and hums, while vocal output from the 24L animals was significantly suppressed. Neither group revealed a significant endogenous rhythm but simply seemed to be vocally potentiated or damped by darkness or light, respectively.

After this general survey of the seasonal, photoperiodic and activity-dependent sensitivity of the midshipman vocal motor system, the inevitable next step was a

deeper investigation into the rhythmic, neurological mechanisms that underlie it. Given the extensive GABAergic innervation of the vocal motor nucleus and VPP (Marchaterre et al., 1989; AH Bass and J Zee, unpublished data) and the integral role of inhibition in the synchronizing and timing of network activity, the influence of GABAergic inhibition on the generation and modulation of fictive vocalizations evoked from territorial male midshipman fish was investigated (Chapter 3 and refs. therein for review). It was hypothesized that shifts between natural and fictive call types reflect, in part, rapid modulation of GABAergic activity in VPP and VPN-VMN that contributes to activity-dependent plasticity of synaptic and/or intrinsic neuronal properties. Some 20 years ago and more recently, electron microscopy, electrophysiology and immunohistochemistry have revealed the dense GABAergic innervation of VMN by VPN afferents as well as the presence of mixed synapses on vocal motoneurons from VPN afferents that combine inhibitory potentials with electrotonic coupling (Bass and Marchaterre, 1989, J. Zee, unpublished data; see Fig. 1). Without yet a complete understanding of how this neurochemical/morphological architecture contributes to the generation of dynamic vocal motor output, focal microinjections of GABA, the GABA_A receptor agonist muscimol and antagonist gabazine were a first step towards understanding the role of GABAergic modulation, at least for the GABA_A receptor specifically, in the discrete regions of the vocal circuit.



Scale bars = 20 μ m. (A. Bass and M. Marchaterre, unpubl. observ.)

Figure 1.1 A. GABAergic neurons (arrowheads point to soma) are positioned along the periphery of the vocal motor nucleus. B. The vocal motor nucleus receives a dense GABAergic input as shown here for terminals and fibers surrounding the soma (s) of a single motor neuron.

Almost immediately it became clear that the same seasonal and diurnal sensitivity that affected vocal motor output in pharmacologically un-manipulated fish, was also determining the efficacy of up or down-regulation of inhibition in VPP and VPN-VMN. While the responses in VPP to GABA, muscimol and gabazine were neatly segregated by a reproductive or nonreproductive state, VPN-VMN was more finicky. Instead of overall reproductive state determining output, the more transient *vocal state* of each fish was the defining factor. Thus, the animals in this group of experiments were separated into a continuum of “grunters” and “grunt-hummers”, designated “states 1-3”, based upon the initial excitability of the circuit and evidence of their potential or not to produce longer duration calls, which generally, but not exclusively, correlated with night and day and reproductive condition. Baseline recordings tended to be flat and non-potentiating in grunters (“state 1”) while incipient grunt-hummers (state 2) quickly increased grunt duration and could even subsequently produce some early sporadic, roughly patterned hums. “State 3” males were already producing some grunt-hums at baseline.

In VPP of state 1 and 3 animals, bilateral injections of GABA rapidly (at 1 min) decreased duration of the fictive call, eliminating hums if they were present, upon which calling rebounded slightly above baseline by 5 and 10 min, ultimately increasing grunt duration or stabilizing hum production. Muscimol, on the other hand, simply had a reducing effect with a slow (30 min) recovery to baseline duration. This contrasted with the dramatic augmenting effect of gabazine in this nucleus in state 1 grunTERS or state 3 grunt-hummers. Grunt duration could increase by up to 600%, while tripling the injection volume or increasing its concentration disinhibited the circuit entirely, such that it was able to fire independently for over an hour, emitting intermittent long duration buzzes in non-reproductive animals, and syncopated grunt trains interspersed with buzzes in reproductive animals.

Downstream in VPN-VMN, GABA injections had a similar and yet unique effect. In grunTERS, fictive grunt duration and amplitude were also reduced, but not as far as in VPP, and duration only slowly recovered to baseline by 30 minutes with no rapid, overshooting rebound. To determine the receptor specificity of this effect, muscimol was injected in both grunTERS and hummers with an opposite, yet complementary outcome. Muscimol had little or no effect on grunt patterning or duration, while it completely eliminated hums. Gabazine, on the other hand, disrupted grunt patterning and reduced grunt duration while rapidly (5-10 min) and dramatically *enhancing* the production of hums and the regularity of their lower firing frequency. Occasionally, gabazine would eliminate the grunt portion of grunt-hums entirely, revealing the divergent and opposing facilitation of call type by GABA and GABA_AR in this nucleus. Although reducing inhibition via GABA_AR enhanced hum production, too much or a complete block of inhibition via this receptor could obliterate all vocal motor output, such that increased stimulation time and intensity were required to retrieve the calls.

GABAergic inhibition, explored via GABA_AR in this study, clearly has distinct effects in the two vocal circuit regions and is modulated by season, time of day and a more nuanced and dynamic vocal state of which this human researcher was not ultimately the master. GABA in VPP appears to primarily control call duration, which may be a byproduct of the fundamental grunt train rhythm: call rate or rhythm is linked to call or burst duration such that shorter bursts create a faster rhythm or longer bursts a slower rhythm (that is essentially non-existent in the hum). The data presented here from VPN-VMN, on the other hand, reveals a fine frequency control or modulation of intra-call firing rate by GABA_AR, distinguishing faster broadband grunts from the slower and highly regular hum.

Interestingly enough, GABA_AR is well known for its modulation and regulation by various progestins in mammals, but not yet in fish. The rapid and sustained allosteric modulation of the GABA_A receptor (GABA_AR) by reduced progesterone metabolites in mammals, such as allopregnanolone, can affect not only reproductive behavior, but also mood, anxiety, cognition, aggression, seizure susceptibility and recovery from traumatic brain injury (Frye and Vongher, 1999; Witt et al., 1995; Van Wingen et al., 2008; Reddy 2004; VanLandingham et al 2006; Kaura et al 2007). Fish have a different progesterone metabolite, (17 α , 20 β , 21-trihydroxy-4-pregnen-3-one or 20 β -S), which is responsible for gamete maturation in both sexes and the general control of reproductive behavior (Thomas et al, 2004). It has been shown to bind a peripheral membrane progestin receptor, mPR, to rapidly enable spawning behaviors (Thomas et al., 2005, Tubbs et al., 2010), but in spite of its identification in fish and mammalian brain and spinal cord, little is known of its central role (Labombarda et al., 2010; Zhu et al., 2003).

Thus, after pilot experiments with systemic injections of 20 β -S in the *in vivo* fictive call preparation revealed an unpredicted growl and hum-promoting effect in

reproductive type I males at night, as early as 45 min post-baseline (Chapter 4), two intriguing hypotheses arose from these data, namely 1) 20β -S modulates $GABA_A$ R at some level of the vocal motor circuit, from the forebrain to the motor nucleus, to enhance production of advertisement hums concurrent with gamete maturation and spawning, or 2) 20β -S binds mPR to the same effect. Radioimmunoassays for plasma 20β -S were undertaken in the lab of Peter Thomas (U. Texas at Austin, Marine Science Institute), to compare progestin levels in reproductive males sampled day vs. night less than 48 hours after removal from their shoreline nests. These assays would potentially corroborate the diel-dependent enhancement of long duration calls from the neurophysiological experiments and validate the progestin concentrations measured in treated vs. control fish. Indeed, the wild-sampled fish revealed undetectable levels of 20β -S during the day vs. 0.37 ng/ml, thus suggesting that this progestin may support the nocturnal production of hums. Fictive grunt-hums could not be evoked from reproductive males treated during the day, but the progestin did have a modest grunt-enhancing effect in non-reproductive males, day or night. Finally, to determine if mPR might be the receptor responsible for these effects, a preliminary western blot of forebrain, midbrain, hindbrain, and testes for mPR was also performed in the lab of Peter Thomas, revealing the presence of this receptor in every tissue. Subsequent blots intended to compare reproductive vs. non-reproductive tissue and night vs. day, however, revealed no reliable difference in expression. Possible explanations for this are discussed in Chapter 5.

In sum, the studies presented here are indeed like the movements of a symphony: 1st movement, seasonal and diurnal rhythms in the vocal circuit; 2nd movement, dynamic GABAergic modulation of vocal output (also dependent upon the background rhythms); and 3rd movement, progestin modulation and support of fictive calling. While the research was conducted as a consecutive and partially overlapping

series, the investigated phenomena are obviously, in real time, more like the instrumental components of a chamber orchestra. The potential synergistic relationships between the rhythmic levels explored here have yet to be established, but the possibilities are tantalizing. Perhaps, if one accumulated enough pieces, investigated from enough angles, used a little perfect pitch and played a little by ear-- the musical composition would eventually emerge.

CHAPTER 2
REPRODUCTIVE AND DIURNAL RHYTHMS REGULATE VOCAL MOTOR
PLASTICITY IN A TELEOST FISH

Abstract

Seasonal and circadian rhythms control fundamental physiological processes including neural excitability and synaptic plasticity that can lead to the periodic modulation of motor behaviors like social vocalizations. Parental male midshipman fish produce three call types during the breeding season: long duration (min - > 1h) advertisement “hums”, frequency and amplitude modulated agonistic “growls” (s), and very brief (ms) agonistic “grunts” produced either singly or repetitively as “grunt trains” for up to several minutes. Fictive grunts that establish the temporal properties of natural grunts are readily evoked and recorded *in vivo* from vocal occipital nerve roots at any time of day or year by electrical microstimulation in either the midbrain periaqueductal grey or a hindbrain vocal pre-pacemaker nucleus. Now, as shown here, the longer duration fictive growls and hums can also be elicited, but are restricted to the nocturnal reproductive season. A significant drop in call threshold accompanies the fictive growls and hums that are distinguished by their much longer duration and lower and more regular firing frequency. Lastly, the long duration fictive calls are dependent upon increased stimulation time and intensity and hence may result from activity-dependent changes in the vocal motor circuit that are themselves modulated by seasonal and circadian rhythms.

Introduction

Synchronizing seasonal and circadian patterns of behavior with relevant social and abiotic cues must ultimately depend upon plasticity in the morpho-physiological properties of neural networks (Herzog 2007; Panda, 2008). The avian song control system has been a model for periodic anatomical and functional plasticity in the adult central nervous system across the longest time span, the reproductive year (e.g., Arnold et al., 1976; Ball et al., 2004; Brenowitz, 2004; Meitzen et al., 2007; Park et al., 2005). Birdsong has also been studied to demonstrate state-dependent auditory activity during the 24 h sleep/wake cycle (Dave et al., 1998; Schmidt and Konishi, 1998), as well as the role of sleep in song learning (Shank and Margoliash, 2008). However, while many animals clearly exhibit daily as well as seasonal patterns of vocal production, few organisms have allowed the comprehensive exploration of a single rhythmic behavior that extends from the broadest neural and neuroendocrine cycles to the oscillating activity of a dedicated circuit. Now, as shown here, a teleost fish, the plainfin midshipman (*Porichthys notatus*, family Batrachoididae), presents a seasonally and diurnally rhythmic vocal behavior readily accessible to neurophysiological and behavioral study.

Essentially the entire life history of adult midshipman fish is characterized by dramatic patterns of seasonal and daily periodicity in reproductive behavior. From non-reproductive winters spent in deep waters off the Pacific coastline, they migrate to tidal spawning grounds in the spring where males excavate nests under rocks from which to attract females and guard offspring (Bass, 1996). Nesting males court females primarily at night with long duration (~350 ms - >1 h) advertisement calls known as “hums” that are generated by the rhythmic contraction of muscles attached to the walls of the swim bladder (Fig. 1A1, A2a) (Bass et al., 1999; Brantley and Bass, 1994; Ibara et al., 1983). The multiharmonic hum has a highly stable fundamental

frequency around 100 Hz at ambient temperatures that does not vary across the duration of the call (Figs. 1A2a; 2A1) and shows almost no amplitude modulation (Fig. 1A2a). The hum contrasts sharply with the very brief (50-100 ms), higher frequency (~110 Hz) and broadband agonistic grunt produced singly by nesting parental males (type I), an alternative male morphotype (type II) that either sneaks or satellite spawns, and females. During agonistic encounters with other males, the grunt is also produced repetitively as a “grunt train” by nesting males at rates of 1.5-3 Hz for as long as several minutes (Fig. 1A2b, 2A2; Bass et al., 1999; Brantley and Bass, 1994; Cohen and Winn, 1967; McKibben and Bass, 1998). A second agonistic call, the “growl”, is exclusive to the nest-building males and most frequently recorded at night (Bass et al., 1999). Growls are the most complex call; they overlap hums in duration (~200 ms – 5 s) and are reiterative sequences of grunt- and hum-like signals (Figs. 1A2cd; 2A3). Only the nesting, type I male morph employs all call types and thus has been the focus of the present study.

The rhythmic properties of midshipman vocalizations are determined by the activity of a vocal pattern generator that shares evolutionary origins with comparable networks in tetrapods (Bass and Baker, 1990; Bass et al., 1994, 2008). The pattern generator includes a rostral, hindbrain vocal pre-pacemaker nucleus (VPP, formerly the ventral medullary nucleus) that projects to paired columns of vocal pacemaker neurons (VPN) that lie ventrolateral to the paired vocal motor nuclei (VMN) found along the midline of the caudal medulla and the rostral spinal cord (Fig. 1B1). Vocal motoneurons receive input from the pacemaker neurons that set their discharge frequency, the subsequent contraction rate of the muscles and either the fundamental frequency of harmonic, or the pulse repetition rate (PRR) of non-harmonic, vocalizations (Bass and Baker, 1990; see Cohen and Winn, 1967 and Skoglund, 1961 for one-to-one correspondence between each complex action potential in the nerve

volley, muscle contraction and sound pulse in midshipman and the closely related toadfish, *Opsanus tau*).

Vocal motoneurons can be activated in a neurophysiological preparation of midshipman and toadfish by electrical microstimulation in the forebrain's preoptic area-anterior hypothalamus (POA-AH, Fig. 1B1), the midbrain's periaqueductal gray (PAG, Fig. 1B1) and the hindbrain's VPP (Fig. 1B1) (Bass and Baker 1990; Goodson and Bass, 2000a,b, 2002; Kittelberger et al., 2006; Ramage-Healey and Bass, 2004, 2006). Electrical microstimulation in each of the above regions can produce a rhythmic vocal motor volley known as a fictive vocalization that is readily monitored with electrodes placed on ventral occipital nerve roots that form the vocal nerve innervating the ipsilateral vocal muscle (see Fig. 1B1; Bass and Baker, 1990). Surgical isolation of the hindbrain-spinal region containing the VPP-VPN-VMN circuitry further shows that this region alone can produce and modulate the duration of fictive grunts with discharge frequencies independent of the stimulus frequency (Bass and Baker, 1990; Ramage-Healey and Bass, 2004, 2006; Kittelberger et al., 2006). Thus, the firing pattern of the vocal motor circuit directly determines easily quantified temporal properties such as the fundamental frequency/ PRR and duration of natural calls that together with amplitude modulation (AM) can be used to characterize fictive calls. Midshipman fish behaviorally discriminate and neurally encode vocalizations that vary in duration, frequency, and AM; hence, the behavioral saliency of these neuro-behavioral traits (Bass and McKibben, 2003).

Vocalizations play a crucial role in the seasonal reproductive behaviors of midshipman and toadfish as they do in songbirds and anurans (Bass and McKibben, 2003; Kelley and Brenowitz, 2002). Because of the one-to-one correlation between the temporal features of the vocal motor volley/ fictive call and natural calls, the fictive *in vivo* preparation becomes a reliable measure of the probability of the fish producing

each type of natural vocalization in a particular physiological condition. The simplest and briefest fictive call type, the grunt, has been electrically and neurochemically (glutamate) evoked from midshipman at any time of day or year (Bass and Baker, 1990; Goodson and Bass, 2000a; Ramage-Healey and Bass, 2004; Weeg et al., 2005; Kittelberger et al., 2006). However, long duration fictive calls with firing patterns suggestive of natural growls and hums have only been occasionally recorded (Goodson and Bass, 2000b). Now for the first time, using a new stimulation paradigm, we show that long duration fictive growls and hums can indeed be readily evoked in parental males, but almost exclusively at night and only when they are in reproductive condition. Similarly, long duration fictive grunt trains have now been evoked for the first time in reproductive males, although they can occur either during the day or at night. Thus, as shown here, the seasonally- and nocturnally-dependent vocal behaviors of midshipman fish are clearly supported by periodic changes of basal activity in the vocal motor system. With this analysis of the state-dependence of long duration fictive calls and their comparison to the natural calls, we can further dissect how either system or local circuit changes in neurophysiology ultimately dictate the natural rhythmicity of a behavior.

Materials and Methods

During April-August 2007-2008, type I males (12 cm-20 cm, standard length) were hand collected from nest sites in the intertidal zone of Washington State and California and shipped within 6-72 h to Cornell University where they were housed in a 14 light (L):10 dark (D) light cycle with lights out at 17:00 Eastern Standard Time (EST). Type I males are unambiguously identified on the basis of their body size and coloration upon collection as well as visual inspection of their vocal muscle and testes (Bass, 1996). A subset of males were collected in July and August and shifted to a

winter photoperiod of 10L:14D in October. By this time in the fall, this group had reverted to a non-reproductive state with either partially or fully regressed gonads, reflecting duration in captivity as well as a response to natural shifts in photoperiod (Sisneros et al., 2004, 2008). The gonadosomatic indices (GSIs, ratio of gonad mass to body mass – gonad mass) were determined for a subset of males at the end of neurophysiological experiments following deep anesthetization in 0.025% benzocaine (Sigma, St. Louis, MO). The mean GSIs for mean (\pm s.e.m.) reproductive and non-reproductive males were, respectively, 1.84 ± 0.21 and 0.77 ± 0.12 (N = 6 animals per group). All methods were approved by the Institutional Animal Care and Use Committee at Cornell University.

Neurophysiological experiments

The fictive vocalization preparation used here has been thoroughly described elsewhere (Bass and Baker 1990; Goodson and Bass, 2000a; Ramage-Healey and Bass, 2004). Briefly, brain and rostral spinal cord with occipital nerve roots were exposed by dorsal craniotomy under general anesthesia with 0.025% benzocaine (Sigma, St. Louis, MO) and a local injection at the wound site of a long-lasting analgesic (0.25% bupivacaine; Abbot Laboratories, Chicago, IL) with 0.01 mg/ml epinephrine (International Medication Systems, El Monte, CA). After surgery, fish were immobilized with an intramuscular injection of pancuronium bromide (0.5 mg/kg, Astra Pharmaceutical, Westborough, MA) and stabilized in a plexiglass tank with aged, chilled (16-17°C) saltwater perfused through the mouth. One hour after surgery, an insulated tungsten electrode (125 μ m diameter, 8° tip angle, 5 M Ω impedance, 20 μ m exposed tips; A-M Systems, Sequim, WA) was used to evoke the vocal/ occipital nerve's motor volley (fictive vocalization) through delivery of 40 brief (30 ms) trains of 200 Hz stimuli (0.1 ms pulse width, 50-75 μ A positive current) at 1/s

via a WPI stimulus isolation unit (Model 850S, World Precision Instruments, Sarasota, FL) to either the midbrain PAG region which connects to the hindbrain pattern generator or the hindbrain VPP region that projects to the VPN-VMN circuit (Fig. 1B1). The same low current intensity was used for all fish at all time points, whether it was at or above the call threshold for each individual. When only the threshold current (minimum current to elicit a call) was used, the probability of evoking long duration calls was much reduced (see Results). Well-documented surface landmarks and depth measurements based on previous mapping studies of the vocal motor system provided guides for electrode placement (Goodson and Bass, 2002; Kittelberger et al., 2006; Ramage-Healey and Bass, 2004). As noted earlier, fictive vocalizations reflect the firing properties of the VPN-VMN circuit that directly determines a natural call's duration and fundamental frequency (harmonic call)/ pulse repetition rate (non-harmonic call); hence, its designation as a fictive call/ vocalization. Fictive calls were recorded unilaterally from an occipital nerve with an extracellular electrode (Teflon-coated silver wire with exposed ball tip; 50-100 μm diameter) and digitized using MATLAB software designed by Dr. Bruce Land (Department of Neurobiology and Behavior, Cornell University). Both sides of the brain fire together so that a unilateral recording represents bilateral synchrony of the descending vocal motor volley (Bass and Baker, 1991) that leads to the natural, simultaneous contraction of the paired vocal muscles (Skoglund, 1961; Cohen and Winn, 1967).

Neurophysiological and Statistical Analysis

Previously, fictive vocalization preparations performed during the day typically evoked grunts with 15 brief (30 ms) stimulus trains presented at one-second intervals (1/s) at each of several time-points over the course of 120 min (Goodson and Bass, 2000a,b, 2002; Ramage-Healey and Bass, 2004, 2006, 2007). However, it was

found here during pilot studies with reproductive males that they were highly responsive to a longer stimulation time at night, consistent with the time of day that they mainly produce long duration calls (Bass et al., 1999; Brantley and Bass, 1994, Ibara et al., 1983). Thus, if the number of stimulus trains was increased to 40 at every recording, long duration calls could be readily evoked from some males by 60 min post-baseline recordings. Hence, the first set of studies in this investigation delivered 40 brief stimulus trains at 1/s at eight time-points (baseline/ 0, 5, 15, 30, 45, 60, 90, and 120 min) to different groups of reproductive and non-reproductive males at different times of the day.

It was also found during the course of the latter experiments that at the 120 min time point, presentation of an additional 60 stimulus trains at 1/s, continuous with the initial 40, had an especially robust effect on the ability to evoke long duration calls at night in reproductive males. We subsequently tested reproductive males in the day and non-reproductive males day and night in the same way. To further evaluate the effect of the prolonged stimulation on evoking long calls before any slower physiological changes were incurred during the 120 min experiment, we compared the previous results to a separate group of reproductive animals that received 100 s of stimulation trains at baseline.

The minimum current or threshold for evoking fictive calls, call duration and the ratio of the number of fictive growls/grunts were averaged for each time point (5-120 min) and normalized against the baseline (0) of each fish. As reported in the Results, natural and fictive growls are a hybrid of grunt- and hum-like calls. For duration measurements of grunt-hums, the duration of the initial grunt-like response (≥ 3 pulses) and any subsequent response (≥ 3 pulses) were added for the complete value but did not include the silent gap between the two. The repetition rates of the motor volley that mimics the fundamental frequency of natural calls were determined

by the peak-to-peak interval between compound action potentials or “interpulse interval” (IPI).

Call duration, grunt-hum probability and threshold change (reported as means with s.e.m.) were analyzed in JMP (7.0) using repeated-measures ANOVA followed by planned individual contrast post-hoc tests for between subjects comparisons from 30 to 120 min. Statistical analysis of baseline grunt duration, based on comparisons of mean values between each study group (see results), was performed in Graphpad Prism (5.0) with a one-way ANOVA followed by Tukey’s post-hoc tests. To expand the database for this analysis beyond the number of animals comprising the experimental groups (3-6) in the main body of this study, we included values from a larger sample size of animals treated identically at baseline (20 brief stimulus trains at 1/s rather than the 40 stimulus trains at 1/s used throughout the remainder of the study). A one-way ANOVA followed by Tukey’s post-hoc tests was also used for duration change (log transformed) after presenting 100 stimulus trains (values normalized against the first 20 s of stimulation). Comparison of IPIs between fictive call types produced by the same fish was performed in Graphpad Prism (5.0) with paired t-tests, while unpaired t-tests were used for comparisons between the IPIs of fictive calls and the fundamental frequencies of natural calls. The IPI/frequency of a particular call type from any single fish is highly consistent, thus an average of 40 calls is not significantly different than one. A general linear mixed model was used to evaluate differences in duration between fictive grunts and growls, and between fictive grunts and natural grunts in order to account for a greater variation in call duration measured from individual fish. Statistical comparisons were always based on the mean values obtained for each animal in a group, not on the total call number for all animals in the group.

Photoperiod Manipulation

We wanted to determine if the nocturnal dependence of the male's fictive grunt-hums and hums reflected either an endogenous rhythm or was dependent upon external light cues. Thus, reproductive type I males shipped to the lab in either July or August 2008 were subjected to 24 h of either dark (D) or light (L) for five days after an initial exposure for one to five days to the 14L:10D light cycle. These animals were then tested for the ability to produce long duration fictive calls. Taking advantage of the midshipman's typical lack of feeding during the first one to two weeks of acclimation to captive conditions (Sisneros et al., 2008), food was withheld from these animals so as not to confound the effect of the photoperiod regime with food entrainable rhythms. Of the six fish in each treatment group, three were tested between 11:00-12:00 EST of the circadian day, while three were tested after 18:00 EST of the circadian night. Subjects of night experiments and all 24D fish were exposed to 30 min of white light during surgery with eyes covered, after which the rest of the neurophysiology experiment was conducted in red light only that does not inhibit the nocturnal behavioral activity of midshipman fish (see McKibben and Bass, 1998).

Sound Recordings

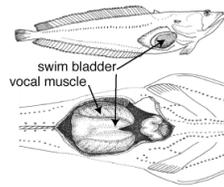
Recordings of midshipman vocalizations (courtesy of Margaret Marchaterre, Department of Neurobiology and Behavior, Cornell University) were made directly from spawning sites in the intertidal zone of Brinnon, Washington using hydrophones (Bioacoustics Research Program, Cornell Laboratory of Ornithology, Ithaca, NY) placed directly adjacent to nests, which are excavations under large rocks (see Bass, 1996; Bass et al., 1999; Bass and Clark, 2003). Since the fundamental frequency/pulse repetition rate of natural harmonic (hums and growls)/ non-harmonic (grunts) calls and the discharge frequency of fictive calls vary directly with ambient

temperature (Bass and Baker, 1991; Brantley and Bass, 1994; McKibben and Bass, 1998), temperature was also recorded (temperature loggers from DataLoggers, Onset Computer Corp., Pocasset, MA). All sound recordings were made between dusk and dawn when spawning and vocal activity peaks (Brantley and Bass 1994; Bass et al., 1999). Recordings were digitized at 2 kHz and 16-bit resolution and waveforms visualized and analyzed using Raven Pro 1.3 (Bioacoustics Program, Cornell Laboratory of Ornithology, Ithaca, NY).

Results

As demonstrated in earlier studies and repeated here, brief fictive grunts can be evoked any time of year or day by electrical microstimulation in either midbrain or hindbrain vocal nuclei and predict the temporal properties of the natural call (see Fig. 1A2b, B2d; Introduction). We now show using a new stimulation paradigm (see Materials and Methods, *Neurophysiological and Statistical Analysis*) that the long duration, fictive hum and growl (Fig. 1B2a, c, d) are almost exclusively evoked from parental males during the scotophase of the reproductive season (14L: 10D housed animals), reflecting the nocturnal occurrence of the natural calls during the spawning season (Brantley and Bass, 1994; Bass et al., 1999). Below, we address first seasonal and diurnal differences in fictive call duration, frequency (measured as interpulse intervals) and call threshold. We then present a more detailed analysis of the temporal properties of long duration fictive and natural calls, revealing the dramatic and combined effects of reproductive state, time of day and stimulation time on call type and probability. We conclude with the effects of photoperiod manipulation on fictive call production.

A1. Swim bladder-sound production



A2. Natural calls

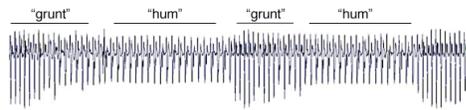
a. Advertisement hum



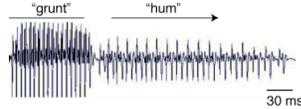
b. Natural grunt train



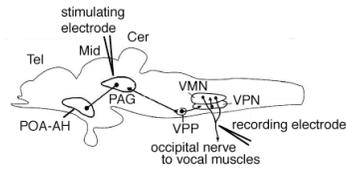
c. Agonistic growls (grunt-hum)



d.



B1. Central vocal motor circuit



B2. Fictive calls

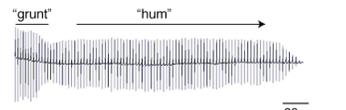
a. Fictive hum



b. Fictive grunt train



c. Fictive growls (grunt-hum)



d.

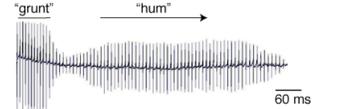


Figure 2.1. Natural and fictive vocalizations of midshipman fish (*Porichthys notatus*). Note that time scales differ between natural and fictive calls to allow visualization of more complex waveforms in natural calls. A1. Vocalizations are produced by the simultaneous contraction of a pair of vocal muscles attached to the lateral walls of the swim bladder. A2. Representative natural calls of parental, type I male. A2 a. The advertisement hum has sound pulses produced at a highly regular frequency for the entire duration of the call, ~ 400 ms - > 1h. A2 b. Agonistic grunt trains are repetitions of brief grunts at a rate of 1.5-3 Hz. A2 c-d. Agonistic growls are the most complex vocalization with amplitude and frequency modulation. They are an amalgam of brief grunts (~ 50-150 ms) and longer duration, multiharmonic hums and range from 300 ms to several seconds in duration. The grunt portion of the call in 'd' is clipped in the original recording because of the proximity of the fish to the hydrophone. B1. Sagittal view of the central network responsible for vocal production (modified from Bass and McKibben, 2003; see Bass et al., 1994; Goodson and Bass, 2002 for details). Stimulation in the midbrain periaqueductal gray (PAG), which receives afferents from the forebrain preoptic area and anterior hypothalamus (POA-AH) and projects to the hindbrain/spinal vocal pattern generator (VPP-VPN-VMN), evokes fictive vocalizations that are recorded from the occipital nerves that innervate each vocal muscle. B2 a. Fictive hums also have a regular discharge frequency with average durations of 400 ms-1 s. B2 b. Fictive grunt trains are repetitions of fictive grunts, like the natural call. B2 c-d. The fictive growl or "grunt-hum" averages 400 ms - 800 ms in duration. Other abbreviations for B1: Cer, cerebellum; Mid, midbrain; Tel, telencephalon; VMN, vocal motor nucleus; VPN, vocal pacemaker nucleus; VPP, vocal prepacemaker nucleus.

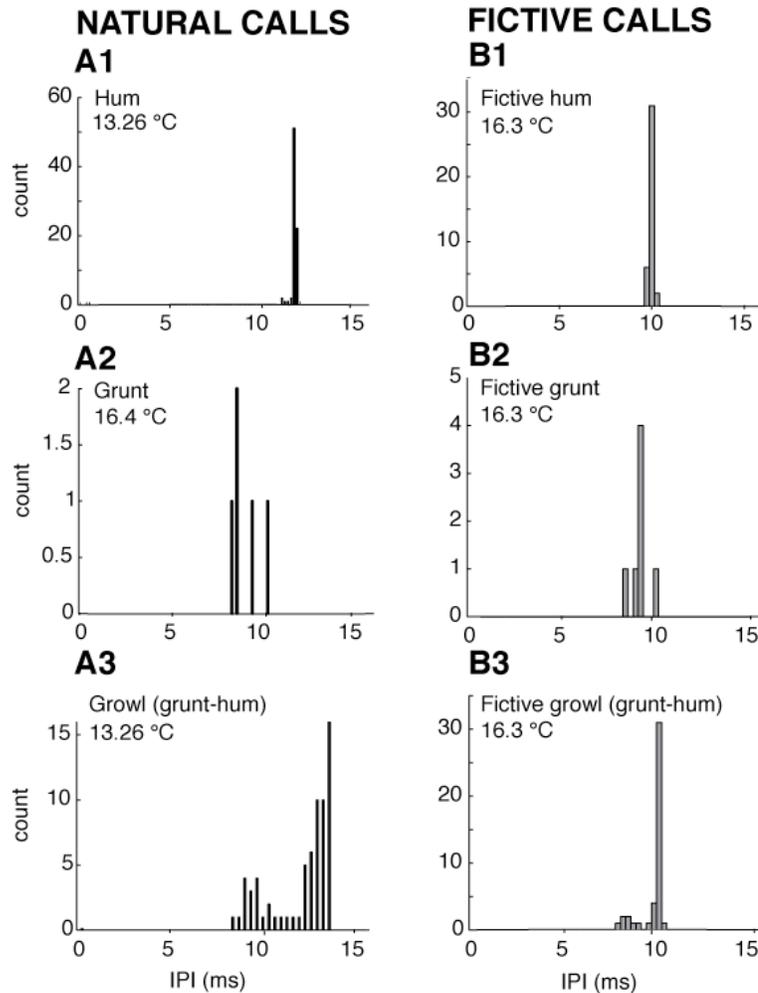


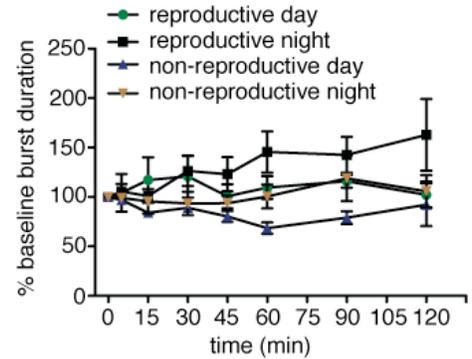
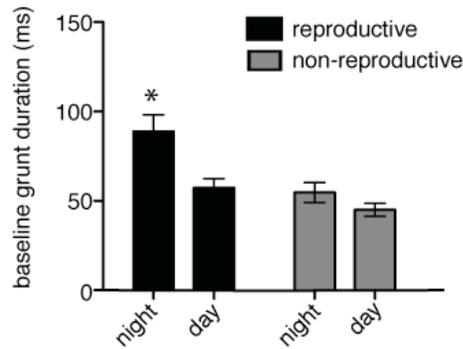
Figure 2.2. Interpulse intervals (IPI) for individual natural and fictive vocalizations. Shown here are the IPIs for representative examples of each call type studied. Recording temperatures are indicated since the repetition rate of natural sound pulses and the discharge frequency of the vocal motor volley/ fictive call are temperature-sensitive (Bass and Baker, 1991; Brantley and Bass, 1994). A1. The IPI of a natural hum (1 s) centers tightly at 12 ms. A2. A natural grunt (~50 ms) with an average IPI of 8.5 ms. A3. A natural growl (800 ms) can have considerable frequency modulation but with a bimodal distribution: the shorter, faster grunt-like portion of this growl has an IPI of ~9 ms, while the longer, more regular hum-like portion averages 13 ms. B1. Like the natural hum, a fictive hum (400 ms) can have an extremely regular IPI (briefer at ~10 ms than the natural one at ~12 ms because of higher recording temperature). B2. The IPI of a fictive grunt averages 8.5 ms (like the natural one because of similar recording temperatures). B3. The IPIs of this fictive growl or “grunt-hum” (470 ms), like the natural one, are bimodally distributed (between 8.5 and 10 ms that is also briefer than the natural call because of higher temperature).

Diurnal and seasonal changes in call duration and frequency

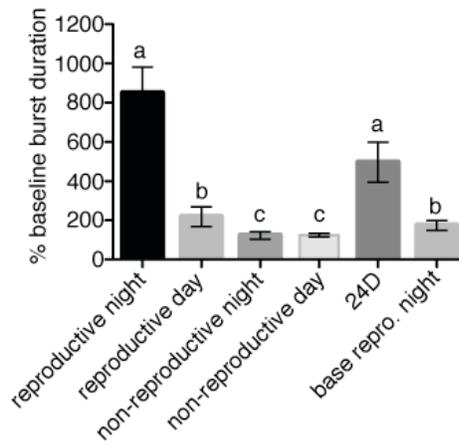
The mean baseline fictive grunt duration of reproductive and non-reproductive males reflects seasonal and daily changes in basal vocal motor excitability. Baseline fictive grunt values were determined for separate day and night groups of reproductive and non-reproductive males. Mean grunt duration of reproductive males tested at night was significantly greater ($p < 0.05$) than all other groups (reproductive night: $88.79 \text{ ms} \pm 9.28$; reproductive day: $57.16 \text{ ms} \pm 5.33$; non-reproductive night: 54.7 ± 5.63 ; non-reproductive day: $44.96 \text{ ms} \pm 3.66$; $N=10$ animals/ group, 20 calls/animal).

Reproductive males at night showed a subtle but significant effect ($p < 0.05$) of reproductive state on the duration of fictive calls evoked over the 120 min stimulus trial (Fig. 3B; the data at each time point are the average of 40 fictive calls evoked by 40 stimulus trains). Figure 4A1-2 shows 20 s segments of representative stimulus trials to better illustrate the time dependent shifts in the temporal properties of fictive calls. Increased call duration was mainly dependent upon an increase in duration of an initial short latency, grunt-like response (Fig. 4A1-2). However, by 120 min, a much lower amplitude, but typically longer duration component sometimes followed the initial grunt-like response (Fig. 4A2, see Materials and Methods for determination of total duration). Reproductive males tested in the daytime were less affected by stimulation than animals at night, but still showed signs of being more responsive than either day or night non-reproductive fish.

A. BASELINE GRUNT DURATION B. DURATION CHANGE



C. 100 s STIMULATION



D. CALL THRESHOLD

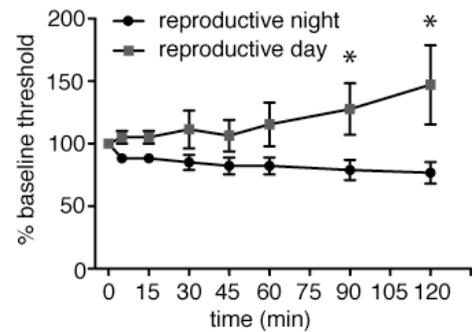


Figure 2.3. Diel and seasonal variation in fictive calls. A. A night vs. day comparison of mean baseline grunt duration in reproductive and non-reproductive males. Reproductive males at night have a significantly higher (*) fictive grunt duration than all other groups (see text). B. Call duration change over 120 min with 40 stimulus trains at each recording. Reproductive males were housed in 14L:10D, and non-reproductive in 10L:14D (all N = 3). There was a significant, overall effect of reproductive state (see text). C. 100 stimulus trains (1/s) at baseline in reproductive males at night vs. 100 stimulus trains at 120 min in all groups (N = 5 for reproductive night; 3 each for reproductive day, non-reproductive night and non-reproductive day; 6 each for 24D and baseline reproductive night). Letters (a, b, c) denote significant differences (see text). D. The threshold stimulus current significantly decreases in reproductive (14L:10) males at night, but rises during the day (asterisks indicate significant differences, see text)

When a stimulus trial of 100 stimulus trains (1/s) was presented at 120 min, call duration increased dramatically in reproductive males at night compared to both reproductive males in the day and to non-reproductive males (day and night), coincident with the evocation of long duration growl-like calls ($p < 0.05$; Figs. 3C; 4A3). Most of the long duration calls had two components as sometimes observed with 40 stimulus trains at the 120 min time point (see above). However, the grunt-like part of the response was typically followed by a long hum-like response: the combined response resembling natural growls (Fig. 4A3; also see Fig. 1A2d). Two of six reproductive males tested at night and given 100 stimulus trains at baseline were able to produce long duration calls as opposed to five of five at 120 min, suggesting both short and long-term activity-dependent changes in the vocal motor circuit. Non-reproductive males (day or night) increased call duration significantly less than all other groups ($p < 0.05$, Fig. 3C), reflecting the absence of fictive growls and hums. At night, reproductive males tested at 120 min produced significantly more long duration calls than either the reproductive males tested during the day at 120 min or the reproductive males tested during the night at baseline (Fig. 3C). Thus, long duration call production peaked in the group that permitted both short- and long-term, activity-dependent changes to occur in vocal circuits already primed by a nocturnal, reproductive condition.

The interpulse interval (IPI), which reflects the fictive call's discharge frequency, was also increased in the longer duration calls evoked with 100 stimulus trains at 120 min. The IPI analysis is shown in Figure 2B1-B3 for single calls in comparison to single natural calls and in Figure 4B1-B3 for a mean of 20 fictive calls to show cumulative results. The shift from grunts to growls that was potentiated by the 100 stimulus trains (Fig. 4A) was accompanied by the appearance of a bimodal

distribution of IPIs, composed of the growl's faster grunt-like and slower hum-like components (Fig. 4B).

In sum, the facilitation of vocal motor excitability, as reflected in the increased production of long duration calls, depended upon reproductive state, time of day and degree of stimulation.

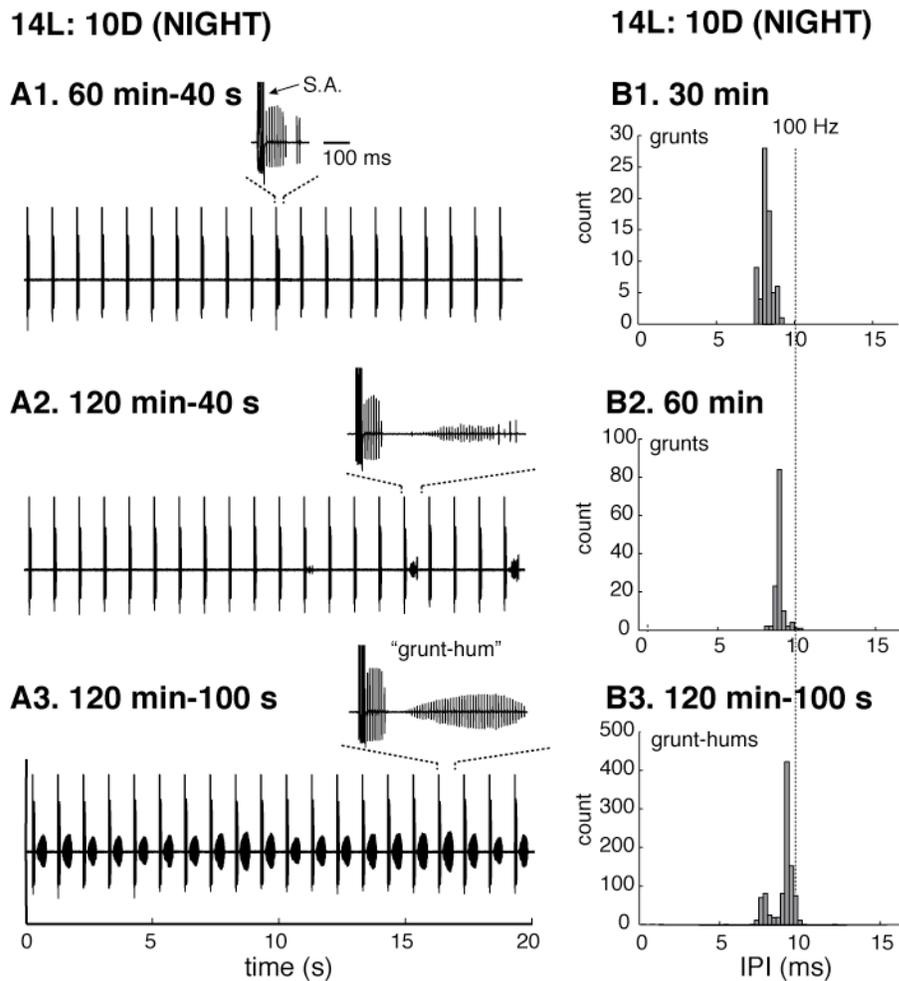


Figure 2.4. Fictive call stimulus-response trains. A1-3. Example of the time and stimulation-dependent progression of fictive grunts to longer duration calls from a reproductive male housed in 14L: 10D and tested at night. Shown here are 20 s excerpts from 40 stimulus trains with stimulus artifacts (S. A., A1 shown at 1 s intervals followed by the fictive call (see text for details). B1-3. As fictive grunts transition to fictive grunt-hums and duration increases, mean firing frequency (20 calls, one male) decreases. Recording temperatures at 16.3°C.

Diurnal and seasonal changes in call threshold

As shown above, fictive growls and hums are distinguished from grunts by their physical attributes (duration, frequency, amplitude modulation) and the time of day and year they can be evoked. Fictive growls and hums are also distinguished by a decreased response threshold (minimum current to elicit a call), and a paradoxical dependence on *increased* stimulation intensity. Amongst reproductive males, there was a conspicuous and significant drop (25-40%) in call threshold ($p = 0.036$) at night, compared to the rise seen during the day (Fig. 3D; 90 min ($p = 0.01$) and 120 min ($p = 0.0006$)) that paralleled the steady, time-dependent increase in duration and IPIs (Fig. 4). While fictive grunts can follow a stimulus at the very low threshold current, fictive growls and hums are elicited from reproductive males at night (and to a much lesser degree during the day) with a slightly elevated stimulation current (25-50 μA above threshold). Thus, in spite of the decrease in burst threshold and this evidence for the vocal circuit's heightened excitability, the likelihood of evoking the longer calls with every stimulus pulse was still greater if the current remained slightly above threshold.

Fine temporal properties of fictive and natural calls

As we note earlier, the fictive growl was designated as a “grunt-hum” due to its hybrid nature, namely a grunt-like beginning followed by a longer, hum-like portion with damped amplitude at either end. Thus, both natural and fictive growls have two distinguishable parts that are either continuous or separated by a sudden, brief change in amplitude (Fig. 1A2c-d, B2c-d; also see Fig. 4A3) and exhibit a bimodal distribution of IPIs (Fig. 2A3, B3; also see Fig. 4B3). The duration and mean frequency of natural growls can range broadly in even one animal (e.g., 542 ms-8 s; 59-116 Hz; N=10 calls), with durations that obviously exceed our fictive recordings.

However, naturally brief growls (e.g., Fig. 1A2d) appear to be a fundamental unit or pattern for the longer calls and the fictive growl is its neural correlate. For reproductive males tested at night and presented with 100 stimulus trains at 120 min, the mean duration of fictive growls ($444.67 \text{ ms} \pm 41.67$, $N = 6$ animals, 5 calls/animal) was significantly longer than that of the grunts evoked at baseline from the same fish ($67.36 \text{ ms} \pm 6.81$; $N = 6$ animals, 5 calls/animal; $p < 0.0001$). The mean frequency (at $16.4 \text{ }^\circ\text{C}$) of the hum-like portions of the fictive growls was significantly lower than that of the grunts (mean grunt frequency = $106.84 \text{ Hz} \pm 1.81$; mean hum frequency = $97.88 \text{ Hz} \pm 0.53$; $p = 0.003$).

Fictive hums alone, although rarely produced *de novo* (one animal, 3 calls, $1140 \text{ ms} \pm 332.21$, continuous through two stimulus trains with little amplitude modulation), resembled brief natural hums in IPIs (Fig. 2A1, B1; differences in recording temperatures can account for different absolute values for IPIs of both natural and fictive calls, see Brantley and Bass, 1994; Bass and Baker, 1991). The more common hum-like portions of fictive growls also had a very regular, low firing frequency ($97.88 \text{ Hz} \pm 0.53$; $N = 7$ animals, 5-15 calls/animal) that was not significantly different ($p = 0.87$) from that of the natural hum (mean fundamental frequency = $97.44 \text{ Hz} \pm 2.76$; $N = 5$ animals, 1 call/animal; same recording temperature). Interpulse intervals strikingly differentiated all fictive and natural hums from even the longest fictive or natural grunts ($\sim 200 \text{ ms}$) that exhibit a higher, irregular IPI (Fig. 2A1, A2; B1, B2; also see Brantley and Bass, 1994; Bass et al., 1999; Bass and Clark, 2003 for natural grunts). The distribution of IPIs ($\sim 10 \text{ ms}$) in all fictive hums, either singular or part of a grunt-hum, was the tightest of any of the natural or fictive calls (Fig. 2B1). Fictive hums and the hum-like portions of fictive growls were also similar to brief natural hums in duration (see Brantley and Bass, 1994, for hums as brief as 370 ms). However, a statistical comparison is not warranted

because the duration of naturally produced hums are highly context dependent (A. Bass and M. Marchaterre, unpublished observations) while the evoked correlates are strictly electrophysiological phenomena that reflect the state of the pattern generator.

Unlike the fictive hums and growls, fictive grunt trains were easily triggered during *both* night and day trials, but like hums and growls, only in reproductive males. Natural grunt trains consist of individual grunts repeated at a rate of 1.5-3 Hz that can persist for several minutes (Fig. 1A2b, see Introduction). After the 120 min recording period, free-running grunt trains were readily triggered with 3-20 s of stimulus trials in the hindbrain VPP region (Fig. 1B1) and continued independently for more than 5 min without further stimulation, mimicking the natural call with a mean grunt repetition rate of $1.9 \text{ Hz} \pm 0.1$ ($N = 5$ animals, one grunt train/animal). For individual grunts within the trains, the pulse repetition rate averaged $113 \text{ Hz} \pm 2.17$, with a mean grunt duration of $46.56 \text{ ms} \pm 7$ ($N = 5$ animals, 5 grunts/animal). This was not significantly different than the intra-grunt frequency ($p = 0.13$) and duration ($p > 0.89$) of grunts from natural grunt trains (mean frequency = $108.52 \text{ Hz} \pm 1.53$; mean duration = $47 \text{ ms} \pm 1.98$; $N = 5$ animals, 5 grunts/animal). While fictive growls and hums could only be evoked from the midbrain's PAG region that projects to the VPP (Fig. 1B1), grunt trains could only be evoked with stimulation in the VPP or VPN region.

Photoperiod manipulation

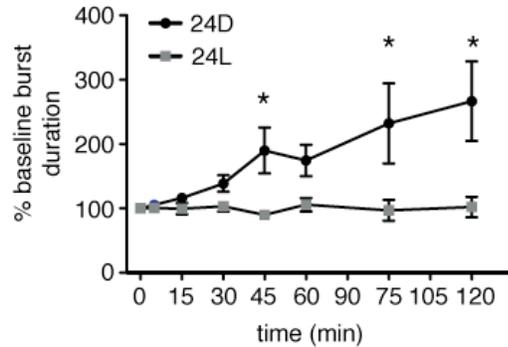
After housing reproductive males in a 24D light cycle for five days, there was a significant effect of photo-treatment ($p = 0.0075$). The low frequency/ long duration calls could be readily evoked during both the natural day and night (day/night test groups were thus pooled) and only required 40 stimulus trains as opposed to 100 s for the 14L: 10D fish (Figs. 3C, 5A, 6A1-2; compare to 4A2). This resulted in an increasingly significant time*photo-treatment effect on call duration at 45 ($p = 0.01$),

90 ($p = 0.001$) and 120 min ($p = 0.0001$) compared to 24L (Fig. 5A). Figure 6A1-2 shows traces from a 24D fish from which fictive grunt-hums were already evoked by 30-60 min post baseline. There was also an increasingly significant effect of time*photo-treatment ($p = 0.0037$) on the proportion of grunt-hums to grunts after 40 stimulus trains in the 24D group compared to 24L and 14L:10D groups (Fig. 5B; 60 min, $p = 0.007$; 90 min, $p = 0.0001$; 120 min, $p = 0.00007$). Thus, instead of the 24D treatment revealing an endogenous circadian rhythm in vocal excitability, increasing at night and decreasing during the day in conjunction with their natural behavior, the constant darkness appeared to tonically facilitate vocal motor output.

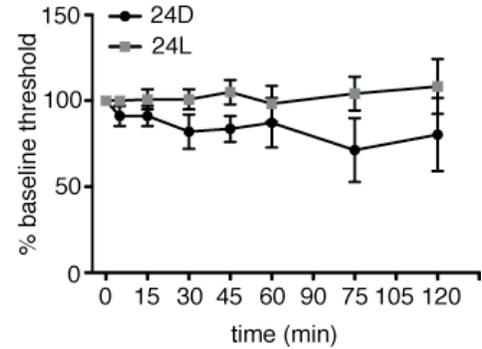
Interpulse intervals also changed gradually with time, but tended to start longer in 24D fish and then decreased slightly rather than showing the increase found in 14L:10D males (see Fig. 6B1-2 for shift in mean IPI between the 30 and 120 min records in a 24D male tested at night; compare to Fig. 4B1-2). In concert with these results, five days of 24L had the opposite effect: fictive vocal output was suppressed day and night such that the probability of inducing long duration calls was nearly eliminated. When call threshold was compared between all 24D and all 24L males, there was a non-significant trend ($p > 0.05$) for threshold to fall in 24D males and to rise in 24L males similar to the day/night contrast found in 14L:10D housed animals (Fig. 5C, compare to Fig. 3D). While the results suggested that light exposure may directly affect excitability in the circuit, the question remains if there is also a persistent endogenous rhythm in call threshold. The number of animals tested so far was too small to reveal any significant differences between the day and night groups in each phototreatment.

24D VS. 24L

A. DURATION CHANGE



C. CALL THRESHOLD



B. GRUNT-HUMS/GRUNTS

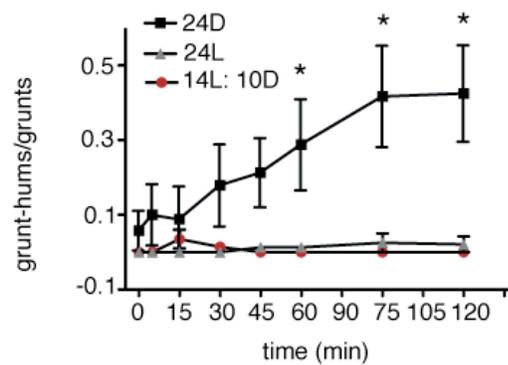
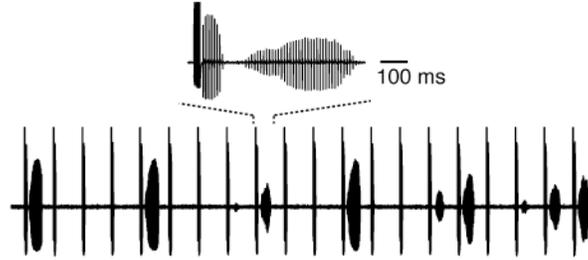


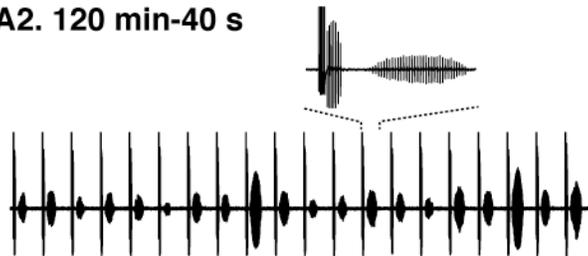
Figure 2.5. Photoperiod-dependent plasticity of fictive calls. A. Call duration change in reproductive males subjected to 24 h of darkness (24D) versus 24 h of light (24L) (N = 6 animals/ group). Asterisks indicate significant differences (see text). B. The ratio of fictive grunt-hums to grunts in reproductive males housed in 24D, 24L and 14L:10D (same animals as in “reproductive night” test group). Asterisks indicate significant differences (see text). C. There was an insignificant trend for call threshold in 24D males (day/night pooled) to fall, while call threshold in 24L (pooled) animals rose (see text).

24D (DAY)

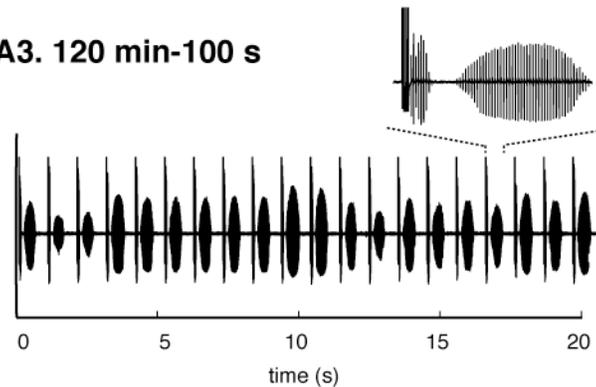
A1. 60 min-40 s



A2. 120 min-40 s

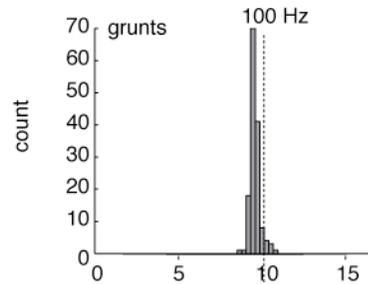


A3. 120 min-100 s

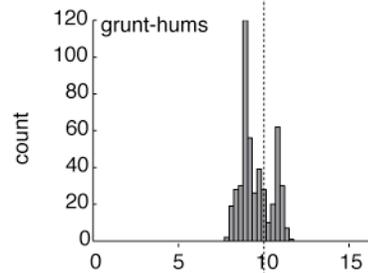


24D (NIGHT)

B1. 30 min



B2. 60 min



B3. 120 min-100 s

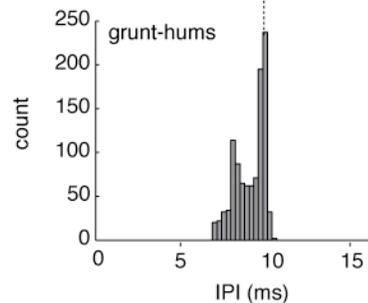


Figure 2.6. Photoperiod-dependent plasticity of stimulus-response trains. A1-3. Examples of the time and stimulation-dependent progression of fictive grunts to longer duration calls from a reproductive male housed in 24D and tested during the circadian day. B1-3. Although fictive grunt-hums appeared earlier in 24D males compared to 14L: 10D animals (compare to Fig. 4A1, B2), the overall firing frequency (mean 20 bursts, one male) started low and increased slightly by 120 min (shown here for one animal tested during the circadian night). Recording temperatures at 16.3°C.

Discussion

The current study emphasizes that it is as important to consider endogenous or environmentally driven biological rhythms when investigating the neurophysiology of a behavior as one does when studying the natural behavior itself. Agonistic grunts, the most elemental midshipman vocalization emitted by males and females, are neither diurnally nor seasonally dependent and reflect a minimal activation of the vocal motor system. By contrast, the much longer growls and hums are temporally confined to the spawning season and produced by parental males mainly at night. Likewise, the probability of eliciting fictive growls and hums in a neurophysiological preparation is much greater in reproductive males at night, while fictive grunts are evoked at any time of day. Clearly, neurophysiological preparations other than those involving the extensively studied circadian pacemaker, the suprachiasmatic nucleus, are subject to diurnal and seasonal changes (see below). By paying attention to naturally occurring behavioral rhythms, the full potential of a neural network, as in the midshipman vocal motor system, is revealed.

Long duration fictive vocalization in midshipman fish

The evocation of fictive growls and hums, the neural correlates of the natural, long duration calls used during the breeding season, depends upon reproductive state and time of day. These calls are accompanied by several distinct neurophysiological changes that reflect the altered state of the vocal motor system at night in a reproductive male. First, either shortly preceding or in tandem with the evocation of the first fictive growl or hum, the call threshold drops by as much as 40% as these longer calls increase in number and length. Second, in addition to the significant (up to 1000%) increase from baseline duration with added stimulation, the firing rate concomitantly falls. Third, fictive calls become more regular in their IPIs, also like

natural hums and the hum-like parts of growls. Interestingly, even though the fictive grunt threshold significantly decreases in conjunction with the first fictive hums, the kindling and evocation of these long duration calls still rely upon a greater current intensity. This might reflect the recruitment of neurons with lower input resistance, as in those exhibiting more electrotonic coupling necessary for synchronous firing (Christie et al., 1989; Christie and Jelinek, 1993).

All of the above characteristics - duration increase, frequency and call threshold decrease, and firing rate constancy - may be considered the outcome of short- (40-100 stimulus trains at 1/s) and long- (120 min trials) term, activity-dependent plasticity in the vocal motor circuit. Furthermore, this network or cellular plasticity is *itself* susceptible to seasonal and daily modulation, such that prolonged stimulation (100 stimulus trains) in a reproductive male during the day evokes only a small fraction of the number of fictive hums that can be elicited from another male at night. However, if the stimulation is not increased from 40 to 100 stimulus trains, the potential to produce long duration calls from reproductive males is not entirely revealed in any group. This strongly suggests that activity-dependent plasticity in a circuit emerges from behaviorally relevant network activity, or electrical stimulation of sufficient duration to mimic naturally occurring network activation (Buchanan, 1996; Parker and Grillner, 1999). Future experiments need to further explore the interaction between short and long term activity-dependent changes that give rise to the vocal circuit plasticity studied here. However, these initial studies clearly reveal the dramatic effects of increased stimulation on the probability of evoking long duration calls. Similarly, with prolonged stimulation in the motor cortex of monkeys, muscle twitches evolve into complex movements reflecting natural behaviors (Graziano et al., 2005).

Compared to previous studies in midshipman (see Introduction), these current experiments increased the number of stimulus trains from 15 to 40 (at 1/s) during each stimulus trial, but did not increase the duration of the individual stimulus trains (30 ms). This may be one reason that the recorded fictive hum rarely exceeded one second, while parental males will hum without pause for up to an hour. In comparison, it is remarkable that the spontaneous fictive grunt train fired independently for many minutes in reproductive animals after only 3-20 s of hindbrain stimulation. It would suggest that rhythmic, oscillatory-like output from the hindbrain vocal circuit can produce the grunt train, while the hum relies upon added upstream drive from the midbrain PAG and the forebrain's POA-AH that is a major integration site for neuroendocrine and vocal mechanisms (Goodson and Bass, 2002).

The induction of different classes of long duration calls also shows site-specificity, namely stimulation in the midbrain PAG region for growls and hums and the hindbrain VPP region for grunt trains. In general, the results are consistent with earlier studies, showing that multiple sites in the vocal motor system can modulate the activity pattern of the pacemaker-motoneuron circuit (see results and other reviews in Goodson and Bass, 2002; Kittelberger et al., 2006). However, the current study shows for the first time in the teleost fictive call preparation, the site-dependent induction of vocal patterns that reflect the greatest divergence in vocal patterning. These new results are further consistent with studies of the vocal brainstem in mammals, including primates (e.g., Fenzl and Schuller, 2005; Jurgens and Hage, 2007).

Unlike teleosts, call patterning in tetrapods depends upon the integration of vocal and respiratory mechanisms (Bass and Baker, 1997; Wild, 2004; Zornik and Kelley, 2008). Like studies in toadfishes and other vocal teleosts (Bass and Baker, 1990, 1991; Barber and Mowbray, 1956; Packard, 1960; Skoglund, 1961), recordings of vocal motor volleys in frogs (in this case from a laryngeal branch of the vagus

nerve) essentially show a 1:1 correspondence between each complex potential, muscle contraction and sound pulse (Yamaguchi and Kelley, 2000). *In vitro* studies of isolated brain preparations from the terrestrial frog *Lithobates pipiens* (formerly *Rana pipiens*, see Frost, 2006) identify two “semi-independent” call pattern generators, one at isthmal levels and one (“the classical respiration generator”) at caudal hindbrain-spinal levels (Schmidt, 1992). Recent *in vitro* studies in *Xenopus laevis*, a fully aquatic frog with a vocal circuit like that of terrestrial species (see Zornik and Kelley, 2007, 2008), show that bath application of serotonin can evoke fictive responses that mimic the temporal properties of natural vocalizations (Rhodes et al., 2007). *In vitro* brain stimulation studies of frogs have been less conclusive. As Zornik and Kelley (2008) point out, the temporal properties of the electrically evoked responses are typically *not* independent of the stimulus frequency, in contrast to studies like the current one of vocal fish (Figs. 4A, 6A; also see Introduction). Rather, in studies of *Xenopus*, each electrical stimulus pulse evokes a single complex potential in the nerve; responses that mimic a natural call have only occasionally been obtained (see Rhodes et al., 2007). The nuances of evoking fictive calls with electrical microstimulation in frogs and in terrestrial vertebrates in general are likely dependent, in part, on a more complex call circuitry that involves the integration of respiratory rhythms (Bass and Baker, 1997; Zornik and Kelley, 2008).

Steroid and Melatonin-Dependent Rhythmicity

What allows the observed neurophysiological changes in fictive calling to occur in a nighttime but not a noontime brain, let alone in a reproductive versus a non-reproductive animal? No doubt gonadal hormones play an enormous role in the seasonal cycles of vocal activity, or any number of other rhythmic behaviors. Indeed, increases in the degree of temporal encoding of the higher harmonics of male hums by

the peripheral auditory system of female midshipman fish during the reproductive season can be induced in nonreproductive females with either testosterone or estradiol treatments over a period of about three to four weeks (Sisneros et al., 2004b). The seasonal rhythmicity in vocal neurophysiology reported here is also reminiscent of the steroid-dependent, morphometric changes in vocal nuclei in songbirds (e.g., Arnold et al., 1976; Ball et al., 2004; Brenowitz, 2004) and midshipman fish (Forlano and Bass, 2005a, b; Bass and Forlano, 2008). As in songbirds, plasma levels of steroid hormones cycle with reproductive state in midshipman, while androgen and estrogen receptors are found in the midshipman's vocal control system in conjunction with the expression of brain aromatase that converts testosterone to estradiol (reviewed in Bass and Ramage-Healey, 2008; Forlano et al., 2006).

While intramuscular injections of androgens in midshipman fish increase the probability of evoking longer duration grunts, they do not evoke fictive growls and hums with the temporal attributes described here (Ramage-Healey and Bass, 2004). Thus, other aspects of reproductive state and time of day are apparently key factors in the natural production of long duration calls during the breeding season. In songbirds as well, there is evidence for testis-independent effects on song production (without accounting for centrally-synthesized neurosteroids), since both sham-operated and castrated sparrows under long day conditions have enlarged song control nuclei and exogenous melatonin decreases the size of telencephalic vocal nuclei (Bernard et al., 1997; Bentley et al., 1999). Finally, the basal rate of the electric organ discharge (EOD) of weakly electric fish increases at night independent of water temperature or breeding status, although EOD rate in breeding males coupled with females is still the greatest (Silva et al., 2007; Stoddard et al., 2007). Thus, steroid hormones, with their effect on the morphology as well as synaptic and intrinsic firing properties of neurons,

may be necessary, but not sufficient, for the maximum upregulation of seasonally dependent vocal behaviors.

Diurnal changes in neuronal activity have been documented in brain regions less typically linked to the motor components of reproductive behaviors, such as the hippocampus (Barnes et al., 1977; Chaudhury et al., 2005). Excitatory postsynaptic potentials (EPSPs) in response to perforant pathway stimulation, recorded *in vivo* in rats and monkeys at different times of day, were as much as 30% larger in the dark phase than the light phase of nocturnal rats, while the opposite effect was observed in diurnal monkeys. Barnes et al. (1977) hypothesized a circadian cycle of synaptic transmission in the hippocampus that covaries with natural behavioral fluctuations, while Chaudhury et al. (2005) concluded that an endogenous circadian oscillator modulates long-term potentiation in the mouse hippocampus.

Sometimes such rhythmic changes in behavior and neural systems can be directly controlled by melatonin binding to regionally abundant receptors (Whitfield-Rucker and Cassone, 2000; Gahr and Kosar, 1996; Musshoff et al., 2002; Rosenstein and Cardinali, 1990; Wan et al., 1999). For example, melatonin applied to brain slices of the avian vocal circuit decreases firing rate in a telencephalic vocal nucleus where the inhibitory G protein-coupled melatonin 1b receptor is expressed (Jansen et al., 2005). In teleost fish, melatonin is rhythmically secreted from the retina and pineal gland in intact and isolated preparations under various light conditions (Bolliet et al., 1996; Cahill, 1996; Migaud et al., 2007). Our exposure of the midshipman to 24D or 24L for five days produced neurophysiological results that correlate with the light manipulated *in vivo* melatonin rhythm found in several temperate teleost species (Migaud et al., unpublished observations reported in Martinez-Chavez et al., 2008) and in one subtropical species, the common dentex (Pavlidis et al., 1999). Common dentex (*Dentex dentex*) acclimatized to 12L:12D and thereafter exposed to 24D did

not exhibit an endogenous melatonin rhythm (low in the day, high at night); rather, levels were maintained as high as during the natural nighttime. If melatonin naturally enhances vocal circuit function in the midshipman at night and 24D stimulates tonically high levels as found in the aforementioned fish, then it may explain our ability to as easily elicit fictive growls and hums from the 24D treated fish tested during the circadian day as during the circadian night. Likewise, 24L can inhibit melatonin production (and rhythmicity) altogether (Martinez-Chavez et al., 2008), thus explaining the almost complete loss of long duration fictive calling in our 24L fish during both natural day and night. Future studies in midshipman need to assess shifting melatonin levels through natural and manipulated photo regimes to more directly investigate the above scenarios. Given the extensive GABAergic innervation of the vocal motor nucleus (Marchaterre et al., 1989), and the evidence for melatonin modulation of GABAergic activity in mammalian cortex (Musshoff et al 2002; Wan et al 1999), an interaction between this hormone and levels of inhibition in the vocal motor circuit may contribute to the transition from short grunts to long duration, lower frequency hums.

Future studies in midshipman need to assess shifting melatonin levels through natural and manipulated photo regimes to more directly investigate the above scenarios. This will include further evaluation of fluctuating fictive call threshold during natural day and night of both photo regimes. At this point, with a limited number of animals tested, there was only a trend for a persistent call threshold rhythm: lower in the natural night compared to day in constant darkness (24D), but not apparent in constant light (24L).

Concluding Comments

The mechanisms underlying the observed neurophysiological changes in the production of long duration fictive growls and hums from parental male midshipman fish likely include a periodic modulation of both excitatory and inhibitory activity in one or more vocal nuclei, as well as modulation of ion channels (e.g., see Pennartz et al., 2002, Teshima et al., 2003, Meredith et al., 2006 for the SCN). Such natural fluctuations could be the downstream effects of steroidal and/ or non-steroidal (e.g., melatonin) hormone activation of either local membrane or nuclear receptors, or even the product of local oscillating clock gene transcription. Midshipman fish now offer the opportunity to integrate the physiological mechanisms underlying stereotyped, oscillatory-like vocalizations with the prevailing rhythms that shape them. Lastly, given the shared origins of vocal pattern generators in fish and tetrapods (Bass et al., 2008), the functional principles revealed by these and other studies will prove informative to the vocal systems of vertebrates in general.

We thank Dr. Bruce Land (Cornell University) for designing the MATLAB data acquisition software, Margaret Marchaterre for recording and providing the natural sounds, Françoise Vermeulen of the Cornell Statistical Consulting Unit for advice on statistics, and Drs. Bruce Johnson and Boris Chagnaud for helpful comments on the manuscript. Support from a Cornell University Fellowship and NIH predoctoral training grants 5-T32-MH15793 and GM007469 (TKR), and NSF IOB 0516748 (AHB).

CHAPTER 3
GABAERGIC INHIBITION FACILITATES CALL SWITCHING VIA
DIFFERENTIAL MODULATION OF DISCRETE VOCAL PATTERN
GENERATOR NUCLEI

Abstract

A discrete hindbrain-spinal circuit in a teleost fish, the plainfin midshipman (*Porichthys notatus*), determines the basic temporal pattern of several stereotyped vocalizations used for advertisement and agonistic displays. Just as primitive locomotor circuits from aquatic species have revealed principles of pattern generation that apply to more complex terrestrial locomotion, this hindbrain-spinal vocal pattern generator (VPG) offers insight into the fundamental mechanisms controlling rhythm and frequency generation that may ultimately apply to other motor circuits and even the patterning of cortical activity. The VPG's output is easily monitored by recording the oscillatory-like vocal motor volley, or fictive vocalization, from occipital nerve roots that directly determines the duration and fundamental frequency/pulse repetition rate of natural calls. By manipulating levels of GABAergic inhibition in the VPG's vocal pre-pacemaker nucleus (VPP) and vocal pacemaker-motoneuron complex (VPN-VMN), *in vivo* midbrain electrical microstimulation evoked fictive vocalizations that mimicked the temporal properties of five natural calls: "grunt", "grunt train", "buzz", "growl" and "hum". Microinjections into VPP of the GABA_A receptor-specific antagonist gabazine increased the duration of a single brief grunt (50-200 ms), while a complete block disinhibited the circuit revealing a spontaneous grunt train rhythm (0.5-5 Hz; 20-60 s) interspersed with long (≤ 6 s) buzzes. A gabazine-induced decrease of GABA_A receptor activity in the downstream VPN-VMN of

reproductive males promoted the generation of grunt-hums, the fictive equivalent of growls, each composed of a grunt and a lower frequency hum. With modestly prolonged stimulation following the pharmacological manipulations, grunts were abolished altogether, leaving only long duration (300 ms - 1 s), nearly constant frequency hums. Shifting levels of GABAergic inhibition in discrete VPG nuclei may be a fundamental determinant of vocal output, responsible for not only motoneuron synchronization, but also for the precise timing that underlies the distinct rhythms or frequencies that compose such vocalizations as the syncopated, locomotor-like grunt train or the profoundly regular long duration hum.

Introduction

Inhibitory interneurons control levels of excitation in the central nervous system and can contribute to the synchronized and precisely timed firing of neural networks underlying sensory processing, memory formation and motor patterning (Alitto and Dan, 2010; Mann and Paulsen, 2007; Roberts et al, 2008). At their most cosmopolitan, GABAergic interneuron networks provide the temporal structure for cortical oscillations (gamma and theta) that bind the activity of anatomically distributed cell populations into synchronous coalitions underlying specific functions and behavior (Buszaki and Chrobak, 1995; Gray et al., 1989; Csicsvari et al., 2003). In a more local manner, the timing of syllable-like activity recorded in the songbird telencephalic vocal nucleus, HVC, depends upon inhibitory synaptic transmission, such that complete block or excessively enhanced fast GABAergic inhibition abolishes evoked rhythmic excitatory postsynaptic potentials and local field potentials (Solis and Perkel, 2005). Within RA, one of HVC's targets in the telencephalon, GABAergic interneurons convert the tonic input to a phasic and temporally precise hindbrain-projecting output predictive of the occurrence of individual notes (Spiro et

al., 1999; Vicario and Raskin, 2000). Finally, brainstem inhibition coordinates vocal/respiratory activity in birds as well as in other terrestrial animals (Kubke et al., 2005; Smotherman et al., 2006), while both GABAergic and glycinergic inhibition modulate the frequency, timing, and patterning of spinal networks that pattern terrestrial and aquatic locomotion (mammals reviewed in Kiehn, 2006; Grillner, 2006; Tegner et al., 1993).

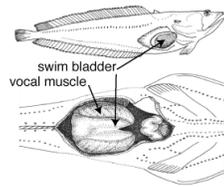
A hindbrain-spinal pacemaker circuit with extensive GABAergic innervation controls the rhythmic contraction of paired muscles attached to the walls of the swim bladder that is responsible for the generation of a variety of social vocalizations in members of the toadfish family (*Batrachoididae*), which include the plainfin midshipman, *Porichthys notatus* (Fig. 1A1) (reviewed in Bass and Ramage-Healey, 2008; also see Marchaterre et al., 1989). The vocal hindbrain-spinal circuit of toadfishes, which shares evolutionary origins with comparable networks in tetrapods (Bass et al., 2008), includes a rostral, hindbrain vocal pre-pacemaker nucleus (VPP, formerly the ventral medullary nucleus) that projects to paired columns of vocal pacemaker neurons (VPN) that lie ventrolateral to the paired vocal motor nuclei (VMN) found along the midline of the caudal medulla and the rostral spinal cord (Fig. 1B1) (also see Bass et al., 1994; Goodson and Bass, 2002). Vocal motoneurons receive input from the pacemaker neurons that set their discharge frequency, the subsequent contraction rate of the muscles and either the fundamental frequency of harmonic, or the pulse repetition rate (PRR) of non-harmonic, vocalizations (Bass and Baker, 1990; Cohen and Winn, 1967; Skoglund, 1961).

The vocal hindbrain of midshipman and toadfish receives descending input from a midbrain region that has been compared to the periaqueductal gray in tetrapods (PAG, Fig. 1B) and receives direct input from the anterior hypothalamus-preoptic area (AH-POA, Fig. 1B1) (Goodson and Bass, 2002; Kittelberger et al., 2006). Vocal

motoneurons can be activated in a neurophysiological preparation by electrical microstimulation in the POA-AH, the PAG, and the VPP (Bass and Baker 1990; Goodson and Bass, 2000a,b, 2002; Kittelberger et al., 2006; Remage-Healey and Bass, 2004, 2006; Rubow and Bass, 2009). Electrical microstimulation in each of the above regions can produce a rhythmic vocal motor volley known as a fictive vocalization because its temporal pattern determines the duration and frequency of natural calls. Fictive calls are readily monitored with electrodes placed on ventral occipital nerve roots that form the vocal nerve innervating the ipsilateral vocal muscle (see Fig. 1B1; Bass and Baker, 1990). Surgical isolation of the hindbrain-spinal region containing the VPP-VPN-VMN circuitry further shows that this region alone can produce and modulate the duration of fictive grunts with discharge frequencies independent of the stimulus frequency (Remage-Healey and Bass, 2004, 2006). Thus, the firing pattern of the vocal motor circuit directly determines easily quantified temporal properties such as the fundamental frequency/ PRR and duration of natural calls that together with amplitude modulation comprise the vocal traits used by midshipman fish to behaviorally discriminate and neurally encode vocalizations (reviewed in Bass and McKibben, 2003).

Territorial males, also known as type I males, build nests in the intertidal zone from where they acoustically court females with advertisement calls (Brantley and Bass, 1994; Bass et al., 1999), unlike the alternative male reproductive morph (type II) that only sneak-spawns to steal fertilizations from nesting type I males (Brantley and Bass, 1994). Type I males have the most extensive repertoire of calls (Bass et al., 1999; Brantley and Bass, 1994; Cohen and Winn, 1967; Ibara et al., 1983; Rubow and Bass, 2009). This includes a multiharmonic, long duration advertisement “hum” (350 ms – 1h) that has a highly stable fundamental frequency around 90-100 Hz at ambient temperatures (see Fig. 1A2 for natural calls). The briefest call is a broadband “grunt”

A1. Swim bladder-sound production



A2. Natural calls

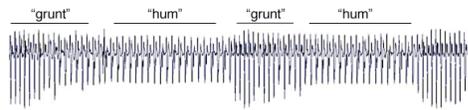
a. Advertisement hum



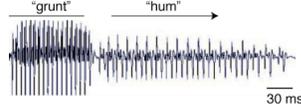
b. Natural grunt train



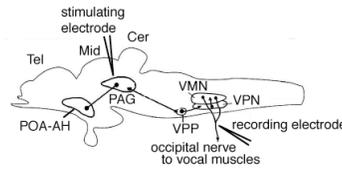
c. Agonistic growls (grunt-hum)



d.



B1. Central vocal motor circuit

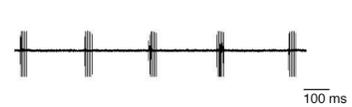


B2. Fictive calls

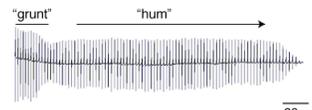
a. Fictive hum



b. Fictive grunt train



c. Fictive growls (grunt-hum)



d.

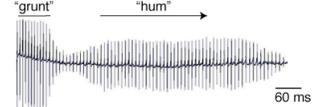


Figure 3.1. Natural and fictive vocalizations of midshipman fish (*Porichthys notatus*). Note that time scales differ between natural and fictive calls to allow visualization of more complex waveforms in natural calls. A1. Vocalizations are produced by the simultaneous contraction of a pair of vocal muscles attached to the lateral walls of the swim bladder. A2. Representative natural calls of parental, type I male. A2 a. The advertisement hum has sound pulses produced at a highly regular frequency for the entire duration of the call, ~ 400 ms - > 1h. A2 b. Agonistic grunt trains are repetitions of brief grunts at a rate of 1.5-3 Hz. A2 c-d. Agonistic growls are the most complex vocalization with amplitude and frequency modulation. They are an amalgam of brief grunts (~ 50-150 ms) and longer duration, multiharmonic hums and range from 300 ms to several seconds in duration. The grunt portion of the call in 'd' is clipped in the original recording because of the proximity of the fish to the hydrophone. B1. Sagittal view of the central network responsible for vocal production (modified from Bass and McKibben, 2003; see Bass et al., 1994; Goodson and Bass, 2002 for details). Stimulation in the midbrain periaqueductal gray (PAG), which receives afferents from the forebrain preoptic area and anterior hypothalamus (POA-AH) and projects to the hindbrain/spinal vocal pattern generator (VPP-VPN-VMN), evokes fictive vocalizations that are recorded from the occipital nerves that innervate each vocal muscle. B2 a. Fictive hums also have a regular discharge frequency with average durations of 400 ms-1 s. B2 b. Fictive grunt trains are repetitions of fictive grunts, like the natural call. B2 c-d. The fictive growl or "grunt-hum" averages 400 ms - 800 ms in duration. Other abbreviations for B1: Cer, cerebellum; Mid, midbrain; Tel, telencephalon; VMN, vocal motor nucleus; VPN, vocal pacemaker nucleus; VPP, vocal prepacemaker nucleus.

(50-100 ms) that is produced in agonistic encounters either singly or as part of a repetitive “grunt train” (~2 Hz) lasting many minutes. “Buzzes”, essentially a longer duration grunt (1- 3.3 s), appear to be anti-predator agonistic calls that have been heard during human territorial trespass or manual capture (A. Bass and T. Rubow, unpublished observations; Brantley and Bass, 1994; Cohen and Winn, 1967). Finally, frequency and amplitude modulated, agonistic “growls” are the most complex call; they overlap hums in duration (~200 ms – 5 s) and are reiterative sequences of grunt- and hum-like signals.

We recently demonstrated the reproductive and nocturnal dependency of midshipman fictive calls that closely mimic the temporal properties of naturally long duration growls and hums (see Fig. 1B2 for fictive calls). Spontaneously firing grunt trains, on the other hand, can be evoked either day or night in a reproductive male (Rubow and Bass, 2009). Given the extensive GABAergic innervation of the VMN and GABAergic somata within VPP (Marchaterre et al., 1989; M. C. Zee and A. H. Bass, unpub observ), and the integral role of inhibition in the synchronizing and timing of network activity (see earlier references), we investigated the influence of GABAergic inhibition on the generation and modulation of fictive vocalizations evoked from territorial male midshipman fish. We hypothesized that shifts between natural and fictive call types reflect, in part, rapid (seconds to minutes) modulation of GABAergic activity in VPP and VPN-VMN that contributes to activity-dependent plasticity of synaptic and/or intrinsic neuronal properties. The slower seasonal and diurnal rhythms, which may include modulation of inhibition along with other permissive physiological changes involving neurosteroids and neuropeptides (Goodson and Bass, 2000a; Remage-Healey and Bass, 2004), are ultimately responsible for bringing the parental male to this labile, vocal state necessary for successful reproduction. We now show that GABAergic inhibition does indeed

provide a potent temporal scaffolding for vocalizations such that differential modulation of inhibition levels in the anatomically discrete, vocal pattern generator nuclei, VPP and VPN-VMN, promotes rapid switching between call types that are distinguished primarily by duration and frequency.

Materials and Methods

During April-August 2008-2009, type I males (12 cm-20 cm, standard length) were hand collected from nest sites in the intertidal zone of Washington State and California and shipped within 6-72 h to Cornell University where they were housed at 16-17°C in a 14 light (L):10 dark (D) light cycle with lights out at 17:00 Eastern Standard Time (EST). Type I males were unambiguously identified on the basis of their body size and coloration upon collection as well as visual inspection of their vocal muscle and testes (Bass, 1996). Males collected late in the summer were used for experiments that continued into the fall as the fish gradually shifted into a non-reproductive state (see Rubow and Bass, 2009). All methods were approved by the Institutional Animal Care and Use Committee at Cornell University.

Neurophysiological experiments

The fictive vocalization preparation used here has been thoroughly described elsewhere (Bass and Baker 1990; Goodson and Bass, 2000a; Ramage-Healey and Bass, 2004; Rubow and Bass, 2009). Briefly, brain and rostral spinal cord with occipital nerve roots were exposed by dorsal craniotomy under general anesthesia with 0.025% benzocaine (Sigma, St. Louis, MO) and a local injection at the wound site of a long-lasting analgesic (0.25% bupivacaine; Abbot Laboratories, Chicago, IL) with 0.01 mg/ ml epinephrine (International Medication Systems, El Monte, CA). After surgery, fish were immobilized with an intramuscular injection of pancuronium

bromide (0.5 mg/kg, Astra Pharmaceutical, Westborough, MA) and stabilized in a plexiglass tank with aged, chilled (16-17°C, same as housing temperature) saltwater perfused through the mouth. The electrical microstimulation protocol followed that used in our previous study that evoked long duration calls typical of reproductive type I males (Rubow and Bass, 2009). One hour after surgery, an insulated tungsten electrode (125 μm diameter, 20 μm exposed tips) was used to evoke the vocal/occipital nerve's motor volley (fictive vocalization) through delivery of 40 brief (30 ms) trains of 200 Hz stimuli (0.1 ms pulse width) at 1/s to either the medial midbrain PAG region which connects to the hindbrain VPP region or VPP itself that projects to the VPN-VMN circuit (Fig. 1B). Well-documented surface landmarks and depth measurements based on previous mapping studies of the vocal motor system provided guides for electrode placement (Goodson and Bass, 2002; Kittelberger et al., 2006; Remage-Healey and Bass, 2004; Rubow and Bass, 2009). The same low current intensity (75 μA) was used for all fish at all time points, whether it was at or above the call threshold for each individual. In our previous study, the probability of evoking long duration calls was much reduced when only the threshold current was used (Rubow and Bass, 2009). The slightly elevated stimulation intensity appeared to mimic a more natural drive in the circuit, dramatically increasing its vocal range. Thus, in the present study, we consistently used this optimal current intensity as the standard stimulation in order to allow the occurrence of fictive hums whenever possible. Call threshold, the minimum current needed to elicit a call, was ascertained at the start of each stimulation trial and then the intensity was subsequently increased to 75 μA for all recorded calls.

As noted earlier, fictive vocalizations reflect the firing properties of the vocal hindbrain-spinal region that includes the VPP-VPN-VMN circuit and directly determines a natural call's duration and fundamental frequency (harmonic call)/ pulse

repetition rate (non-harmonic call); hence, its designation as a fictive call or vocalization. Fictive calls were recorded unilaterally from an occipital nerve with an extracellular electrode (Teflon-coated silver wire with exposed ball tip; 50-100 μm diameter) and digitized using MATLAB software designed by Dr. Bruce Land (Department of Neurobiology and Behavior, Cornell University). Both sides of the brain fire together so that a unilateral recording represents bilateral synchrony of the descending vocal motor volley (Bass and Baker, 1990, 1991) that leads to the natural, simultaneous contraction of the paired vocal muscles (Skoglund, 1961; Cohen and Winn, 1967).

Neurophysiological and Statistical Analyses

All neurophysiological and statistical analyses essentially follow those of Rubow and Bass (2009) but are outlined here as well for convenience (note only the shorter intervals between recordings and the duration of each experiment). Briefly, fictive call duration or amplitude were averaged for each time point (0, 1, 5, 10, 20, 30, or up to 60 min) and normalized against the baseline (0) of each fish. For all treatment groups, each time point was an average of 40 fictive calls from 40 stimulus trains presented at one-second intervals (1 s^{-1}). This stimulation protocol was developed in the earlier study (Rubow and Bass, 2009) where it was found that fictive growls and hums are more dependent upon stimulation time than grunts and that 40 s of stimulation or 40 stimulation trains are optimal for revealing the actual vocal capacity of a reproductive fish at any specific time point. Since natural and fictive growls are a hybrid of grunt- and hum-like calls, we refer to them as “grunt-hums” (see Fig. 1). For duration measurements of grunt-hums, the duration of the initial grunt-like response (≥ 3 pulses) and any subsequent response (≥ 3 pulses) were added for the complete value but did not include the silent gap between the two. Call

duration and amplitude change (reported as means with s.e.m.) were analyzed in Graphpad Prism (5.0) using repeated-measures ANOVA followed by Bonferroni posttests. Interpulse interval (IPI) was the time between peaks of two sequential compound action potentials (fictive pulses within the motor volley). Amplitude was the voltage measured between the negative and positive peaks of a compound action potential.

Pharmacological agents and delivery

As in prior studies using this preparation (Kittelberger et al., 2006), pharmacological agents were injected by picospritzer (Biomedical Engineering, Thornwood, NY) into VPP or VPN-VMN using glass microelectrodes (WPI, 1/0.58 OD/ID mm) pulled on a Flaming/Brown micropipette puller (Model P-97, Sutter Instrument Co., Novato, CA) with tips broken back to 10 μ m. The volume ejected varied by treatment and nucleus (1-4 pulses, 100 ms duration at 20 psi). After initial trials with GABA (0.25M, 0.5M) to show its potent inhibition of vocal output, we further investigated the effect of increased or decreased inhibition in VPP and VPN-VMN by comparing the outcomes of injecting the GABA_AR-specific agonist muscimol (2 mM, 10 mM) and antagonist gabazine (0.1 mM, 1 mM) (all chemicals from Sigma-Aldrich, St. Louis, Mo) to controls, which received an equal volume of only the 9% saline vehicle.

Visualization of Injection Sites

As in previous studies, injection sites were marked by microinjections of fluorescein dextran (Goodson and Bass, 2000a; Kittelberger et al., 2006). 4% fluorescein dextran (Invitrogen) was pressure injected into VPP or VMN using glass microelectrodes (WPI, 1/0.58 OD/ID mm) pulled on a Flaming/Brown micropipette

puller (see above) with tips broken back to 10 μm . Also like the previous studies, the dye was ejected (4 pulses, 100 ms duration at 20 psi) using a picospritzer (see above). Immediately after the injection, the braincase was sealed and fish were returned to an anesthetizing water bath of 0.025% benzocaine for 10 min. Whole brains were removed and stored in 10% buffered (0.1M phosphate buffer/ PB) formalin for at least one week, incubated overnight in 30% sucrose-PB before sectioning frozen at either 30 μm or 50 μm in the transverse plane, mounted on slides, dried overnight and then coverslipped with a fluorescent mounting medium (Vectashield, Vector Laboratories). Sections were viewed on a Nikon Eclipse E800 microscope and photographed using a Nikon digital camera system.

Results

Overview

Modulation of GABAergic inhibition in the vocal pre-pacemaker nucleus (VPP) and the vocal pacemaker-motor circuit (VPN-VMN) may contribute to the plastic temporal properties of fictive vocalizations. By manipulating levels of inhibition with pressure injection of either GABA or GABA_A receptor (R) agonists and antagonists into the two different regions of the hindbrain vocal pattern generator, we were able to almost instantaneously evoke the entire suite of fictive vocalizations that were previously shown to more rigidly depend upon season, time of day and prolonged electrical stimulation from the midbrain or VPP region (Rubow and Bass, 2009). Two overlapping dichotomies organize the current data: vocal region, VPP or VPN-VMN, and baseline physiological or “vocal state”. Injections targeted either VPP or VPN-VMN, the latter so dubbed because of VPN’s close proximity and dense innervation of the paired VMNs (Bass and Baker, 1990; Bass et al., 1994). VPN-VMN

injections were close to the midline, aiming to target the paired VMN that are contiguous along the midline.

For nearly all of the experiments, fictive vocal responses, both control and those with pharmacological manipulation, were evoked with electrical microstimulation in the midbrain (two final sets of experiments present data from cases with spontaneous fictive calls). While pharmacological manipulations of VPP produced fictive call types consistent with reproductive condition, occasionally GABAergic manipulation in VPN-VMN could not evoke low frequency fictive grunt-hums from a reproductive animal (mimics of natural growls; see Materials and Methods, Fig. 1), but only grunts and buzzes. Therefore, to reflect the vocal profiles of treated and control animals at the beginning of each experiment, instead of “non-reproductive” vs. “reproductive” we have defined the broadest physiological categories by vocal state, beginning with 1) baseline grunters that after pharmacological manipulation can only grunt or buzz (“state 1”), 2) baseline grunters that can, with electrical/pharmacological manipulation, produce lower frequency grunt-hums (“state 2”), and finally 3) animals from which grunt-hums are already being evoked at baseline (“state 3”). Figure 2 portrays the distinctions between different vocal baselines and to which experiments they pertained. When the grunt-hum state was specifically being investigated, experiments were performed after 18:00h EST with males that were recently collected from nest sites during the reproductive months to increase the probability of either potentiating or inhibiting the evoking of growl-like and hum-like responses characteristic of this vocal/ reproductive state (see Rubow and Bass, 2009). In sum, vocal state, to either grunt or to grunt-hum, becomes a “transient equilibrium condition” (after Buzsaki and Draguhn, 2004; Buzsaki, 2006), depending upon the neurophysiological sum of its recent history as well as its background reproductive condition.

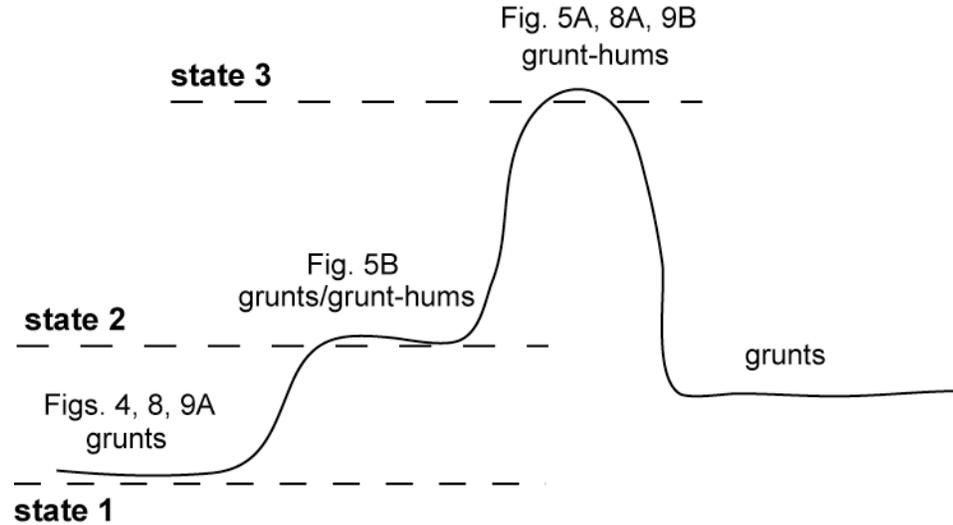
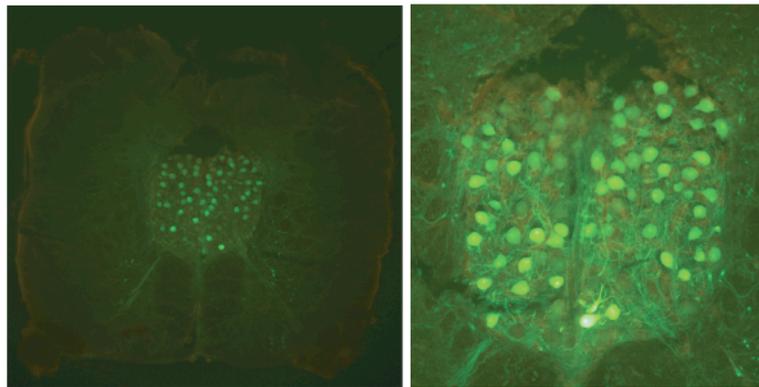


Figure 3.2: Vocal states. Reproductive condition generally promotes greater excitability in the vocal circuit and a larger repertoire of calling in the type I male, with concomitantly divergent responses to GABAergic modulation. However, reproductive condition did not guarantee that an upregulated state was always present, thus the animals used in this study were categorized by the more transient “vocal state” in which they were found at baseline. The most basal condition, or “state 1”, describes animals from which only grunts or buzzes could be evoked at baseline and after pharmacological manipulation. “State 2” males also grunted at baseline but were capable, with microstimulation or neurochemical modulation, of producing grunt-hums. Their baseline state was more volatile, usually indicated by a rapid increase in grunt duration with less than 40 s of stimulation. Finally, “state 3” males were those from which grunt-hums were already being evoked at baseline. Animals that were originally “state 2” grunters but with electrical stimulation or pharmacological treatment became grunt-hummers also belong to this group. Figure numbers are included to indicate the experiments to which the different vocal states pertain.

We begin with the results (duration, amplitude and IPI) of increased and decreased GABAergic inhibition in VPN-VMN of state 1 grunters with injections of GABA, the GABA_AR agonist muscimol or antagonist gabazine, followed by the effects of muscimol or gabazine in state 2 and 3 grunt-hummers. Finally, we report the outcome in VPP from the same pharmacological manipulations. These experiments include two groups of controls, those with and without saline injections. The initial

studies of VPN-VMN and VPP showed that saline injections had no significant effects on calls. Hence, some of the later and more extensive studies of VPN-VMN in state 2 and 3 animals used control animals without saline injections. This allowed us to both conserve the number of animals used and reliably compare the long duration output of pharmacologically manipulated state 2 or 3 animals with the unmanipulated vocal state of state 2 or 3 animals that have an underlying propensity to make long duration calls at baseline.

A. VPN-VMN



B. VPP

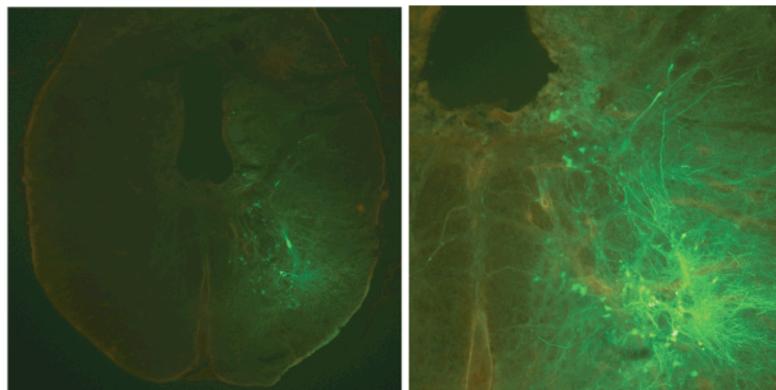


Figure 3.3: Localization of injection sites in the vocal motor nucleus (VMN) and the vocal pre-pacemaker nucleus (VPP). A. Fluorescein was injected along the midline of VMN, from where it was transported throughout the nucleus and picked up by nearby pacemaker (VPN) neurons (see text for details). B. A unilateral injection of fluorescein into VPP was contained around the injection site.

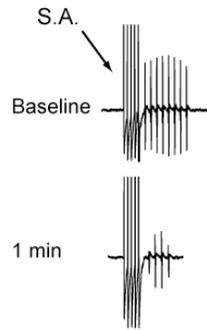
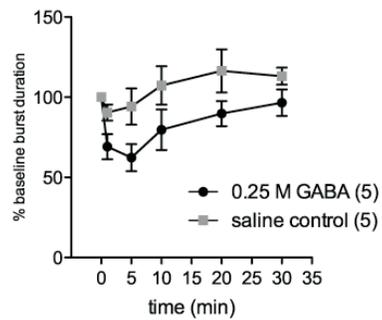
VPN-VMN

Injections of 4% fluorescein dextran into VPN-VMN were used to visualize the chemical injection sites and assure their confinement to the target region (Fig. 3a; similar as level shown in Fig. 3A, Bass et al., 1994). Fluorescein uptake by motoneurons clearly delineated VMN and, as intended and expected, also labeled VPN neurons that are directly adjacent to and densely innervate VMN (see Overview).

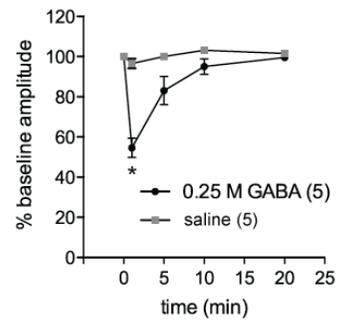
Increasing and decreasing GABAergic inhibition in state 1 (see Fig. 2):

Initial exploratory injections of 0.5 M GABA into central VPN-VMN of state 1 males (grunts at baseline and after pharmacological/electrical manipulation) almost immediately (< 1 min) abolished all motor output for up to 30 min, which no amount of increased current could overcome (not shown). A more moderate GABA concentration, 0.25 M (used for the rest of the study), resulted in a rapid but not significant reduction in call duration (max 40 % of baseline at 5 min, $p > 0.05$) that recovered by 30 min post-injection (Fig. 4A1 and traces of fictive calls). The amplitude of the fictive grunts, on the other hand, instantly (1 min) and significantly decreased by as much as 50% of baseline ($p < 0.05$) and recovered after 20 min, more rapidly than duration (Fig. 4A2 and vocal traces). The change in IPI between baseline ($9.3 \text{ ms} \pm 0.35$) and 1 min ($9 \text{ ms} \pm 0.42$) in GABA-treated animals ($n = 3$ in both groups) was not significantly different ($p > 0.05$) than the IPI change in saline controls at the same time points (baseline, $9.2 \text{ ms} \pm 0.3$; 1 min, $8.9 \text{ ms} \pm 0.19$), although a slight increase in IPI variability did occur (see Fig. 51A, B for cumulative IPIs (20 s) from one GABA-treated animal and one control). Even though the call threshold increased during the recovery time, it never exceeded the standard stimulation current (see materials and methods).

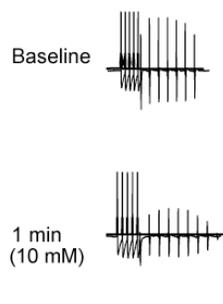
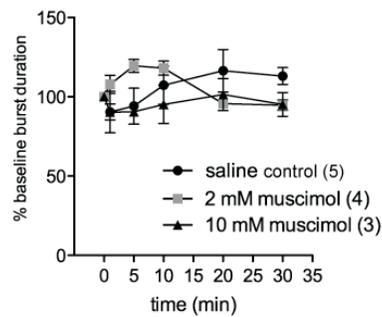
A1. VPN-VMN state 1: GABA



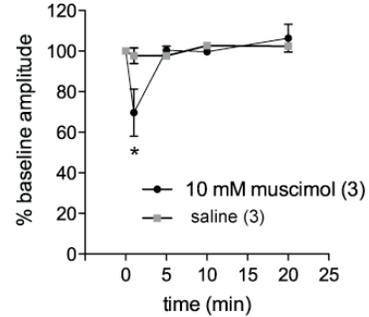
A2.



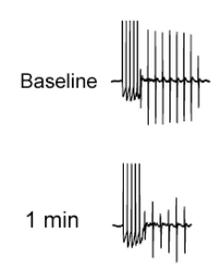
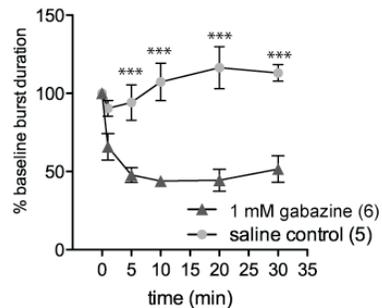
B1. VPN-VMN state 1: muscimol



B2.



C1. VPN-VMN state 1: gabazine



C2.

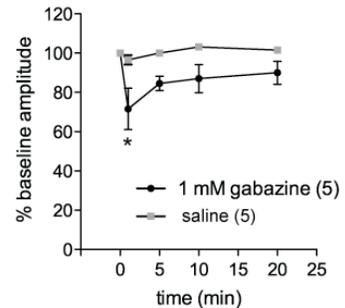


Figure 3.4: A-C. Microinjections of GABA and GABA_AR agonist and antagonist, muscimol and gabazine, into vocal motor-pacemaker circuit (VPN-VMN) of “state 1” grunters. A. 0.25M GABA microinjected along the midline between the paired VMNs (see Fig. 3A) decreased fictive grunt duration (A1) and significantly reduced burst amplitude at 1 min (see traces of fictive calls and A2). B. 2 mM and 10 mM muscimol did not significantly affect call duration (B1), but the higher concentration did significantly reduce amplitude at 1 min (traces and B2). (C) 1 mM gabazine rapidly and significantly decreased call duration (C1) and amplitude (C2), as well as disrupting general patterning (see 1 min trace). Stimulus artifact (S. A.) is indicated.

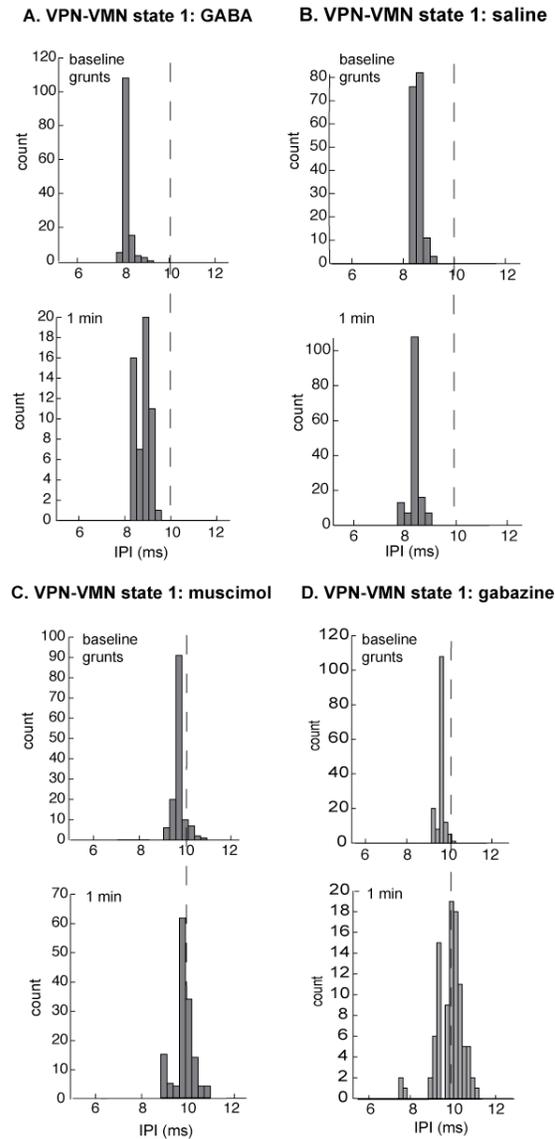


Figure 3.5: Average interpulse intervals (IPIs) for 20 vocal bursts in representative animals with increased or decreased GABAergic inhibition in vocal pacemaker-motor circuit (VPN-VMN). In state 1 males, neither 0.25M GABA (A) saline injections (B), nor 2 mM muscimol (C) significantly changed the average grunt IPI ($p > 0.05$). Decreasing inhibition with 1 mM gabazine (D) could not change average grunt frequency, but did increase IPI variability.

To determine to what extent the GABA_A receptor was responsible for the suppression of vocal motor activity in grunters via this region, we injected the GABA_AR agonist muscimol (2 mM, 10 mM). Neither muscimol concentration had a

significant effect on grunt duration or on IPIs (Fig. 4B1; $p > 0.05$ and Supp. Fig. 1C), but the higher concentration did significantly reduce amplitude by as much as 50% at 1 min, upon which it rapidly recovered (Fig. 4B2 and vocal traces; $p < 0.05$). This suggests that the inhibitory effect of GABA on grunt duration in state 1 animals may occur primarily via the GABA_B receptor (see Fig. 6 for results of microinjections of baclofen, a GABA_B receptor agonist). The drop in amplitude and duration seen in the GABA-treated animals, but not in either the saline controls or muscimol-treated animals, reappeared in the baclofen experiments.

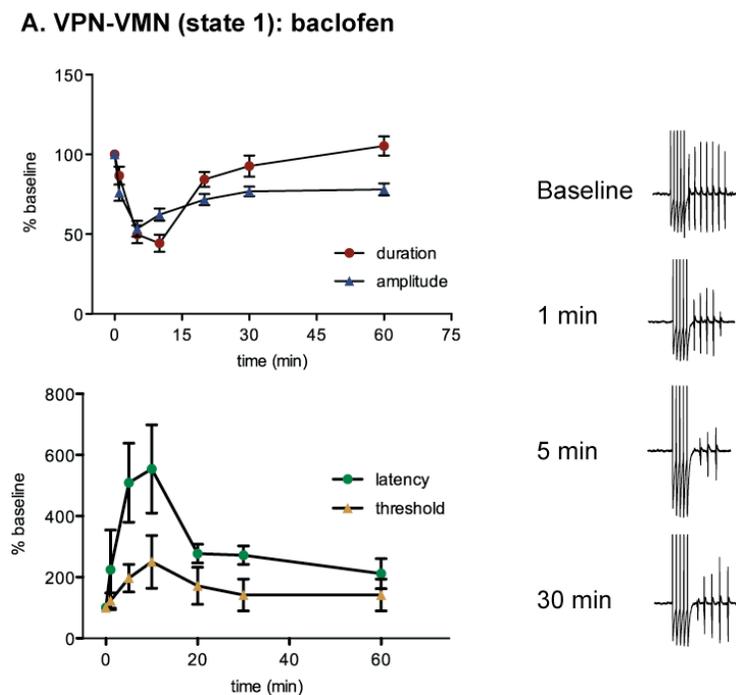
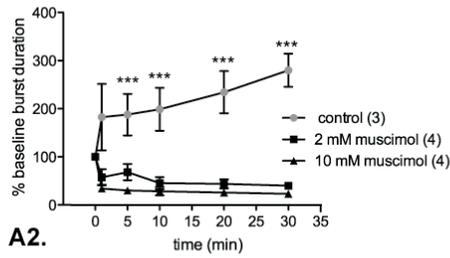


Figure 3.6: Increasing GABA_B receptor activity in vocal pacemaker-motor circuit (VPN-VMN) of state 1 males with 10 mM baclofen. Baclofen injections ($n = 4$) maximally and significantly reduced grunt duration at 5 and 10 min ($p < 0.01$) and amplitude from 1 to 60 min ($p < 0.001$), while latency ($p < 0.01$ from 1 to 20 min) and threshold ($p < 0.01$ from 5 to 20 min) significantly increased.

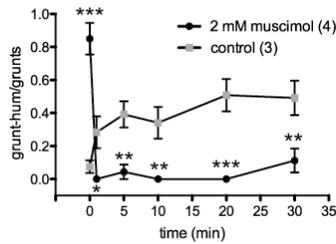
Unlike the inability of muscimol to significantly affect duration in this region, injection of the GABA_AR-specific antagonist gabazine (1 mM) into VPN-VMN of state 1 males decreased fictive grunt duration (~50 % at 5 min, $p < 0.001$) and amplitude (~ 70% at 1 min, $p < 0.05$) (Fig. 4C1, C2). Gabazine also generally disrupted grunt patterning (Fig. 4C vocal traces), while increasing frequency and amplitude variability (Fig. 4C vocal traces; Fig. 5D). Call threshold did not change unless the injected volume was doubled, nearly eliminating all fictive calling (data not shown). Increasing stimulation intensity beyond the standard stimulation current could rarely counteract the suppression of vocal output.

Increasing and decreasing GABAergic inhibition in states 2 and 3 (see Fig. 2): In contrast to muscimol's negligible effect on grunt duration, amplitude and IPI, when either 2 mM or 10 mM muscimol were injected into the VPN-VMN of state 3 males (grunt-hums at baseline), the hum component of each evoked grunt-hum was rapidly extinguished by 1 min, and significantly by 5 min ($p < 0.001$), leaving only the grunt component (Fig. 7A1, A2, Fig. 8A1, Fig. 9A, B). By 20 and 30 minutes, grunt-hums began to recover with an extra 20s of stimulation for a total of 60s in one 2 mM treated fish (Fig. 7A3), but in none of the 10 mM group, likely reflecting the lack of a rapid *in vivo* clearance mechanism for a synthetic GABA analog. Repeated stimulation trials in the control animals, on the other hand (Figs. 7A1, 8A2), significantly potentiated and maintained the long duration calls similar to the circuit's activity-dependent plasticity demonstrated in Rubow and Bass (2009).

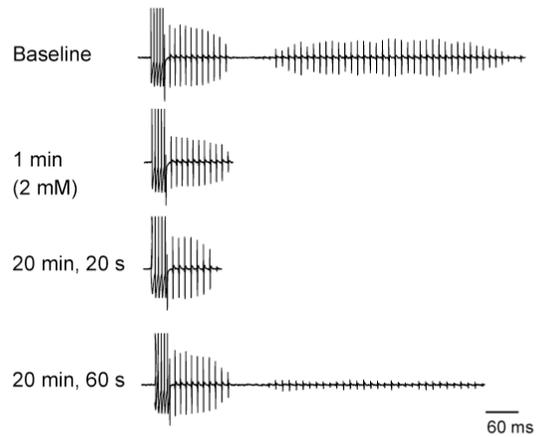
A1. VPN-VMN state 3: muscimol



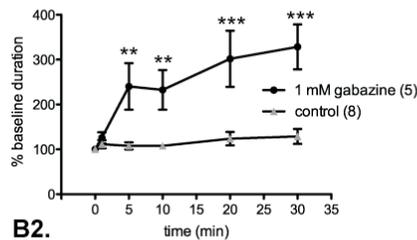
A2.



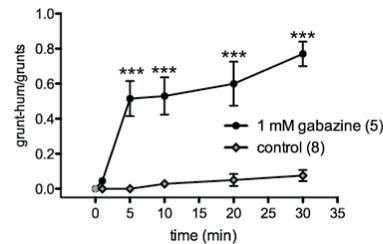
A3.



B1. VPN-VMN state 2: gabazine



B2.



B3.

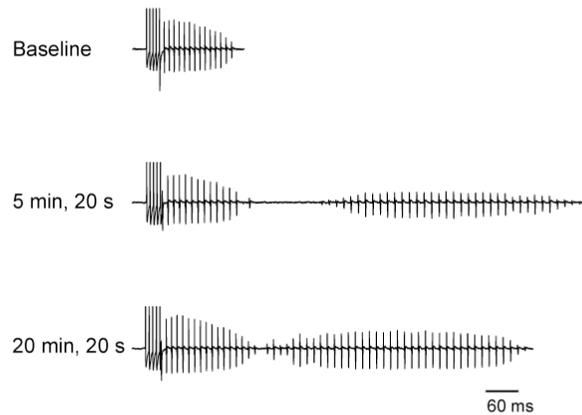


Figure 3.7: Microinjections of muscimol and gabazine into vocal pacemaker-motor circuit (VPN-VMN) of “state 2” grunters and “state 3” grunt-hummers.

A. In state 3 males from which grunt-hums were being evoked at baseline, both 2 mM and 10 mM muscimol reduced call duration (A1) and grunt-hum probability (A2) by eliminating the hum component of the grunt-hum. Control animals, on the other hand, began with grunt-hums that increased in probability and duration with stimulation and time. (A3) Only one 2 mM fish out of four began to recover grunt-hums at 20 min with extra stimulation (vocal traces). B. Injections of 1 mM gabazine into VPN-VMN of state 2 grunters, or fish whose baseline recordings indicated the ability to potentiate, significantly increased the duration of the calls (B1) by increasing the probability of evoking grunt-hums (B2). In one fish (B3, vocal traces), gabazine facilitated the production of the first low amplitude hum components at 5 min post-injection, which then increased in amplitude and number by 20 min. Stimulus artifact (S. A.) is indicated.

In dramatic contrast to the deleterious effect of blocking GABA_AR activity in grunting males (Fig. 4C1), injection of 1 mM gabazine into the VPN-VMN of state 2 animals (i.e., reproductive males with grunts at baseline and the potential to produce grunt-hums, see Overview) led to a significant increase in overall call duration by 5 min and up to 400% of baseline by 20 min in some cases ($p < 0.001$; Fig. 7B1). The duration increase reflected the increased probability of evoking grunt-hums with every stimulus train by 5 min post injection ($p < 0.001$; Figs. 7B2, 8B1). There was also an enhancement of the bimodal frequency distribution as well as the regularity in the hum-like component (Figs. 8B1, 9C, D). Male subjects in this treatment group and their controls (Fig. 8B2; see Overview) demonstrated the potential to produce long duration calls by the presence of rapidly potentiating (< 40 s) grunts at or near baseline with occasional irregular fictive pulses occurring after the grunts. At 5 min post injection, the earliest fictive hum components tended to appear as very low amplitude, lower frequency bursts following the initial stimulus-evoked grunts that slowly gained amplitude and duration during the 40 stimulation trains (Fig. 8B1).

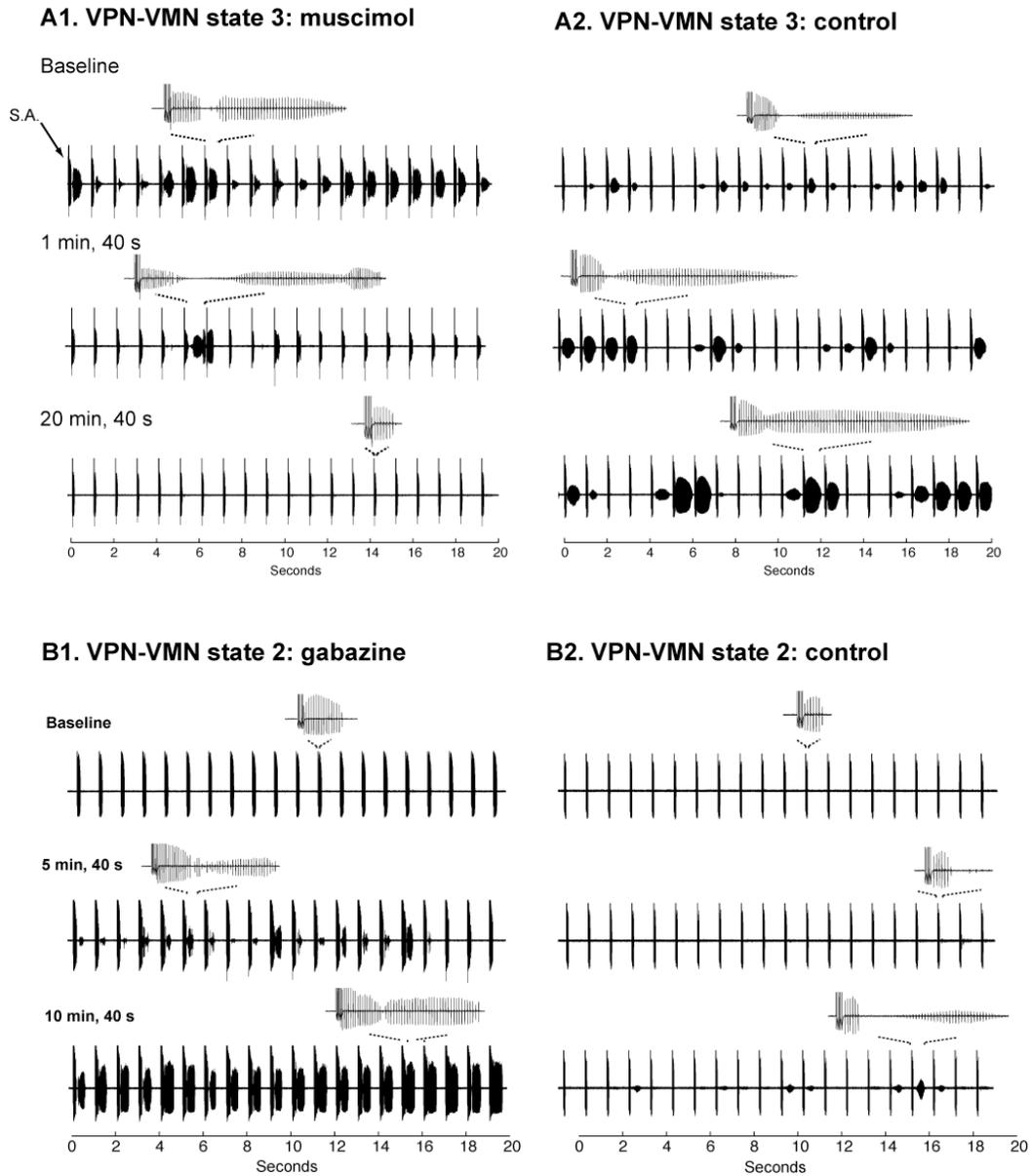


Figure 3.8: Fictive-call stimulus-response trains in “state 2” and “state 3” males injected with either muscimol or gabazine in vocal pacemaker-motor circuit (VPN-VMN). Examples of the time, stimulation and inhibitory state- dependent progression or regression of grunt-hums with 20 s excerpts from 40 s stimulation trains with stimulus artifacts (S. A.) at 1 s intervals. A. Microinjections of 2 mM muscimol in VPN-VMN of a state 3 male, rapidly (1 min) removed the hum components of the calls (A1), while microstimulation alone in a control animal augmented the grunt-hums already recorded at baseline (A2). B. Microinjections of 1 mM gabazine in state 2 males facilitated the production of grunt-hums, which first appeared at 5 min post-injection and rapidly increased in amplitude, duration and number by 10 min (B1). Six low-amplitude grunt-hums were evoked from a state 2 control male at 10 min post-injection with stimulation alone (B2).

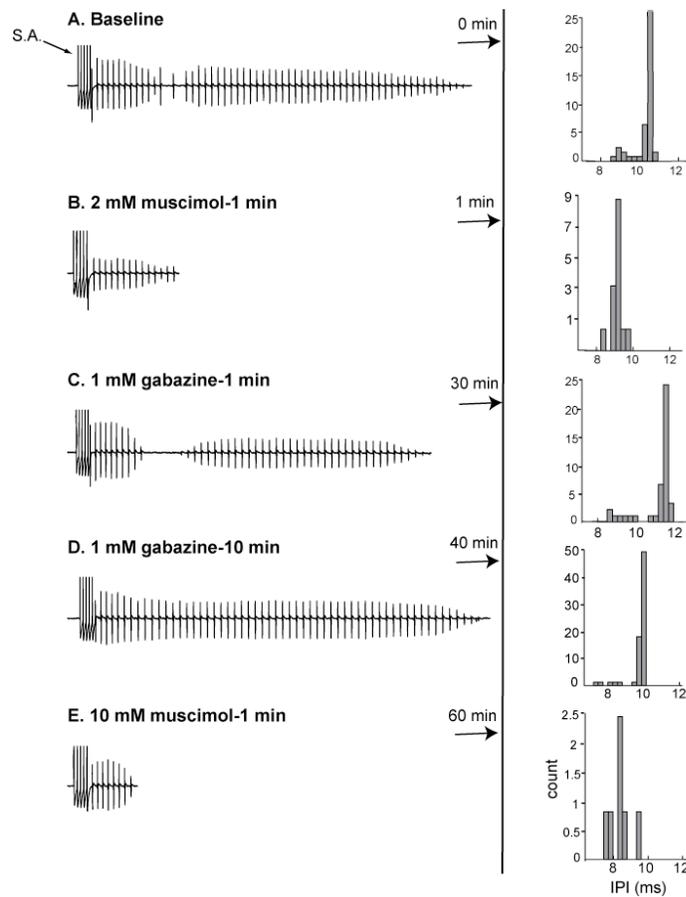


Figure 3.9: Timeline of sequential muscimol and gabazine injections into vocal pacemaker-motor circuit in one “state 3” grunt-hummer with associated changes in frequency/ interpulse intervals (IPIs) shown in accompanying histograms. A. Grunt-hums have a bimodal frequency distribution with the grunt IPI around 9 ms and the hum IPI around 10.5 ms. B. 2 mM muscimol removed the hums in 1 min which did not recover during the subsequent four recordings. C. At 30 min, when 1 mM gabazine was injected, the hum components were restored after 1 min and with 40 s of stimulation, producing a strongly bimodal grunt-hum. D. At the 10 min recording and another 40 s of stimulation, the gabazine-restored grunt-hums were elongated and more continuous. E. Finally, after the 20 min recording when 10 mM muscimol was injected, the hums were again eliminated in less than 1 min. Stimulus artifact (S. A.) is indicated.

The effects of gabazine were even more potent in two state 3 males, not included in the state 2 study group from which a few small grunt-hums could already be evoked at baseline after the delivery of 20 to 40 stimulation trains (Fig. 10). In this “vocal state”, at least 20 s of briefly, two-fold higher intensity stimulation at 20 min post-injection could rekindle the long duration, highly regular, low frequency calls, but at a much reduced amplitude. However, after 40 stimulation trials or 40 s, their full amplitude was regained, the current intensity could be reduced again, and the initial grunt portion of each call completely dropped out until only the hum component remained. This never occurred in animals without gabazine microinjections in VPN-VMN. The apparent tradeoff with reducing inhibition in animals already primed for hums, was the sensitivity of the nucleus to a hyper-reduction of GABAergic inhibition. The results suggest an optimal, intermediate block of GABA_AR for promoting fictive grunt-hums. Too little and the fish still grunted, too much and the grunt-hums were delayed and preceded by variable latency and irregular spiking with a higher threshold.

A. VPN-VMN (state 3): gabazine

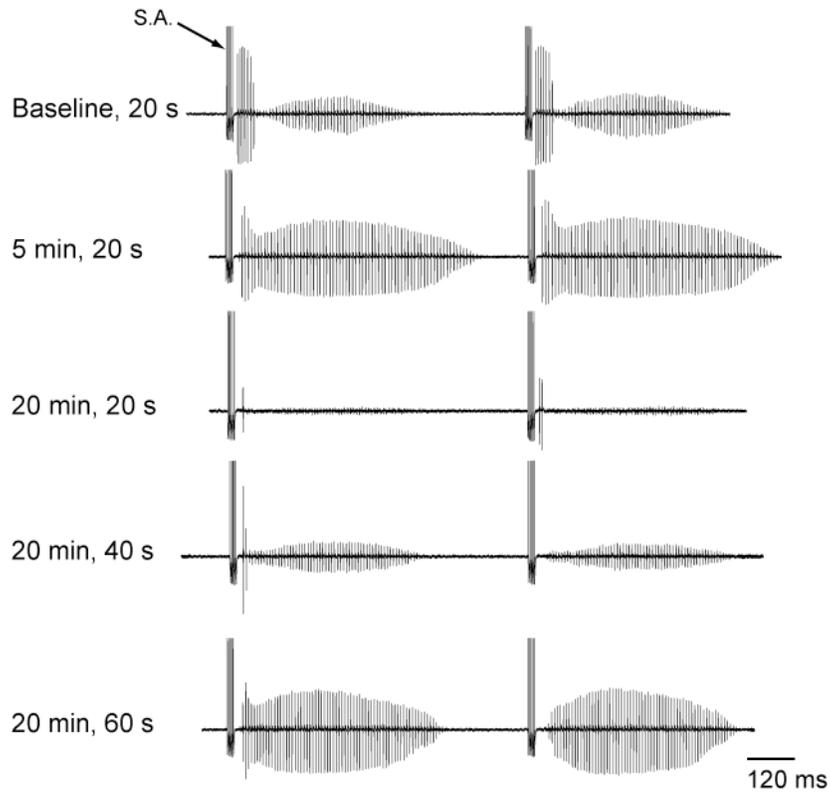


Figure 3.10: Gabazine injections in vocal pacemaker-motor circuit (VPN-VMN) of “state 3” male subtracted grunts from grunt-hums. In one male shown here, grunt components of the grunt-hums diminished after a gabazine injection until only the hum component remained at 20 min. In the first 20 s of stimulation, call threshold was increased and almost no bursts could be evoked. After the standard 40 s of stimulation, the grunt portions intermittently disappeared while the low amplitude hum component began to recover. With 20 s more of stimulation for a total of 60 s, the grunts vanished altogether and the hums again reached their maximum amplitude. Stimulus artifact (S. A.) is indicated.

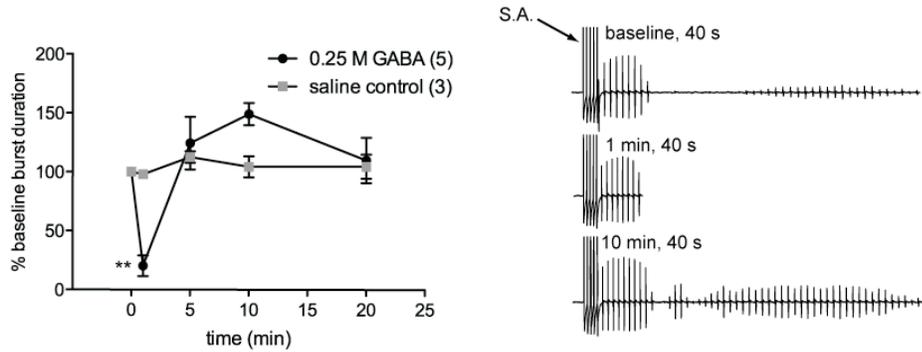
VPP

Unilateral injections of 4% fluorescein dextran were used to establish the precision of the chemical injections in VPP just as in VPN-VMN (Fig. 3B; similar as level shown in Fig. 2K, Bass et al., 1994). The dye showed no signs of diffusing into either the VPN-VMN region or PAG, but rather was confined to the VPP region after

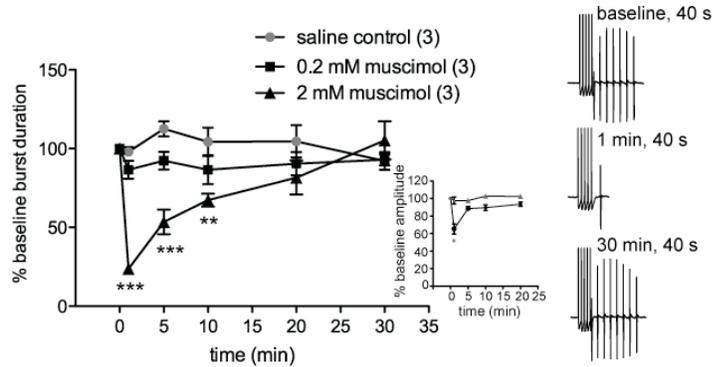
the elapsed 25 minutes between injection and the immersion of the isolated brain in 10% formalin. Despite evidence of some spread into the area surrounding VPP, the most intense label was in VPP-labeled somata and their immediate surrounds. A few small somata labeled near the fourth ventricle were likely due to minimal leakage during either initial penetration or retraction of the electrode from the medulla. This confirmed the site-specific effects of the chemical injections in this nucleus.

Increasing and decreasing GABAergic inhibition in states 1, 2 and 3 (see Fig. 2): Bilateral injections of 0.25 M GABA into the VPP of state 1 and state 3 males induced a rapid decrease in call duration (~20% of baseline; $p < 0.01$; Fig. 11A) by 1 min, reflected in the disappearance of grunt-hums and/or a significant decrease in grunt duration. Overall call duration then rebounded slightly above baseline (~ 150 %; $p > 0.05$) with increased number and duration of grunt-hums at 5 and 10 min in the case of grunt-hummers (Fig. 11A vocal traces), or increased grunt duration in the case of grunTERS (traces not shown), upon which it returned to baseline values by 20 min. Unlike GABA's null effect on grunt IPIs in VPN-VMN, in VPP grunt IPIs did briefly and significantly increase ($p < 0.05$, $n=3$ in both groups) from $8.7 \text{ ms} \pm 0.29$ at baseline to $9.9 \text{ ms} \pm 0.23$ at 1 min, compared to saline controls (baseline, $8.6 \text{ ms} \pm 0.09$; 1 min, $8.8 \text{ ms} \pm 0.12$) (Fig. 12A, B). Grunt amplitude was not significantly affected at any time point ($p > 0.05$; Fig. 13A).

A. VPP state 1 and 3: GABA



B. VPP state 1: muscimol



C. VPP state 1: gabazine

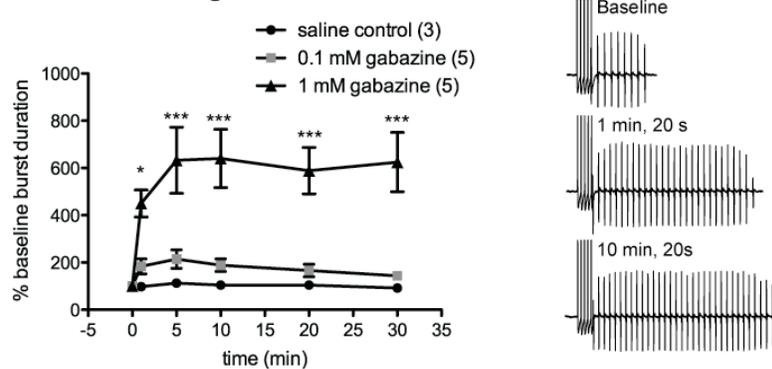


Figure 3.11: Microinjections of GABA, muscimol and gabazine into vocal prepacemaker nucleus (VPP) of “state 1” and “state 3” males. A. Bilateral injections of 0.25 M GABA significantly reduced grunt duration in state 1 animals or eliminated the hum components from grunt-hums of state 3 animals by 1 min. Grunt duration or hums quickly recovered or increased (vocal traces) by 10 min. There was no significant change in amplitude ($p > 0.05$, see Supp. Fig. 4A). B. Bilateral injections of 2 mM muscimol into VPP of state 1 grunTERS significantly decreased call duration and amplitude (see traces and inset), recovering by 30 min. C. Injections of gabazine into VPP of state 1 males significantly increased call duration in the first 20 s of stimulation while maintaining the high frequency. There was no change in amplitude ($p > 0.05$, Supp. Fig. 4B). Stimulus artifact is indicated (S. A.)

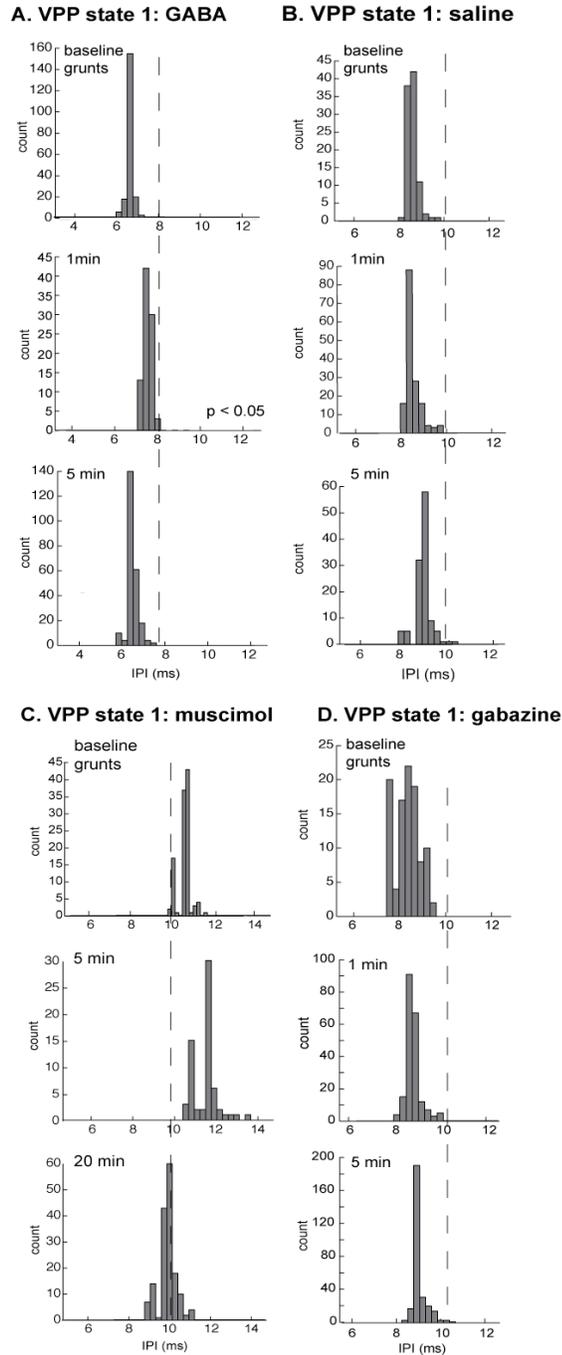


Figure 3.12: Average interpulse intervals (IPIs) for 20 vocal bursts in representative animals with increased or decreased GABAergic inhibition in vocal prepacemaker nucleus (VPP). In state 1 males, 0.25M GABA (A) significantly increased grunt IPI at 1 min post-injection ($p < 0.05$), while saline injections (B) had no effect ($p > 0.05$). Neither muscimol (2 mM) nor gabazine (1 mM) significantly changed mean grunt IPI ($p > 0.05$), but gabazine did reduce IPI variability, in contrast with its effect in state 1 VPN-VMN, where IPI variability was increased (see Supp. Fig. 1D).

Similar to GABA injections in state 1 and state 3 animals (grunters and grunt-hummers), bilateral injections of 2 mM muscimol (but not 0.2 mM) into state 1 males only rapidly and significantly reduced call duration at 1 min ($p < 0.001$), which then remained significantly reduced until 20 min post-injection ($p < 0.01$; Fig. 11B). This contrasted with grunt amplitude, which was also briefly and significantly reduced at 1 min ($p < 0.05$) but immediately returned to baseline (Fig. 11B inset), followed by grunt frequency (see Fig. 12C; note slower baseline frequencies in non-reproductive grunter compared to Fig. 12A). The lack of a rapid duration recovery or overshoot may reflect the non-reproductive condition and/or, once again, an inability to rapidly equilibrate or clear a synthetic agonist.

Just as muscimol rapidly inhibited vocal output, bilateral injections of 1 mM gabazine into state 1 males rapidly (< 1 min) and significantly increased call duration, in a dose-dependent manner, by as much as 600% at 5 min ($p < 0.001$), with an effect so robust it was already apparent after 1 min in the first 20 s of stimulation (Fig. 11C, traces). Duration remained high at 30 min post-injection and slowly declined thereafter, while call amplitude never changed ($p > 0.05$; Fig. 13B). Although this duration increase can be even more dramatic than that seen in the transition from fictive grunts to fictive grunt-hums after gabazine injections in VPN-VMN, the frequency remains as high as the typically brief grunt and thus may be the fictive equivalent of a buzz (Fig. 12D; see also Fig. 14A, B for vocal traces with IPIs).

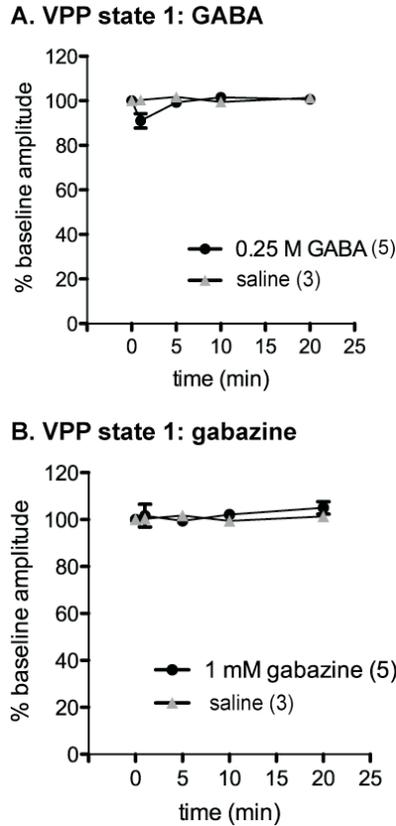


Figure 3.13: Grunt amplitude change after GABA or gabazine injections in VPP of state 1 males. Injections of 0.25M GABA (A) or 1 mM gabazine (B) did not significantly change grunt amplitude compared to controls ($p > 0.05$).

Gabazine disinhibition in VPP after initial injection in VPN-VMN:

Gabazine injections into VPP of state 1 or state 3 males, irrespective of their previous treatment and stimulation history, further revealed the ability of GABAergic mechanisms to facilitate transitions between vocal states. A bilateral gabazine (1mM) injection in VPP immediately after the 30 min recording from a state 1 grunting male that first received gabazine injections in VPN-VMN (see Fig. 4C, $n = 4$), resulted in a dramatic duration increase (up to 400% of 30 min baseline) as well as a consolidation of IPIs to ultimately generate a call resembling the natural buzz, which may be almost

as regular as a hum but much faster (8 ms IPI vs. >10 ms) (Fig. 14A1, A2 for one example: cumulative histogram from 20 bursts with one sample call). When gabazine was injected bilaterally into VPP 30 min after were state 2 males taken to state 3 grunt-hums by gabazine injections in VPN-VMN (see Fig. 7B, n = 6), the resultant call also resembled a buzz. Thus, discharge frequency increased to that of a fictive grunt, while the duration ($278.6 \text{ ms} \pm 50.9$, n = 5 animals, 20 calls/animal) tended to be less than that of a grunt-hum, but at least 3 times greater than that of a grunt (Fig. 14B1, B2; see Fig. 6 for representative vocal traces of grunts and grunt-hums and Rubow and Bass, 2009 for quantitative analysis of temporal properties of these fictive calls). The IPIs in this longer duration call also tended to be more regular than the shorter fictive grunts.

Disinhibition and spontaneous activity: When the bilateral injection volume of gabazine aimed at VPP was at least doubled or tripled in state 1, strictly grunting, non-reproductive males, the vocal system was disinhibited for an hour or more (n = 10; Fig. 15A). Thus, midbrain stimulation could sometimes still evoke buzzes, but most remarkably, the circuit now also fired independently without any stimulation at all. We recognize given these injection volumes that gabazine may have spread to the

GABAZINE

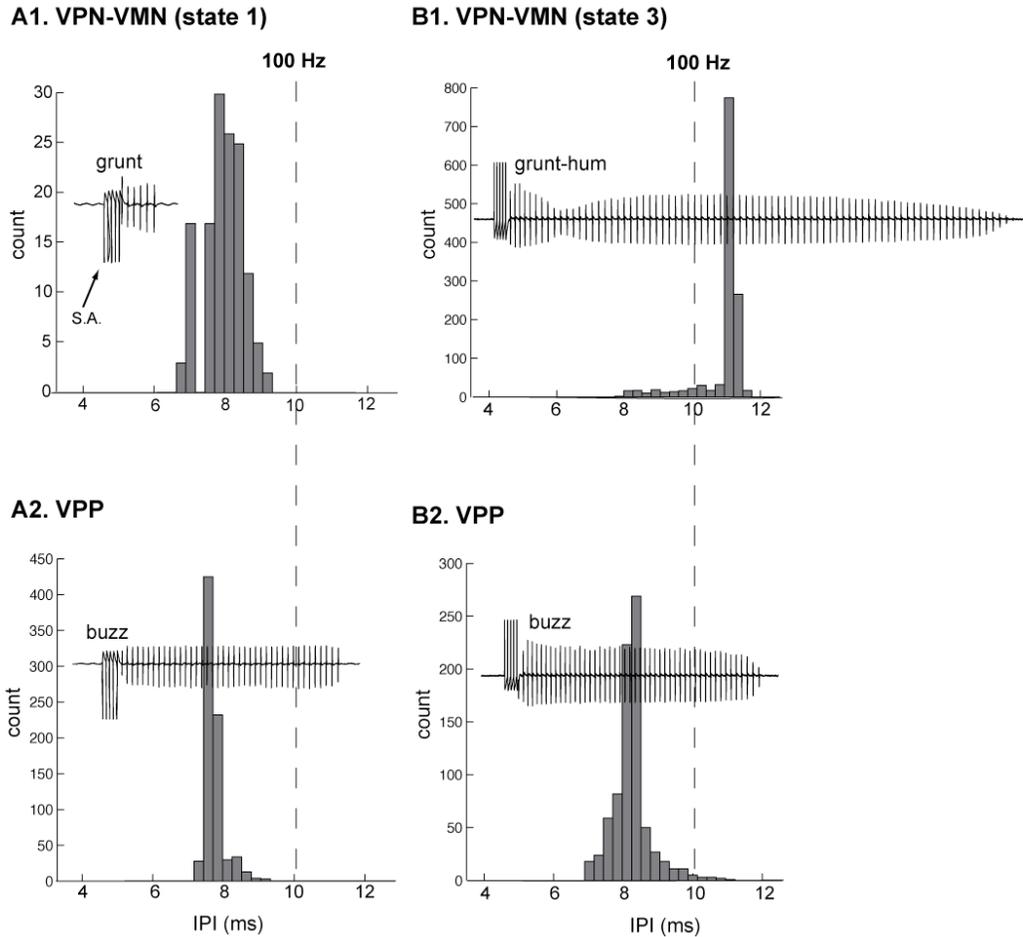


Figure 3.14: Decreasing GABAergic inhibition with gabazine controls different aspects of fictive calling depending upon the nucleus and vocal state of the fish. A. Fictive grunt duration and patterning in a **state 1** male was partially recovered 30 min after a gabazine injection in VPN-VMN (average of 20 vocal bursts) (A1). However, IPIs still tended to be more irregular than at baseline (see also Supp. Fig. 1D). When gabazine was subsequently injected into VPP, IPIs immediately became more regular and duration dramatically increased (up to 400% of 30 min baseline) (A2). The regular, long duration, high frequency fictive calls resembled the natural buzz. B. Gabazine injections in VPN-VMN of a **state 2** male supported and enhanced the ability to produce grunt-hums with a slow, regular discharge frequency at 30 min post-injection (B1). B. When gabazine was injected into VPP of this now **state 3** grunt-humming male, the discharge frequency increased to that of a fictive grunt with a duration less than that of a hum but at least 3-fold greater than that of a grunt, thus resembling a natural, agonistic buzz (B2). The IPIs in this longer duration call was also more regular than the shorter fictive grunts (see A1).

VPN-VMN circuit (but see Discussion). Nonetheless, these injections revealed the powerful effects of GABA on the hindbrain vocal network. Whether the animal received gabazine injections before any other manipulations or at the close of another treatment and series of recordings, generally did not affect the ability of the circuit to fire spontaneously. These injections essentially funneled fish into one overriding mode of independent activity. Buzzes of 1 to 6 s in duration periodically erupted followed by intervals of silence (20-40 s) (Fig. 15A for example of one 3.5 s buzz). This could continue for an hour or longer while gradually running down, but was renewable by repeating the same injections.

In state 1 strictly reproductive males, tripling the volume of the bilateral gabazine injection aimed at VPP ($n = 22$) also disinhibited the vocal system (Fig. 15B trace). However, instead of only spontaneous, intermittent “buzzes”, the free-running output was more likely to begin with rhythmic grunt trains, exhibiting burst rates faster or slower than the natural grunt train or the fictive grunt train evoked only by stimulation (grunt repetition rate: 1.5-3 Hz) (Fig. 15B, C). In some cases the inter-grunt interval would gradually increase with increasing grunt duration until longer duration buzzes were produced. After a few buzzes, the circuit could begin this sequence again, but more often the buzzes then dominated with decreasing probability until calling ceased. Figure 15 also shows the relationship between burst or grunt duration and cycle time (the interval between the peaks of the first pulses of two consecutive bursts) in stimulated, free-running grunt trains vs. disinhibited grunt trains. The relationship was roughly linear, such that as cycle period increased, so too

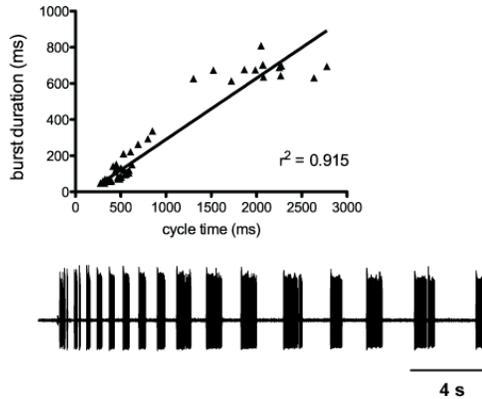
did grunt duration. The mean fraction of the cycle period occupied by the grunt (duty cycle) did not systematically change across a considerable range of grunt repetition rates (<0.5-3 Hz) in both groups, but was smaller in stimulated grunt trains ($9.3\% \pm 0.71$) than in the disinhibited ones ($25.7\% \pm 4.3$).

In addition to the disinhibition of the vocal circuit via VPP gabazine injections, reducing GABA_AR mediated inhibition via simultaneous gabazine injections in VPN-VMN and PAG, but not VPP, also allowed the circuit to fire spontaneously (n = 9). In reproductive males following previous treatments or none at all, this produced a great variety of output, with bursts resembling amplitude-modulated growls, grunt trains or the longer, less syncopated buzzes, similar to burst in Fig. 15A. As with gabazine injections aimed at VPP, the net, robust effect was almost always disinhibition, regardless of treatment history.

A. Non-rhythmic buzz



B. Disinhibited spontaneous grunt trains (n = 5 fish, 50 bursts)



C. Stimulated spontaneous grunt trains (n = 5 fish, 50 bursts)

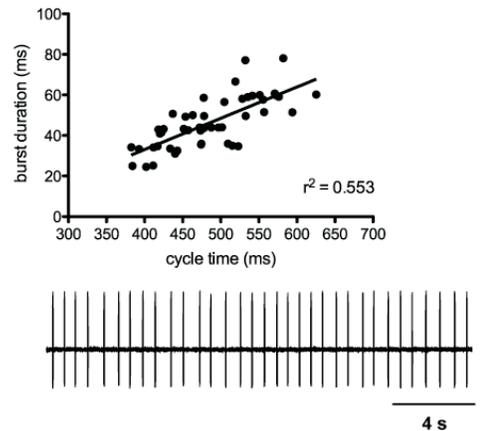


Figure 3.15: Free-running, spontaneous buzzes or grunt trains can be triggered by stimulation in vocal prepacemaker nucleus (VPP) or by increased disinhibition with gabazine injections. Cycle time (time elapsed between first pulses in two consecutive bursts or grunts) vs. burst duration is plotted. A. Bilateral injections of gabazine into VPP of strictly non-reproductive males released spontaneous, non-rhythmic, very long duration (up to 6 s) fictive buzzes that could repeat intermittently (at most 20-40 s pauses) for an hour or more. B. Increased disinhibition in VPP of strictly reproductive males tended to trigger initial grunt trains with increasing grunt duration and grunt interval with a strong linear correlation in both clusters. Ten, consecutive fictive grunt durations with inter-grunt intervals were plotted from five different fish. After 1-2 min, the grunt trains were usually overtaken by the longer duration, non-rhythmic buzzes. C. Stimulated fictive grunt trains are highly regular and burst duration correlates linearly with cycle time.

Discussion

By manipulating levels of inhibition in the hindbrain/spinal vocal pattern generator with microinjections of GABA or GABA_AR agonists and antagonists into VPP or VPN-VMN, we were able to rapidly modulate the duration, frequency, and amplitude of fictive vocalizations that directly predict the temporal properties of five natural call types produced by type I males: single grunts, grunt train, buzz, growl and hum. The fictive correlates of these more complex, long duration calls (except for buzzes) have been previously evoked without pharmacological manipulation in the same neurophysiological preparation after modestly prolonged stimulation and were dependent upon reproductive condition and time of day. As previously shown in reproductive males at night, electrical stimulation in the medial PAG during a 120 min experimental trial (i.e., 40 s of stimulation at multiple intervals over a 120 min period) could produce fictive “grunt-hums” that mimic natural growls and a free-running grunt train occurred when stimulation was briefly (< 40 s) switched from the PAG to VPP (Rubow and Bass, 2009). The two call types were mutually exclusive although the potential for both motor outputs could exist simultaneously in the vocal circuit of an unmanipulated fish. Hence, the output of a vocal pattern generator primed by reproductive condition, a diurnal rhythm, and increased activity, bifurcates depending upon site of stimulation, while the regionally specific, dynamic levels of GABAergic inhibition further control activity in the multifunctional circuit. There was a bidirectional response to either decreasing or increasing GABAergic activity in the VPN-VMN of type I males that roughly correlated with reproductive condition, but more specifically with “vocal state”, and was further enhanced by nighttime experiments.

GABAergic inhibition and synaptic drive are key parameters of a multifunctional circuit

The data presented here strongly suggest that a convergent modulation of inhibitory mechanisms, either by stimulation or direct pharmacological manipulations, contributes to call switching (see Fig. 15). Activity-dependent plasticity in the vocal circuit, or prolonged stimulation, was already shown in Rubow and Bass (2009) to promote the evocation of the long duration, lower frequency calls. Grunt-hums are more stimulation dependent than fictive grunts, and it may be the stimulation itself that increases or decreases the concentration of endogenous or exogenous GABA at the synapses. The sheer repetition of stimulation trials between the baseline recording and 30 min in stable, state 3 control animals in Figures 7A and 8A2 significantly potentiated and maintained the long duration calls while focal gabazine injections in VPN-VMN (Figs. 7B, 8A1) may have simulated this prolonged or tetanic stimulation in the upstream PAG by disinhibiting specific neuronal pools in VPN-VMN to promote grunt-hums. Figure 16 uses bifurcation diagrams to sketch the relationship between the different calls in the more vocally plastic reproductive animals at night and their dependency upon two parameters: degree of GABAergic inhibition and synaptic drive (multistability reviewed in Briggman and Kristan, 2008, see especially Cymbalyuk et al., 2002 for example from leech heart CPG). Dashed lines indicate where, holding one of the parameters constant, increasing or decreasing the other allows multiple states or calls to coexist. The growl or grunt-hum, in which grunt-like and hum-like components alternate continuously, may occupy the most dynamically multistable zone that also gives way to auto-rhythmic grunt trains simply by shifting activation of key neurons in VPP.

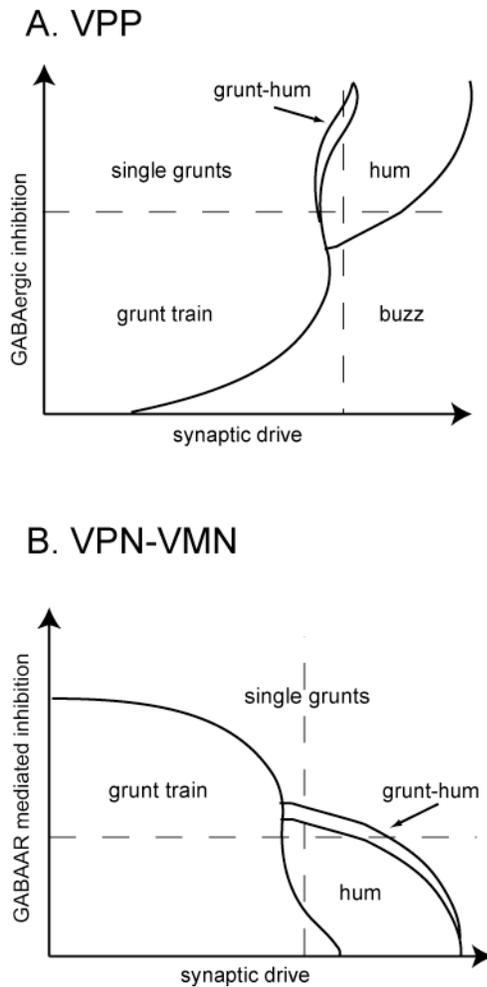


Figure 3.16: Bifurcation diagrams of the multistable midshipman vocal pattern generator. Dashed lines indicate tristable states at specific levels of GABAergic inhibition or synaptic drive. A. Vocal Prepacemaker Nucleus (**VPP**). Low levels of GABAergic inhibition and synaptic drive or activity supports buzzes. VPP stimulation after increased activity or decreased inhibition levels may also trigger a spontaneous grunt train. In contrast, stimulation-dependent fictive grunts can be evoked with much higher levels of inhibition, while much increased activity with adequate levels of inhibition promotes hums in reproductive males at night. B. Vocal pacemaker-motoneuron circuit (**VPN-VMN**). A reflection and rotation of the same curves depicts the GABA_AR and activity dependent tuning of the pace-maker/vocal motoneuron region. Here, grunt trains are supported by varying levels of inhibition and synaptic drive (though triggered upstream in VPP), while single grunts can be evoked at the highest inhibition levels and at any level of activity. Hums and growls depend upon the lowest levels of inhibition in this nucleus and the greatest synaptic drive.

VPN-VMN

A GABA dose of 0.5 M effectively terminated motor output when injected into VPN-VMN of all animals, regardless of reproductive condition or vocal state. Half that concentration, 0.25 M, that presumably raised GABA concentrations within a more physiological range, modestly reduced the duration of fictive grunts in state 1 grunters while the GABA_AR agonist muscimol had little or no effect on grunt duration (although it could decrease amplitude). In state 3 grunt-hummers, increasing inhibition with muscimol likewise preserved grunts but rapidly removed the hum components. Correspondingly, blocking GABA_AR with gabazine disrupted fictive grunt patterning and decreased their duration, whereas in reproductive males on the verge of producing long duration calls or already sporadically capable (state 2--state 3), it rapidly (< 5 min) increased the duration and probability of evoking grunt-hums. Pharmacological reduction of inhibition in this region bypassed the necessity of prolonged stimulation to evoke grunt-hums (Rubow and Bass, 2009), while also enhancing their number and patterning. Furthermore, gabazine microinjections actually split the grunt-hum, augmenting the hum component while shortening and finally removing the grunt portion. This was never seen with stimulation alone. Thus, rather than simply changing the intra-grunt frequency as seen in VPP, increasing or decreasing GABA_AR activity in VPN-VMN (of reproductive males) selects for either grunts or hums, respectively.

Gabazine microinjections had a deleterious effect on grunt duration and the stability of grunt IPI, but dramatically potentiated the long duration, low frequency hums of fictive grunt-hums. GABA_AR activity in this final output region of the vocal circuit appears to up or down-regulate the grunt and the hum by controlling the discharge frequency and secondarily the duration of the fictive call with which it is typically associated (evoked hums of grunt-hums are usually much longer than

grunts). The gradual, amplitude-increasing arrival of the hum component mirrors their amplitude-reducing loss when inhibition is increased in VPN-VMN in humming males and may suggest the recruitment and de-recruitment of call-specific motoneurons or changes in their firing states (Fig. 4A2, B2). In one early spring or recrudescing male these “mini fictive hums” never progressed past this first stage to achieve full regularity in frequency and amplitude (thus resembling locomotor bursts), due to the absence of at least one other permissive factor such as gap junction connectivity, which is present in this nucleus (Bass and Marchaterre, 1989) and may be seasonally modulated. The question concerning circuits dedicated to call types vs. multifunctional neuron pools is discussed further below when comparing the vocal system with spinal networks underlying locomotion.

VPP

Similar to VPN-VMN, a GABA dose of 0.5 M microinjected into VPP in all animals temporarily abolished vocal output. More modest increases in GABAergic activity with 0.25 M GABA rapidly (< 1 min) reduced fictive grunt or grunt-hum duration, which quickly recovered (< 5 min) with a trend towards increase or potentiation of output at 10 min post-injection. Microinjections of 2 mM muscimol also rapidly (1 min) decreased call duration but only slowly returned to baseline at least 30 min after the injection. Native GABA may best approximate and demonstrate the dynamic regulation of inhibition in this nucleus, via at least GABA_AR if not also GABA_BR. Thus, in VPP of state 3 grunt-humming males, dynamic *increases* in endogenous GABA appeared to ultimately support grunt-hums (Fig. 11A vocal traces), while *decreasing* GABA_AR activity via gabazine injections shifted the previous bimodal frequency of grunt-hums into a unimodally higher grunt or buzz-like frequency (Fig. 11C, Fig. 12). When injected in grunting males, this higher frequency

was maintained or even slightly increased while duration was dramatically (up to 600 %) increased over baseline, producing fictive calls that resembled natural buzzes.

Disinhibition of the vocal pattern generator

Partially reducing inhibition in VPP had an overall augmenting effect on call duration that was always accompanied either by a maintenance or shift into higher grunt frequencies. When the injected volume of gabazine was tripled, however, the hindbrain-spinal pacemaker circuit was disinhibited, releasing two different patterns of spontaneous output that continued for an hour or longer. In strictly non-reproductive (winter) males, disinhibition typically resulted in irregularly repeated intervals of long duration buzzes (up to 6 s, grunt frequency), while in strictly reproductive males (summer), spontaneous bursting would begin with an output resembling a rhythmic grunt train that such males typically produce under natural conditions (Brantley and Bass, 1994). The cycle period and duration of individual “grunts” progressively lengthened (for 20-60s) and finally terminated in long duration buzzes, after which a rhythmic grunt train rarely appeared again.

In sum, long duration buzzes were enhanced by the greatest reduction in GABAergic inhibition levels in VPP, while long duration hums and long-lasting, syncopated grunt trains appeared to depend upon the maintenance and dynamic regulation of at least basal GABA levels in this nucleus for proper patterning. Bilateral gabazine injections in VPP replaced existing grunt-hums with higher frequency buzzes, either by excessive drive to downstream VPN-VMN, or possibly by reconfiguration of actively firing neurons in VPP. Although it is possible that gabazine microinjections diffused from VPP into rostral regions of VPN-VMN, this is unlikely for two reasons. Early trials with 2 mM bicuculline injections required smaller volumes than gabazine (two 10 ms bilateral injections vs. three or four 100 ms

bilateral injections of gabazine) to trigger similar spontaneous firing. These smaller volumes were equal or less than the fluorescein injection that showed no sign of spread into VPN-VMN (Fig. 3). However, if gabazine did leak caudally into the VPN region, this might suggest that the spontaneously firing circuit controlling grunt trains is composed of reciprocal connections between VPP and the pace-making cells in VPN.

Finally, since grunt-hums are primarily evoked at night even with pharmacological manipulations, inhibition levels in VPP as well as in VPN-VMN may fluctuate diurnally to support this demanding, nocturnal, advertisement call. Seasonal expression change in the GABA system coinciding with reproductive development has already been demonstrated in other teleosts (Lariviere et al., 2005; Zhang et al., 2009), while in avian song nuclei there are seasonal and testosterone-dependent changes in noradrenergic receptor distribution (Riters et al., 2002). Concomitant with the shift in neurotransmitter function, at least one other activity-dependent, permissive factor such as increased electrotonic coupling must take place in the midshipman vocal circuit. Gap junction connectivity increases in the goldfish after heightened network activity, ultimately enhancing synchronization of an interneuronal network (Pereda and Faber, 1996).

Inhibitory neurons and network timing

Inhibitory neurons with their higher input resistance and precise “clocking” abilities are undoubtedly critical for the timing and patterning of some circuits. Shifting temporal coalitions of neurons, or “transient assembly synchronization by oscillation” rely upon inhibitory interneurons (reviewed in Buzsaki and Draguhn, 2004), and may be an important component of the rapid call switching demonstrated in the midshipman fictive call preparation that mimics their natural vocal plasticity in

the wild. It is one of the least metabolically demanding solutions for a multistable circuit, whereas slower, activity-dependent modification of inhibitory synapses (i.e. LTP or LTD of inhibitory networks) that arise from changes in synaptic strength may also contribute to circuit plasticity (Charpier et al., 1995; Miles and Wong, 1987; Oda et al., 1998) but depends upon more costly biochemical changes, i.e. changing postsynaptic calcium concentration (modeled in Soto-Treviño et al., 2001). Finally, disinhibition, rather than potentiation of inhibition in a circuit, could result from an increase in intracellular chloride and depolarization of the chloride reversal potential after prolonged or tetanic stimulation, which may even lead to down-regulation of the transmembrane anion transporter, KCC2, that controls the potassium/chloride gradients (Thompson and Gahwiler, 1988; Hewitt et al., 2009). Lastly, augmented activity in the midshipman vocal motor circuit may shift vocal output by increasing extracellular potassium, depress inhibition by receptor desensitization, or increase pre-synaptic inhibition of GABA release via GABA_BR (Thompson and Gahwiler, 1988; Mott and Lewis, 1991).

Since the fictive grunt train can only be evoked in reproductive males by VPP stimulation or by disinhibition of VPP with a GABA_AR blocker, VPP may be responsible for the generation of the grunt train rhythm which linearly correlates with spontaneous call (grunt) duration, just as burst rate correlates with burst duration in other CPGs (Wallén and Williams, 1984; Fetcho and Svoboda, 1993; Biró et al., 2008). Injections of gabazine into VPP, or a decrease in GABA_AR activity, massively increased fictive grunt duration regardless of vocal or reproductive state, concomitantly with maintenance of intra-grunt frequency, which might vary depending upon season and steroid background. This is in stark contrast to VPN-VMN alone, where gabazine *in state 1 reproductive males* never disinhibited the circuit resulting in spontaneous activity. Rather, in these males, gabazine could shift the

default fast grunt frequency to the slower and highly regular fundamental frequency of the hum, with subsequent, secondary changes in call duration. The evoked fictive grunt was replaced by a perfectly regular, low frequency, long duration hum or bimodal grunt-hum.

In other words, modulation of GABA_AR activity in VPN-VMN primarily controls rapid frequency change that determines call type in reproductive males whereas moderate shifts in local VPP inhibition is permissive for at least grunt duration change, while controlling burst rhythm and rate as revealed by the fictive grunt trains. VPP may also, at least indirectly, contribute to intra-burst frequency control, since microinjections of gabazine into this region can abolish already existing grunt-hums by raising the discharge frequency or essentially removing the slower hum component of the grunt-hum while increasing the duration of the grunt. This demonstrates nucleus specific mechanisms or perhaps semi-independent pattern generators as found in a terrestrial frog that must coordinate calling with respiration (Schmidt, 1992). The hindbrain pretrigeminal nucleus was shown to be primarily responsible for calls, but depended upon reciprocal connections with the pulmonary respiratory generator in motor nucleus IX-X for the alternation of expiratory (calling) and inspiratory (call inhibiting) phases. An interdependent, functionally segregated network also promotes variable output from a vocal motor system that doesn't rely on respiration (Zornik et al., 2010). Semi-independent pattern generators dedicated to particular motor behaviors can be included in the greater category of multifunctional circuits in which anatomically defined circuits reconfigure into distinct functional circuits depending upon state of modulation, synaptic input and plasticity of intrinsic membrane properties (reviewed in Briggman and Kristan, 2006; McLean et al., 2008). A variety of multistable architectures may enable switching between call types distinguished by firing rates and duration, just as complex locomotor systems must

coordinate rhythm generation and speed with activation of appropriate motoneuron pools for different movements.

Rhythms and patterns: comparisons with CPGs for locomotion

Recurrent excitatory interneurons with pacemaker-like properties have been proposed as the source of locomotor rhythmogenesis in the spinal cord of both aquatic and terrestrial species, including the lamprey (Cohen and Harris-Warrick, 1984), zebrafish (Gabriel et al., 2008), *Xenopus* tadpole (Roberts and Tunstall, 1990), mouse and cat (reviewed in Kiehn, 2006). In contrast, crossed inhibition (usually glycinergic) controls left/right alternation and flexor/extensor activity, with an overall slowing of the output frequency (McPherson, 1994; Cangiano and Grillner, 2003) Given the data presented here, a similar organization could be proposed for the midshipman hindbrain VPP nucleus, in spite of the fact that the midshipman vocal circuit is distinguished by a *bilaterally* synchronized vocal motor nucleus that drives the simultaneous contraction of the swim bladder muscles. At this point in time, any conclusions about the anatomical and neurochemical basis for the role of VPP in controlling the grunt train rhythm and determining call duration is purely speculative. However, the fundamental role of inhibition in shifting vocal circuit output, and the spontaneous, oscillatory firing that arises when sufficient levels of inhibition are removed in VPP, hints at the possibility of common properties shared with other rhythmic motor systems or central pattern generators (CPGs), including the most primitive crustacean stomatogastric ganglion and a variety of locomotor circuits in aquatic and terrestrial species (see below).

In the pyloric system of the stomatogastric ganglion, an oscillatory pacemaker neuron produces the circuit rhythm while inhibitory connections affect the rate (Marder and Calabrese, 1996 and refs therein). Rhythmicity in the gastric system, on

the other hand, is an emergent network phenomenon based on reciprocal inhibitory connections as well as electrical coupling. Midline-spanning reciprocal inhibitory interneurons also help to control the alternating activity of a number of locomotor behaviors, including the parapodial flapping of the marine mollusk, *Clione* (Arshavsky et al., 1985), the alternating, segmental motor pattern underlying swimming in tadpoles (Roberts and Tunstall, 1990), lamprey (Cohen and Harris-Warrick, 1984; Cangiano and Grillner, 2005) and zebrafish (Gabriel et al., 2008), as well as mammalian terrestrial locomotion (reviewed in Kiehn, 2006). The swim circuit of *Clione* is similar to the pyloric system, with endogenous pacemaking neurons providing the dominant rhythm while reciprocal inhibition and post-inhibitory rebound shape the final pattern that elevates and depresses the parapodia. In spite of the integral role of inhibition in these various CPGs, with inhibitory commissural interneurons required for left/right alternation or determination of the final output frequency by post-inhibitory rebound, they are not necessary in all species for unilateral rhythmogenesis, as shown in hemisected spinal cord preparations from lamprey, zebrafish, *Xenopus* tadpole, and mice (Cangiano and Grillner, 2003; Soffe, 1989; Bonnot et al., 2002; Bracci et al., 1996; see Kiehn review, 2006). Complete block of glycinergic inhibition in an adult zebrafish preparation or *in vitro* lamprey spinal cord transformed left-right alternation into synchronous bilateral firing (Gabriel et al., 2008; McPherson et al., 1994). In the *Xenopus* tadpole, an ipsilateral inhibitory interneuron was shown to be responsible for both unilateral synchrony and control of burst duration. While a commissural interneuron contributed to both rhythm generation and cycle period through postinhibitory rebound, some rhythm generating capacity also remains after removal of both contralateral and ipsilateral inhibition (Roberts and Tunstall, 1990; Roberts et al., 2008, Soffe, 1989).

Similarly, after blockade of glycinergic inhibition in an intact lamprey spinal cord (Cohen and Harris-Warrick, 1984) or with application of NMDA or microstimulation in a hemisected preparation, it was found that the circuitry controlling the locomotor or burst rhythm is intrinsically and independently rhythmic on either side of the cord (Cangiano and Grillner, 2003; 2005). An “excitatory kernel” or positive feedback between excitatory interneurons with pacemaker-like qualities has been suggested to underlie the rhythmic output, and does not rely upon ipsilateral glycinergic inhibition. Excitatory interneurons and motoneurons tend to fire once per cycle in the hemisected preparation such that the interspike interval of a single neuron is roughly equivalent to the interburst interval, and the recorded ventral root burst reflects partially synchronized population activity. When the hemisected cord was briefly stimulated, spontaneous bursting activity could be recorded for several minutes with a slowing cycle period and increased interspike interval in single recorded neurons, both presumably resulting from a progressive depletion of available glutamate.

The free-running midshipman fictive grunt train bears considerable resemblance to the spontaneous locomotor rhythm evoked in the lamprey hemi-spinal cord. Long-lasting (up to 5 min), spontaneous grunt trains triggered by brief stimulation in VPP in our *in vivo* (not pharmacologically manipulated) fictive call preparation began at a faster rate and progressively slowed with diminishing regularity, suggesting a similar rundown of excitatory transmitter (see Rubow and Bass, 2009). The inability of strong electrical stimuli to elicit more than a few *seconds* of spontaneous bursting in *intact* lamprey spinal cord was attributed to strong crossed inhibition (Fagerstedt et al., 2000) that both controls burst rate and shifts the firing pattern, likely due to post-inhibitory rebound (Grillner et al., 2001). There is evidence for similar levels of ipsilateral and/or contralateral inhibition in VPP, but the

stimulation paradigm that preceded release of the grunt train rhythm (at least five 40 s stimulation trials in medial PAG during a recording period of 60 min) may have promoted longer episodes of spontaneous activity by modulation of upstream input from the PAG (Rubow and Bass, 2009).

Our pharmacologically disinhibited preparation also produced spontaneous grunt trains, however, this initial rhythmic output rarely lasted more than a minute before the cycle period slowed, grunt duration increased, and rhythmic grunts were replaced by irregular long duration buzzes (Fig. 11). In this case, the initial grunt train to buzz phase actually suggests a brief *build-up* of excitability after the removal of inhibition that terminates in a long duration buzz. This phenomenon was also observed in the lamprey hemicord when stimulation was barely above threshold (Cangiano and Grillner, 2005). The conclusion that removal of crossed inhibition (Fagerstedt et al., 2000) allowed electrically stimulated hemicords to fire for longer than intact cords (several minutes as opposed to just seconds), echoes the ability of our pharmacologically, rather than surgically, disinhibited circuit to fire for an hour or more compared to just several minutes in preparations triggered only by electrical stimulation.

From experimental data and CPG modeling in terrestrial mammals where more complex locomotor circuits have evolved to coordinate multiple limbs as well as flexor/extensor activity, principles have been proposed that may also support our data from the midshipman vocal circuit. With experimental data and modeling of the cat locomotor circuit (reviewed in McCrea and Rybak, 2008), it was suggested that instead of multiple unit burst generators distributed along the cord that are responsible for the complete CPG output, each unit may be divided into a rhythm generating layer (RG) and a pattern formation layer (PF). The RG layer controls the basic timing of the motor rhythm or burst rate, and by association the duration of the bursts (see also

Fetcho and Svoboda, 1993 for a linear correlation between burst duration and cycle time), while the PF layer is responsible for the spatial and temporal recruitment of specific motoneuron pools, or *binding synergies*, that coordinate appropriate muscle groups for different behaviors. In the midshipman preparation, we were able to demonstrate that VPP controls a fundamental rhythm, the cycle period of the grunt train, and thereby the duration of the fictive grunt, while VPN-VMN primarily controls the frequency that determines call type and only secondarily the duration of the fictive call. In this way, the vocal CPG could be said to be composed of two levels as in spinal locomotor circuits: the rhythm generator and the pattern generator.

The fictive grunt train with its self-generated, syncopated bursting (1-3 Hz) can only be evoked by disinhibition of VPP in reproductive males, suggesting that this nucleus is the fundamental rhythm generator of the circuit. Modestly decreasing inhibition at this level lengthens the duration of stimulus-evoked grunts while maintaining the average intra-grunt frequency, 110 Hz. This distinctly contrasts with VPN-VMN where decreasing GABA_AR mediated inhibition *decreases* the fundamental frequency of the evoked call while also increasing its regularity until its temporal properties mimic the fictive hum. Increasing GABA_AR activity in this region, on the other hand, supports production and patterning of the higher frequency grunt. This suggests a very different role for this final layer of the vocal pattern generator: fine frequency control (which controls the fine temporal structure of distinct vocalizations) rather than burst rate and duration control (which is equivalent to the gross temporal structure or envelope of natural vocalizations). Frequency and duration are two of the primary units of information for decision-making contained in the natural calls (McKibben and Bass, 1998, 2001). While the proposed PF layer in terrestrial vertebrate CPGs coordinates activity in motoneuron pools or muscle synergies for specific movements, perhaps the “PF” layer in the midshipman is

responsible for frequency shifts that distinguish specific call types, particularly the agonistic grunt vs. the advertisement hum, or the fast (grunt) and slow (hum-like) components of a single growl. GABAergic inhibition at the level of the pacemaker-motoneuron circuit could either serve to dynamically suppress or recruit motoneurons responsible for generating the four call types, or alternatively, may modify intrinsic membrane properties to shift firing modes. Upstream, VPP generates the fundamental grunt train rhythm and together with PAG gates call duration.

Concluding comments

In sum, microinjections of gabazine into VPP suggest a rhythmogenic capability in this nucleus that controls the highly syncopated grunt train. It may be generated by pacemaking cells and/or an excitatory recurrent network similar to the excitatory kernels driving circuit rhythms in locomotor systems. Second, altering inhibition levels in this nucleus can modulate the rhythm as well as the duration of the vocal bursts, just as cycle period and burst duration correlate linearly in fictive swimming. Third, although decreasing the inhibition level in VPP can abolish low frequency hums and increasing it supports them (perhaps involving enhanced post-inhibitory rebound to synchronize and slow firing or modulate left/right alternation), the downstream VPN-VMN region appears to only control intra-call frequency as opposed to the burst rate of the grunt train. Decreasing inhibition in this pacemaker-motoneuron region ultimately abolishes high frequency grunts and promotes low frequency hums in a reproductive male, while increasing inhibition produces the opposite effect.

This study focused primarily on GABAergic inhibition mediated by the GABA_A receptor, but pilot studies also indicated a role for the GABA_B receptor in at least the VPN-VMN region, and glycinergic inhibition in VPP. Future studies should

address the more complex (complementary, antagonistic or redundant) interactions between these different modes of inhibition and their importance in controlling the timing, patterning and synchronization of a multifunctional neuronal network.

CHAPTER 4
PROGESTIN MODULATION OF A VOCAL PATTERN GENERATOR IN A
MALE TELEOST FISH

Abstract

Periodic changes in the environment entrain physiological rhythms, mediated in great part by steroid hormones that help to coordinate reproductive cycles with associated behaviors like male advertisement calling. While androgens and estrogen are sufficient to modulate the vocal motor systems of songbirds and anuran amphibians, additional factors may guide the neurophysiology and vocal behavior of a teleost fish, the plainfin midshipman (*Porichthys notatus*). Central activation of a hindbrain-spinal pattern generator using electrical microstimulation typically produces a brief duration and highly stereotyped rhythmic motor volley known as a fictive vocalization that predicts the temporal properties of natural agonistic “grunts” and can be recorded intracranially from occipital nerve roots (homologous to hypoglossal nerve of tetrapods) that innervate vocal muscles attached to the walls of the swim bladder. We now show that cycling progestins, a hormone not typically associated with male behaviors but necessary for gamete maturation in both sexes, support the rhythmic seasonal and diel production of long duration fictive agonistic growls and advertisement hums. Within 45-60 minutes after intramuscular injection of a teleost-specific progestin metabolite ($17\alpha, 20\beta, 21$ -trihydroxy-4-pregnen-3-one, 20β -S), there is a 20-fold increase in duration of the vocal motor volley that coincides with the transition from brief, higher frequency fictive grunts to the long duration, lower frequency fictive growls and hums. In concert with the nocturnal dependency of 20β -S’s ability to enhance vocal output that reflects the use of these vocalizations during

nocturnal spawning, radioimmunoassays show a daily basal rhythm in plasma progestin levels in spawning males, from non-detectable during the day to 0.37 ng/ml at night. A recently cloned membrane progestin receptor (mPR α) that may mediate the observed 20 β -S effects has been identified throughout midshipman brain by Western blot analysis. Altogether, the above evidence supports a progestin-dependent enhancement of central vocal pattern generator output that directly determines the temporal features of long duration male calls essential to territoriality and female courtship during the spawning season. Support from NIMH (TKR) and NSF (AHB).

Introduction

Progesterone's biological importance extends beyond its role as a female reproductive hormone. Its effects are as diverse as the receptors it binds, whether it is the nuclear progesterone receptor (PR) in the gonads and hypothalamus, the GABA_A receptor, or the more recently described membrane progestin receptor (mPR), responsible for gamete maturation in *both sexes* and identified in central and peripheral tissues of numerous vertebrate species from fish to humans (Zhu et al., 2003a, b). In the males of some species, such as lizards, rats and teleost fish, peripheral progestin effects are supported by central progestin activation of courtship and other sexual behavior (Crews et al., 1996; Mayer et al., 1994; Witt et al., 1995). The rapid neuroactive effects of progestins may be mediated by its own membrane receptor or it may occur via interaction with other neurotransmitters, such as glutamate at the NMDA receptor or the inhibitory GABAergic system (Irwin et al., 1992, 1994; Majewska, 1985). The allosteric modulation of the GABA_A receptor (GABA_AR) by reduced progesterone metabolites can affect not only reproductive behavior, but also mood, anxiety, cognition, aggression, seizure susceptibility and recovery from

traumatic brain injury (Frye and Vongher, 1999; Witt et al., 1995; Van Wingen et al., 2008; Reddy 2004; Van Landingham et al 2006; Kaura et al 2007).

In spite of some significant examples of progestins controlling male sexual behavior as well as female, and a whole suite of gender-neutral functions, detailed mechanistic exploration of steroid modulation in the CNS has tended to divide down gender lines (reviewed in Anderson and Tufik, 2006; Witt et al, 1994). In mammals, rat lordosis has been an important bioassay for the investigation of progestins' rapid facilitation of a female sexual behavior via GABA_AR (Frye, 2001), while androgen and estrogen regulation of the avian song system via nuclear steroid receptors has become the prototype for gradual seasonal and steroid-dependent plasticity in male neural morphology and function (e.g., Arnold et al., 1976; Ball et al., 2004; Brenowitz 2004; Meitzen et al., 2007; Park et al., 2005). In addition, *in vivo* microdialysis has been used to monitor acute changes in neurosteroid levels in song nuclei (Ramage-Healey et al., 2008) while rapid neuromodulation of a vocal motor system by androgens, estrogen and glucocorticoids has been demonstrated in male and female members of the toadfish family that includes the plainfin midshipman (*Porichthys notatus*) (Ramage-Healey & Bass, 2004, 2006).

Exogenous and naturally elevated levels of the teleost-specific, non-aromatizable androgen 11-ketotestosterone (11kT, analogue of dihydrotestosterone found in tetrapods), increase the calling rate of wild male toadfish (Ramage-Healey & Bass, 2005), while plasma concentrations of 11kT and estradiol are elevated in midshipman during their reproductive season (Sisneros et al., 2004). Systemic concentrations of the progestin metabolite, 17, 20 β -21-trihydroxy-4-pregnen-3-one (20 β -S), a gamete maturation inducing steroid (MIS) in some teleost fish, rise to levels above that of 11kT in the Lusitanian toadfish during the reproductive season, and, in addition to another MIS, is essential for the promotion of courtship/spawning

behaviors in at least two other species (Modesto et al., 2003; Mayer et al., 1994; Pankhurst, 1990). Although its role in the midshipman may be confined to the gonads, due to the diverse neuroactive effects of other progestin metabolites in mammalian brain, we hypothesized that 20β -S may affect vocal production in the male midshipman via direct and rapid modulation of the vocal motor circuit.

The fictive correlates of all natural recorded midshipman vocalizations (produced by contraction of vocal muscles attached to the lateral walls of the swim bladder), have been evoked in a neurophysiological preparation that monitors the output of the hindbrain vocal pattern generator in response to electrical microstimulation in previously mapped, upstream sites of the vocal circuit (Bass et al., 1999; Brantley and Bass, 1994; Goodson and Bass, 2000a,b, 2002; Rubow and Bass, 2009). Stimulation in the forebrain (anterior hypothalamus-preoptic region, POA-AH), midbrain (periaqueductal gray or PAG) and hindbrain (VPP) produces a rhythmic vocal motor volley known as a fictive vocalization, the firing pattern of which determines the temporal properties of natural vocalizations. These include the long duration, multiharmonic hum used by nesting midshipman males to court females and two agonistic call types- brief “grunts” (comprising the repetitive grunt train) and long duration, amplitude and frequency modulated “growls” or “grunt-hums”, intermediate in duration between grunts and hums. In an *in vivo* neurophysiological preparation, intramuscular injections of 11-kT, cortisol and estradiol rapidly (≤ 5 min) increase the duration of the briefest fictive grunt at any time of day or year (Ramage-Healey and Bass, 2004), while reproductive state and nocturnal, modestly prolonged electrical stimulation are essential for the induction of much longer duration, lower frequency grunt-hums and hums (Rubow and Bass, 2009).

Our understanding of rapid hormonal modulation of neurophysiology and behavior continues to grow. Investigations of the role of teleost progestins, like 20β -S,

in gamete maturation have not only produced a wealth of mechanistic detail, but also introduced a new mediator of rapid steroidal action, the membrane progesterin receptor (mPR) (Zhu et al., 2003ab; Thomas et al., 2005; Thomas 2008). While its function in the gonads is undisputed, much less is known about its role in the CNS beyond anatomically based suggestions that the mPR α isoform has trophic and neuroprotective actions in the mammalian spinal cord while mPR β , with more limited expression in the motoneurons, may contribute to neuronal activity and plasticity (Labombarda et al., 2010; De Nicola et al., 2009a). Here, in showing that 20 β -S may be one component of the seasonal and diel rhythm that supports the production of long duration calls in male midshipman fish, we also offer an excellent model for further investigation of specific mechanisms underlying rapid neuroactive effects of progestins in the brain.

Materials and Methods

Animals

As described in Chapter 2, type I male midshipman fish (12 cm-20 cm, standard length) were hand collected from nest sites in the intertidal zone of Washington State and California during April-August 2006-2008, and shipped within 6-72 h to Cornell University where they were housed in a 14 light (L):10 dark (D) light cycle with lights out at 17:00 Eastern Standard Time (EST). For further details, see Chapter 2.

Neurophysiological experiments

The fictive vocalization preparation used here, a standard technique in the Bass Laboratory at Cornell University, is thoroughly described in Chapter 2. One is also

referred to Chapter 2 for a more complete review of the hindbrain-spinal vocal pattern generator studied here that directly determines the temporal properties of vocalizations, including duration and fundamental frequency.

Drug dosages and delivery

The progesterone metabolite, 20β -S, is a well-documented maturation-inducing steroid in various teleost species responsible for final oocyte maturation, spermiation and increased sperm motility. Seasonal levels of 11kT and 20β -S in the closely related Lusitanian toadfish, were used to predict relevant plasma 20β -S concentrations in the reproductive male midshipman. Using the optimal 11kT dose (0.2mg/kg) from midshipman studies using androgens and the ratio of 11kT to 20β -S in the toadfish during the breeding season (1:1.625), a 20β -S dosage was extrapolated for the males—0.3 mg/kg. This proved to be the most effective dose. To evaluate the effect of the metabolite on the fictive vocalization, either the steroid dissolved in sesame oil vehicle or oil vehicle control alone, was injected via a pre-inserted butterfly needle into the dorsal trunk muscle of the fish after the initial baseline recording was taken. After the hormone or vehicle injection, a series of vocal motor recordings were collected at 5, 15, 30, 45, 60, 90, and 120 minutes and the changes in duration were normalized against the baseline of each fish (e.g., Fig. 1). Blood samples were taken after each experiment to verify absorption of the steroid and behaviorally relevant plasma levels. Whole blood was separated under centrifugation and plasma and frozen for future RIA analysis.

Radioimmunoassays (RIA)

RIAs were performed in Peter Thomas' lab at the Marine Science Institute, University of Texas, Austin following established protocols. Tritiated 20β -S is

prepared in the lab from 17 α -hydroxyprogesterone. Cross reactivities of the 20 β -S antiserum to 17 α , 20 β -P, pregnenolone, progesterone and 11kT are all less than 0.006%⁵⁹. 100uL aliquots of blood plasma were extracted in hexane/ethyl acetate, dried under a stream of nitrogen gas in a 40°C water bath and then resuspended in 300uL of RIA buffer. Samples were incubated overnight in 20 β -S tracer, antibody and charcoal. The following day samples were centrifuged, the supernatant pipetted into scintillation vials and counted.

Western Blotting

Membrane fractions of tissue were boiled in SDS loading buffer (Laemmli, Bio-RAD) for 5 minutes, cooled and resolved on a 10% SDS-PAGE gel. Samples were then transferred onto a nitrocellulose membrane and blocked for 1½ hours with a 5% milk, 0.1 % Tween 20 solution in tris buffered saline. After incubating membranes overnight at 4°C in a solution with the primary mPR α antibody (1:2000) (gift from lab of P. Thomas), the membranes were soaked in a 1:10,000 dilution of a goat anti-rabbit horseradish peroxidase-linked secondary antibody for 1 ½ hours at room temperature. The receptor proteins were finally visualized with the addition of a chemiluminescent substrate (Amersham).

Statistical analyses

All neurophysiological and statistical analyses essentially follow those of Rubow and Bass (2009) but are outlined here as well for convenience. Briefly, fictive call duration or amplitude were averaged for each time point (0, 5, 15, 30, 45, 60, and 120 min) and normalized against the baseline (0) of each fish. For all treatment groups, each time point is an average of 40 fictive calls from 40 stimulus trains presented at one-second intervals (1 s⁻¹). This stimulation protocol was developed in

this and the concurrent study (Rubow and Bass, 2009) where it was found that grunt-hums are more dependent upon stimulation time than grunts and that 40 s of stimulation or 40 stimulation trains are optimal for revealing the actual vocal capacity of a reproductive fish at any specific time point. Since natural and fictive growls are a hybrid of grunt- and hum-like calls, we refer to them as “grunt-hums”. For duration measurements of grunt-hums, the duration of the initial grunt-like response (≥ 3 pulses) and any subsequent response (≥ 3 pulses) were added for the complete value but did not include the silent gap between the two. Call duration and threshold change (reported as means with s.e.m.) were analyzed in JMP (7.0) using repeated-measures ANOVA followed by planned individual contrast post-hoc tests for between subjects comparisons from 30 to 120 min. Plasma 20β -S concentrations from animals sampled in the field during the day vs. night were compared in Graphpad Prism (5.0) using unpaired t-tests.

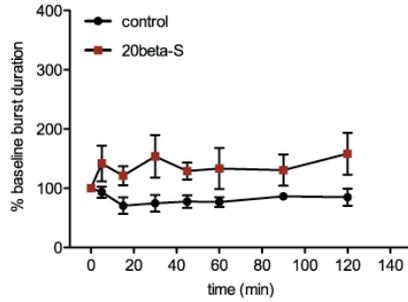
Results

Progestin promotion of fictive vocalizations and a seasonal/diel dependency

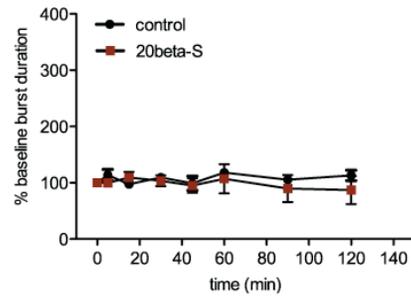
It was recently shown that long duration, low frequency grunt-hums could be evoked at night from type I reproductive males and were dependent upon modestly elevated and prolonged stimulation (Rubow and Bass, 2009). The majority of these fictive calls could only be elicited when at least 100 s of brief (30 ms) stimulation trials (1/s) were presented at 120 min after baseline recordings. In contrast, the duration of brief fictive grunts can be rapidly increased at any time of day or year with systemic injections of 11kT, estradiol or cortisol (Ramage-Healey & Bass, 2004). Now we show that compared to vehicle (oil) controls, the teleost-specific progestin metabolite, 20β -S, also increased grunt duration in *non-reproductive* males across all

time points (5-120 min; $p < 0.0001$). More dramatically, 20β -S enhanced vocal motor output in reproductive males up to 4-fold (Fig. 1A, C). By 45 to 60 minutes post-steroid injection with only 40 s of stimulation trials (1/s), 20β -S facilitated long duration (up to 2-3 fold increase above fictive grunt duration; $p < 0.0001$), low frequency calls that mimic agonistic growls (>250 ms duration) and advertisement hums (>400 ms) (see Fig. 2 for vocal traces and discharge frequency change; Rubow and Bass, 2009 and Chapter 2 for more complete comparison of natural and fictive call types). Fictive growls or grunt-hums were most common and tended to appear by 60 min whereupon duration and patterning continued to strengthen by 120 min. The grunt-hums also exhibited the typical bimodal frequency distribution found in the previous study, with higher frequency grunts (IPI \sim 8 ms) followed by lower frequency hums (IPI \sim 10 ms) (Fig. 2A2, B2, A3, B3). Furthermore, 20β -S could only accelerate and enhance the production of these calls during the fish's night phase when they are naturally most vocally active. During the day, the duration change of fictive calls evoked from 20β -S treated animals was not significantly different than oil controls ($p > 0.05$; Fig. 1B).

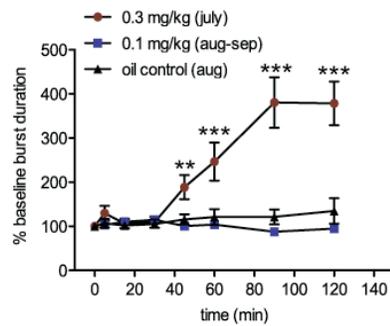
A. Non-reproductive night



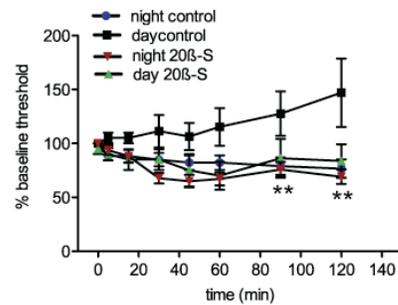
B. Reproductive day



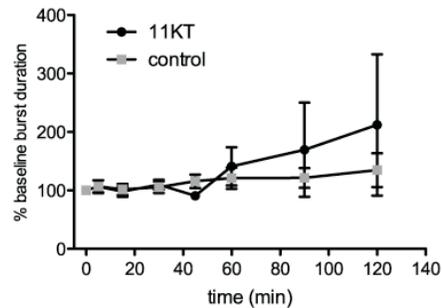
C. Reproductive night



D. Call threshold



E. 11-ketotestosterone at night



F. 11-ketotestosterone thresholds

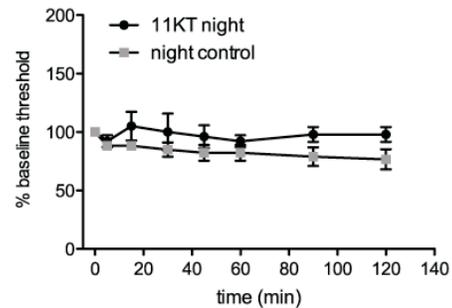


Figure 4.1: Progestin modulation of fictive calls depends upon reproductive condition and time of day. A. In non-reproductive males at night, 20β -S modestly but significantly increased grunt duration across all time points. B. In reproductive males during the day 20β -S had no effect. C. In reproductive males during the night, 0.3 mg/kg 20β -S significantly potentiated fictive calls after 45 min, promoting the production of grunt-hums. D. Oil controls and 20β -S treated fish at night exhibited a significant drop in threshold by 90 and 120 min compared to the day animals. Progestin-treated day animals also showed a significant drop in threshold at 120 min compared to untreated day animals, but were not significantly different from their treated night counterparts. E. While 11-ketotestosterone can increase grunt duration in males during the day (see refs in text), it did not have a significant effect on reproductive males at night and neither affected call threshold (F).

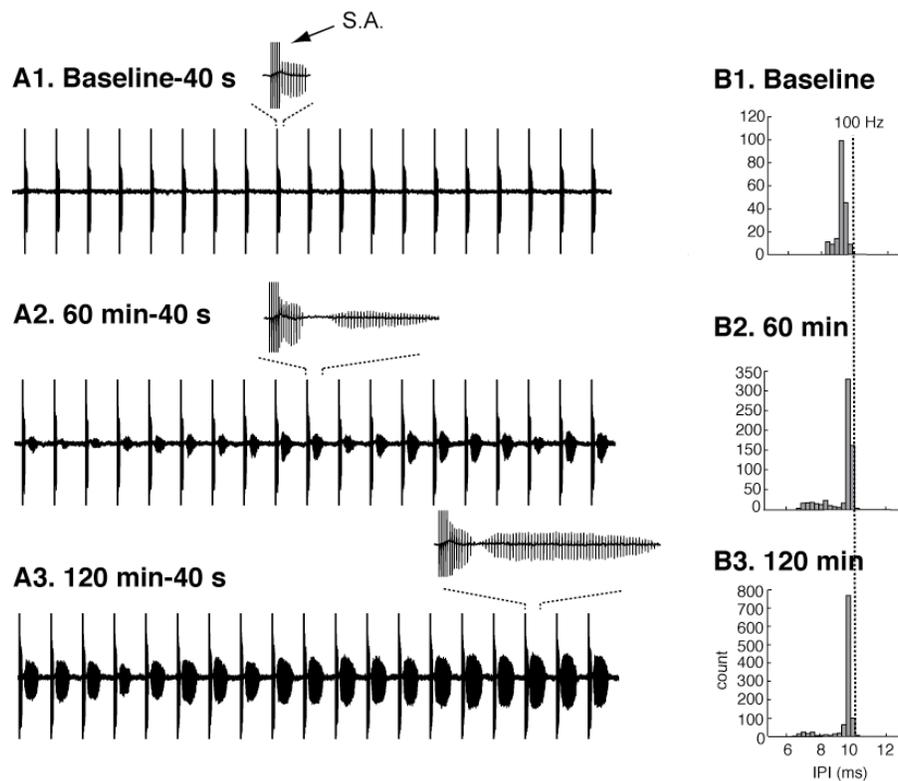


Figure 4.2: Fictive call stimulus-response trains from a 20β -S treated, reproductive male at night with accompanying IPI histograms. A1-A3. Example of progestin facilitation of grunt-hums in one male that followed every stimulus after 20 s of stimulation at 60 min post-injection, and increased in amplitude and duration by 120 min. B1-B3. Histograms of interpulse intervals (IPI) depict a shift to the bimodal and lower mean firing frequency that distinguishes grunt-hums from grunts alone.

The probability of evoking grunt-hums was as dependent upon reproductive state and time of day in 20β -S treated fish as it was in untreated reproductive males from the earlier study (Rubow and Bass, 2009). While progestin treatment removed the requirement for prolonged stimulation (100 s of stimulus trains at 1/s vs. 40 s) and accelerated the arrival of the long duration calls (as early as 45 min post baseline vs. 120 min in untreated reproductive males)—the effectiveness of 20β -S was still augmented by the same, maintained slight *increase* (~ 25 to $50\mu\text{A}$) in stimulus current

above threshold, such that raising or lowering of stimulation intensity during a recording allowed switching between call types. Thus, in spite of a consistent and significant drop in stimulus current threshold just before the first grunt-hum began around 45 minutes post steroid injection, the probability of evoking this call and its ability to follow the stimulus were increased by the slight elevation in stimulus current. Although the decrease in stimulus threshold preceded or coincided with longer calls in this study, it most significantly correlated with time of day, such that night controls and 20 β -S treated fish exhibited a significant drop in threshold by 90 and 120 min compared to the day animals (Figure 1D; $p < 0.001$). Progestin-treated day animals also had a significant drop in threshold at 120 min compared to untreated day animals ($p < 0.05$) but were not significantly different from their treated night counterparts ($p > 0.05$). The pattern is suggestive of a diurnal rhythm in call threshold that is slightly augmented by central progestin levels, but due to considerable variation within the treatment groups, this analysis requires an increase in sample size to be more conclusive.

Steroid-specificity

Injections of the androgen, 11-ketotestosterone (0.2 mg/kg), which as previously reported can induce up to 2-fold increases in fictive grunt duration during the day in either reproductive or non-reproductive animals, did not evoke grunt-hums in four animals tested at night and neither increased grunt duration compared to controls (Fig. 1E; $p > 0.05$). However, long duration calls were evoked from a fifth fish at 90 and 120 min. These experiments raised the currently irresolvable problem of finding adequate controls for these progestin data. Since a small percentage of untreated fish are able to produce grunt-hums, some even with minimal stimulation, it is impossible to conclusively attribute the generation or lack of this call to any

pharmaceutical treatment. Exploratory trials with the C21 progestins, progesterone and allopregnanolone were abandoned for this reason. Without yet access to specific agonists or antagonists for the 20β -S receptor, mPR, and the ability to block the production of grunt-hums, any demonstration of steroid-specific promotion of long duration, low frequency calling, remains on hold.

Call threshold change in the 11KT-treated animals was not significantly different from night or day control groups at any time point ($p > 0.05$), rather, falling somewhere in between the two treatment groups (Figure 1F). The one animal from which grunt-hums were evoked, not surprisingly, did exhibit a threshold drop immediately preceding the appearance of the long duration calls, while the others were simply constant or rose slightly. Again, the grunt-humming animal introduced the variability in the data, and as already suggested for the threshold analysis of control and progestin-treated animals, a larger sample size is necessary to draw any conclusions. Furthermore, the 11KT experiments were conducted at the end of August when type I males are losing the reproductive priming of their vocal system while the control animals were tested between June and early August. The rhythm in excitability, or more specifically, sensitivity to stimulation, may simply flatten as the fish leave their reproductive state.

Plasma 20β -S concentrations and central membrane progestin receptor (mPR α) expression

Although we have previously demonstrated that fictive growls (grunt-hums) and hums can be evoked from reproductive males at night without steroid treatment, 20β -S injections clearly enhanced this vocal rhythm and thus may be one natural component of the periodic changes in the vocal motor circuit that support reproduction. We compared 20β -S concentrations in plasma taken from reproductive

fish during the night and day and investigated the expression of one receptor that may mediate the progestin's effects, mPR α .

Plasma 20 β -S concentrations cycle with nocturnal calling

20 β -S concentrations in plasma collected from fish in the field and at the end of neurophysiological experiments were measured by radioimmunoassay. Fish sampled within two days of collection from nest sites had a mean 20 β -S plasma concentration of 0.36 ng/ml \pm 0.03 at night and undetectable levels during the day (Fig. 3A). Since these are nocturnal fish, wild-caught, reproductive males were expected to have diel fluctuations in circulating plasma progestins, opposite to the daily cycle found in diurnal damselfish whose levels peak in the daytime. Midshipman males given 20 β -S intramuscular (dorsal epaxial) injections of 0.3 mg/kg at night that induced grunt-hums had a mean plasma concentration of 19.8 ng/ml \pm 3.4, while lab controls given oil injections had a mean plasma concentration of 0.41 ng/ml \pm 0.05.

CNS mPR α expression

Western blots of reproductive type I male, membrane-fractionated forebrain, midbrain, and hindbrain-rostral spinal cord (site of VPG) with an antibody to seatrout membrane progestin receptor (stmPR α) produced approximately 80 kD bands, the receptor dimer. This result is similar to mPR α Western blots with Atlantic croaker sperm and testes, goldfish ovary, human sperm and mouse testicular membrane, and thus confirms the presence of mPR α in midshipman CNS.

A. 20 β -S radioimmunoassays B. mPR-like immunoreactivity

Treatment group	[20 β -S] ng/ml	
20 β -S (n = 8) (0.3 mg/kg)	19.8 \pm 3.4	
oil control (n = 8)	0.41 \pm 0.05	
field night (n = 5)	0.36 \pm 0.03	
field day (n = 5)	ND	
intrassay %CV	0.1 %	
interassay % CV	15 %	

Figure 4.3: 20 β -S concentrations in plasma and mPR-like immunoreactivity in three brain compartments. A. Plasma from wild-caught males sampled during the day vs. night exhibited a diel rhythm in 20 β -S concentrations. B. Western blots of reproductive type I male, membrane-fractionated forebrain, midbrain, and hindbrain-rostral spinal cord (site of VPG) with an antibody to seatrout membrane progesterin receptor (stmPR α) produced approximately 80 kD bands, the receptor dimer.

Summary

In sum, 20 β -S accelerates and potentiates the production of long duration fictive calls in reproductive males at night and is paralleled by a daily rhythm in plasma 20 β -S concentrations. Altogether this implies that a diel rhythm in progesterin concentrations is one component of the periodic shift in vocal pattern generator excitability that permits the production of complex and longer duration growls and hums. This progesterin cycle may coordinate peripheral gamete maturation and sperm motility with central activation of social calling to most effectively promote reproductive success. The diel and seasonal dependency of the progesterin facilitation is consistent with the nocturnal habits of midshipman and the use of these vocalizations during spawning.

Discussion

The teleost progestin 20β -S is a potent modulator of fictive vocalizations in the type I male midshipman, with a *potentiating* rather than inhibitory effect. Typically progestins are assumed to have anti-androgen effects in males and to oppose or synergize with estrogenic effects in females. More accurately, physiological levels of both progestins and androgens, as well as estrogen, may be necessary for the full complement of sexual behavior in males just as progestins and estrogens *jointly* (antagonistically or synergistically) control reproductive and receptive behavior in females. Two regions of the rat preoptic area are sexually dimorphic in their expression levels of PR, with males exhibiting a much higher, estrogen-controlled expression than females (Quadros et al., 2002). There is also a strong circadian pattern of progesterone secretion in male rats, peaking at night with their nocturnal activity just as we have shown here with the nocturnal midshipman male (Lauber et al 1991; Kalra & Kalra 1977).

When injected at night in reproductive male midshipman, 20β -S induces, by 45 to 60 minutes post-steroid injection, an up to 2-3 fold increase in fictive call duration, essentially facilitating and accelerating the transition from grunts to grunt-hums. Natural and fictive hums differ from grunts not only in duration but also in their significantly lower, and highly regular fundamental frequency or pulse repetition rate. So while 20β -S and $11kT$ rapidly (≥ 5 min) induce up to a 2-fold increase in grunt duration, maintaining an average frequency of 115 Hz at $16^{\circ}C$, only 20β -S accelerates the more dramatic shift into long duration grunt-hums or hums that are distinguished in part by their lower and more regular frequency, ~ 90 -100 Hz at $17^{\circ}C$.

Importantly, evocation of these long duration calls was entirely dependent on the animal's reproductive state and the time of day, just as it was in untreated animals. Nocturnal spawning and increased vocal behavior in wild midshipman is consistent

with an overall lower stimulus threshold for induction of any call type in a night fictive call preparation. However, it does not appear that 20β -S is the sole driver of this diel shift in excitability, or sensitivity to activity-dependent upregulation of VPG function, rather the steroid may simply support or augment concurrent physiological changes controlled by other factors. An injection of 20β -S during the day did not evoke the longer calls in reproductive males, but appeared to permit a slight drop in call threshold by 120 min compared to day controls. Although 20β -S could increase fictive grunt duration at night in winter-trawled, non-reproductive animals approaching the spawning season, it could not remediate the captivity-induced quiescence of reproductive state seen in animals collected from nesting sites in California and held for more than 4 weeks (data not shown). Type I males only remained sensitive to the progestin's effects for at most three weeks before they subsided into a non-humming, wintry physiological condition, at which point injections of 20β -S could not evoke fictive grunt-hums or hums. Obviously and not surprisingly, the production of the very long duration, lower frequency fictive calls depends upon a more complex suite of physiological changes than simply the elevation of one steroid hormone.

As noted in Rubow and Bass (2009), which described the electrophysiological parameters of the long duration fictive calls, the progestin-facilitated grunt-hums are also dependent on a stimulus intensity slightly elevated above the very low threshold current that evokes fictive grunts at night. Even when the threshold fell 30-45 minutes after the baseline recording, the longer fictive calls depended upon a minimum current of $175 \mu\text{A}$; lower than which only fictive grunts were evoked. By the same token, *too* much current degraded the vocal bursts, reducing overall duration or causing massive firing followed by a refractory period of about 20 s during which no calls could be evoked.

Mechanism of action

Identification by Western blotting of the recently cloned G protein-coupled membrane progesterin receptor (mPR α) in midshipman brain, including the hindbrain-spinal region containing the vocal pattern generator, suggests that mPR may mediate the rapid and sustained, progesterin-dependent changes in fictive vocal output. The presence of mPR α in peripheral reproductive tissues and its role in gamete maturation has been extensively described in teleost fishes (reviewed in Thomas et al., 2004), while both mPR α and another isoform, mPR β have been identified in the mouse spinal cord where it is suggested, based on anatomical location, that they have both trophic and neuroprotective roles (mPR α) and contribute to neuronal activity and plasticity (mPR β) (Labombarda et al., 2010). Much less is known about the role of either receptor in the brain although they have been identified in the rat hypothalamus, cortex and hippocampus as well as throughout the teleost brain (Tischkau et al., 1993; Kazeto et al., 2005). In the female rat PGMRC1 is assumed to facilitate sexual behavior along with the nuclear receptor, PR, although it has not yet been directly demonstrated nor the mechanism characterized (Krebs et al 2000). In fish oocytes, mPR activates an inhibitory G protein to decrease cAMP levels or may initiate MAP kinase activity, while in sperm the olfactory G protein is activated to increase cAMP and intracellular calcium concentrations (Tubbs and Thomas, 2009). Three mPR isoforms have been cloned, with mPR α represented in peripheral reproductive tissues as well as in the brain and mPR β predominantly found in the brain and spinal cord (Zhu et al 2003ab). However, its role there remains mostly unexplored.

Any of the well-documented, peripheral downstream effects of mPR activation by progestins could be taking place in the brain. However, none have yet been demonstrated making the possibility that mPR mediates 20 β -S facilitation of fictive

calls in midshipman an exciting possibility because of the accessibility of this *in vivo* preparation to detailed pharmacological and neurophysiological study in an *intact, awake* animal. Unfortunately, in spite of the expectation that brains from non-humming experimental animals (such as those in captivity past three weeks), or brains from daytime field-collected males, which are vocally less active, would show lower receptor concentrations than that found in brains collected from nighttime field males, we could detect no consistent differences between these animals. If, hypothetically, mPR concentrations in the brain were static and daily/seasonal fluctuating plasma levels of 20β -S controlled the physiological potential of the vocal circuit, then exogenous injections at any time would be sufficient to evoke grunt-hums. This was not the case either. Nonetheless, adequate levels of progestin and appropriate concentrations of the membrane receptor may simply be *necessary* for the production of the long duration fictive calls, but not *sufficient*, and thus cannot be used as a quantifiable, post hoc explanation for a fish's ability or inability to produce grunt-hums in the neurophysiological preparation.

Another, better documented, non-genomic mediator of neuroactive progestins' effects in the brain is the GABA_A receptor. The allosteric modulation of this receptor by progestin metabolites, such as allopregnanolone, has been thoroughly described in mammals, but also documented in frogs and zebrafish (Lambert et al 2003; Hollis et al 2004; Renier et al 2007). There is extensive GABA immunoreactivity in the midshipman vocal motor nucleus and physiological evidence of considerable GABAergic innervation in the vocal pre-pacemaker nucleus (VPP) as well, making GABA_AR another intriguing candidate receptor for 20β -S activity in the midshipman brain (Marchaterre et al., 1989; A.H. Bass and J. Zee, unpublished data). Chapter 3 also presents evidence for GABAergic modulation of fictive calling following

microinjections of GABA agonists and antagonists in VPP and the vocal motor nucleus.

Peripherally and centrally synthesized progestins “fine tune” the GABAergic system in a region-specific, neuron-specific and even receptor-specific manner across vertebrate species (Belelli et al., 2002, Biggio et al., 2006; Biggio et al., 2009). Local enzymatic activity controls the concentration of progestin metabolites in specific nuclei while GABA_AR subunit composition (partially regulated by progestins) determines the degree of sensitivity to neurosteroid modulation and thereby the exact location of progestin modulation, synaptic vs extra-synaptic (Herd et al., 2007; Mitchell et al 2008). Cycling female rats exhibit significantly less edema following brain injury than males while exogenous treatment with progesterone and especially allopregnanolone in males or females significantly improves recovery, presumably exerting its neuroprotective effect via GABAergic modulation. Thus progestins affect GABAergic activity in both sexes and influence a wide range of functions.

Nonetheless, for all the detailed analysis of androgen and estrogen modulation of the vocal motor circuit in song birds, anuran amphibians and of course, in toadfishes, as well as the anatomical and physiological evidence for pervasive GABAergic innervation in particular song nuclei--until now there has been no demonstration of a progestin influence on vocal motor output in any animal, male or female (Spiro et al., 1999; Vicario and Raskin, 2000). It simply may never have been tested. GABAergic inhibition patterns and synchronizes excitatory activity in the brain, from the cortex and the hippocampus to the reticular formation of the hindbrain (Buzsaki and Chrobak, 1995; Mann and Paulsen, 2007; Smotherman et al., 2006). Depending upon receptor subunit composition or the local synthesis or sequestration of neuroactive steroids, this GABAergic tuning or control of rhythmicity in the brain may or may not be modulated by progestins. Considering the extensive GABAergic innervation of the

hindbrain vocal motor nuclei and the at least diel cyclicality of 20β -S plasma concentrations in breeding season males, the daily rhythm of call type production may be controlled by shifts in GABAergic activity modulated by this teleost progesterin metabolite.

Conclusions

With identification of the recently cloned membrane progesterin receptor (mPR α) in midshipman brain, including the hindbrain-spinal region containing the vocal pattern generator, mPR is a candidate mediator of the rapid and sustained, progesterin-dependent changes in fictive vocal output. Alternatively, since various progesterin metabolites act as neuromodulators of the GABA $_A$ receptor and GABAergic interneurons often organize and synchronize rhythmic brain activity, 20β -S may facilitate the production of long duration fictive growls and hums via modulation of GABA $_A$ R. If the effects of 20β -S are mediated by GABA $_A$ R, then this is a novel demonstration of progesterin modulation of a vocal pattern generator or oscillatory network controlled by GABAergic inhibition. Alternatively, if mPR proves to be the primary receptor, this study will for the first time, link the presence of this membrane receptor in the CNS to modulation of dynamic, context-dependent social behavior, in this case, vocalizations.

CHAPTER 5

CONCLUSIONS

When the sun surrenders to the Pacific fog and night darkens the rubbled beaches, one homely fish drones a peculiar mantra--om mani padme HUM. How many rhythms converge in this teleost, in the buoyancy and unbearable lightness of its hums? Out of the three investigations presented here--seasonal/diel dependency of long duration calls, dynamic GABAergic modulation in the vocal motor circuit that promotes call switching, and the possible promotion and maintenance of this ability by the teleost progesterin, 20β -S--there did in fact arise one consistent and immutable theme that united the data. Whether the modulation and enhancement of vocal circuit function was provided by prolonged stimulation (activity dependence), central, focal injections of GABA agonists or antagonists, or systemic injections of 20β -S--the evocation of the more complex, long duration fictive calls, which include the hum, the growl or “grunt-hum”, and the grunt train, almost consistently depended upon reproductive state and time of day. None of the other manipulations were sufficient to override this limitation, *unless, the photoperiod itself was altered*. The 24D/24L treatments appeared to tap a primary, rate-limiting step in the up-regulation of the vocal motor system. Whatever the five days of darkness invoked, it was a governing factor for the tuning of the circuit, while five days of 24-hour light nearly shut the circuit down. *However*, 24D enhancement of vocal output was only effective in reproductive males that had not been in captivity much past a month. Thus, photo-manipulation may be a magic bullet for controlling the diurnal rhythm, but could not control the longer reproductive cycle or subvert the central quiescence that occurs in midshipman after prolonged captivity. The figure below provides an overview of some

of the important environmental and physiological factors that this research attempted to address. Multiple layers of reciprocal interactions regulate the functioning of a seasonally, daily and inherently rhythmic vocal neural network.

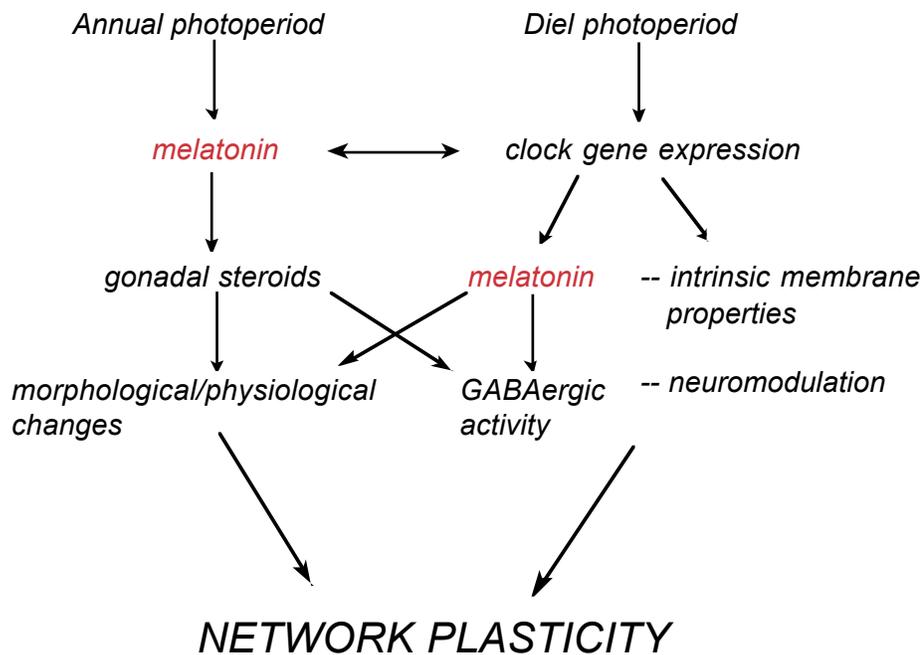


Figure 5.1: Summary of actual and hypothesized molecular, hormonal and neurological factors driving photoperiodic changes in the midshipman vocal motor system.

Seasonal and daily plasticity in a vocal network

Unlike many studies that employed photoperiodic manipulations to reveal endogenous circadian rhythms in teleosts and other vertebrates (Cahill, 2002; Vansteensel et al., 2008 and refs therein), neither 24D nor 24L unmasked a significant neurophysiological rhythm in the midshipman that could be attributed to daily clock gene expression. However, there was a trend for a lower call threshold during the circadian night vs. the circadian day that may have simply lacked statistical power (only three animals per photoperiod treatment group). If this suggestion of an

endogenous rhythm in membrane potential ever bore out, it would compliment the data from reproductive animals housed in 14L: 10D, and begin to pinpoint what governs the significantly lower call threshold in experimental animals at night compared to day. The very significant increase, on the other hand, in long duration fictive calling from 24D males, day and night, raises the possibility of tonically increased melatonin secretion and activity in the brain as has been found in other fish (Pavlidis et al., 1999).

Midshipman confine the majority of their vocal behavior to the dark, and thus their expected nocturnal increase in melatonin secretion, as in all other animals, could well indeed contribute to the enhancement of vocal motor functioning, concomitantly with a change in receptor expression. In nocturnal and highly vocal Atlantic croaker, melatonin increases gonadotropin secretion in the early dark phase to promote spawning and perhaps even facilitate the vocal “drumming” that precedes it (Khan and Thomas, 1996). Melatonin’s dual role as a circadian and seasonal timer, however, can complicate this interpretation. It would be a reasonable prediction that in the midshipman, as in other long day-breeding vertebrates, long nights and prolonged elevation of circulating melatonin are a hormonal signal for winter and the non-reproductive season. How then does one reconcile that mechanism with the proposal that 24 hours of darkness and a tonic elevation in melatonin *stimulates* the vocal system for advertisement calling during spring/summer spawning? If their response to increased melatonin secretion varies depending upon season or reproductive condition (Khan and Thomas, 1996; Goldman, 2001), then melatonin’s antithetic effects may be explained by changes in the encoding of its photoperiodic signal. Although attempts to assay plasma concentrations of melatonin in wild-caught male midshipman sampled day and night did not produce conclusive results, likely because the day samples were taken when melatonin levels were already rising again for the night (A. Bass, N. Feng,

M. Marchaterre, T. Rubow, unpubl observ), it would be highly unusual for this fish to not exhibit the same diurnal melatonin rhythm that has been documented in every other investigated species. Future assays should confirm the expected daily rhythm that would correlate with the experimental results, and perhaps reveal that 24D tonically increased plasma or central melatonin concentrations while 24L damped it.

Short-term activity dependent changes in the vocal circuit depended upon a system already tuned by reproductive condition and time of day. No amount of prolonged stimulation in the medial PAG of a winter-trawled male could evoke a hum, a growl or a grunt train. But come spawning season and nightfall, hormonal, chemical or synaptic changes in the brain rendered it more plastic or responsive to this treatment. Indeed, the occasional fish at night didn't even require extra stimulation to produce hums and already arrived in an ardently operatic state. The possible mechanistic underpinnings are many and there is little point in discussing them all here. However, some obvious hypotheses do arise from these studies beginning with the modulation of neuronal function by a circadian system that includes rhythmic melatonin secretion.

If melatonin increases in the brain at night or during darkness in general, and supports the production of long duration calls, namely the hum, there are several possible mechanisms demonstrated in other species that also need to be explored. Melatonin has been implicated in the functioning of the zebra finch and house sparrow song systems on both a seasonal and daily basis (Bentley et al., 1999; Cassone et al., 2008). Pinealectomized sparrows housed in 24L receiving long duration melatonin injections (simulating winter) had enlarged testes but reduced HVC and RA volumes, suggesting the melatonin can directly affect song nuclei structures independent of the gonads. HVC, RA and the hypoglossal motoneurons all express melatonin receptors (see Jansen et al., 2005 for refs). Application of melatonin to brain slices increased

firing rate of RA neurons, while systemic injections of an antagonist altered song, motif and syllable lengths produced the next day (Jansen et al., 2005). Serendipitous to the midshipman studies, the melatonin receptors cloned in rats, Mel_{1a} and Mel_{1b}, differentially modulated the GABA_A receptor in the SCN and hippocampus, respectively (Wan et al, 1999). Mel_{1a} increased GABA_AR mediated currents and Mel_{1b} decreased them, while in another study, bath-applied melatonin increased firing rates of neurons in the CA1 region of the hippocampus (Musshoff et al., 2002). Another study showed that chronic melatonin treatment increased GABA binding and turnover rate in the hypothalamus, and may affect circadian rhythmicity by modifying GABAergic activity in the SCN (Rosenstein and Cardinali, 1990). Thus it is highly possible that melatonin, mediated by its own G-protein coupled receptors and/or via interactions with GABA_AR, contributes to the vocal motor plasticity already demonstrated here. Future studies could further explore the effects of systemic and central injection of melatonin (along with antagonists for GABA_AR and Mel receptors) in the fictive call preparation, as well as determining central melatonin receptor distribution, in photo-manipulated fish as well as those recently wild-caught and exposed to ambient light conditions. It is possible that melatonin modulates the functioning of the GABA system in a periodic manner thereby altering its response to increased circuit activity. Whether it acts in concert with central or distributed clock gene control of other factors controlling VPG properties, however, remains to be seen (reviewed in Goldman, 1999).

While the teleost SCN has not been shown to contain a biological clock as it does in mammals, robust clock gene rhythms are produced in the pineal and retina as well as in peripheral tissues of the zebrafish (Cahill, 2002), contributing to rhythmic melatonin secretion in this fish as well (Cahill, 1996). The rainbow trout lacks an endogenous melatonin rhythm, but its receptors have been indentified throughout

areas of the brain associated with the visual system, in neurons co-labeled by *clock* and GAD (glutamic acid decarboxylase) probes (Mazurais et al., 2000). The 24D/24L experiments with midshipman also suggest that some aspects of their vocal motor activity are directly controlled by light rather than by any endogenous rhythm, however, this does not rule out other forms of circadian rhythmicity in these benthic, migratory, humming male toadfishes. There may yet be reason to believe that endogenously rhythmic local *clock* genes, along with light-controlled melatonin, contribute to the daily and seasonal rhythms in vocal motor function.

On a daily basis, oscillating clock gene expression could regulate cell membrane excitability in the vocal motor system by controlling expression of enzymes, ion transporters or channels. Potassium and calcium currents are obvious targets for rhythmic regulation of CNS excitability because of their contribution to the membrane oscillations that give rise to spontaneous action potentials; most specifically the currents associated with L-type Ca^{++} channels and BK or SK potassium channels. There is no better example of this than in the SCN itself, where there is a diurnal fluctuation in calcium current that contributes to the oscillating membrane potential underlying greater spontaneous spiking during the day (Pennartz et al., 2002, Teshima et al., 2003, Meredith et al., 2006). The large conductance calcium-activated potassium channel, BK, whose increased expression at night leads to a decrease in nocturnal firing rate is also, of course, dependent upon calcium, rendering the rhythms of these two currents inextricably intertwined. Local clock gene expression can also control neuromodulator function as it does with dopaminergic transmission in the mouse ventral tegmental area (VTA) (McClung et al., 2005). Homozygous *Clock* mutant mice displayed an increase in cocaine reward and in the excitability of dopamine neurons in the ventral tegmental area, associated with increased expression and phosphorylation of tyrosine hydroxylase (TH), the rate-

limiting enzyme in dopamine synthesis. There is an E-box containing enhancer element upstream of the TH gene by which CLOCK can affect its transcription. This is potentially interesting in that earlier immunohistochemistry performed in the Bass lab revealed considerable TH reactivity around the vocal motor nucleus (Bass et al., 2001), suggesting that dopamine is potentially one neuromodulator rhythmically and hierarchically tuned to regulate the vocal motor circuit.

Melatonin, in its transduction of photoperiod into a seasonal and daily timing mechanism, is both a first messenger in the brain and a conductor of the rhythms of gonadal steroid secretion. Already much is known about the steroid-based seasonal changes in the midshipman reproductive anatomy, physiology and behavior. As testes recrudescence in males in preparation for spawning, there is a concomitant hypertrophy of the swim bladder muscle that is paralleled by androgen and estrogen-induced changes in the vocal motor and auditory systems (Sisneros et al., 20004a; Sisneros et al., 2004b; Ramage-Healey and Bass, 2004, 2006, 2007). The non-aromatizable, teleost androgen, 11kT, contributes to vocal muscle growth (Brantley et al., 1993) and a rapid increase in duration and call rate of toadfish vocalizations (Ramage-Healey and Bass, 2005, 2006), no doubt by binding androgen receptors identified throughout the midshipman brain with concentrations in specific vocal nuclei (Forlano et al., 2010). Studies with other fishes, demonstrate not only an androgenic contribution to spawning behaviors, but also and inseparably, a role in the territorial and aggressive behaviors that accompany them (Antunes and Oliveira, 2009). 11kT levels can peak in pre-spawning states in relationship to spermatogenesis, or remain high until the end of spawning (Modesto and Canario, 2003 and refs therein; Barnett and Pankhurst, 1994). Unbeknownst to many of the androgen-centric, teleost progestins like 20 β -S and 17, 20 β -P are also crucial for gamete maturation and spawning behaviors in males and females (Thomas et al., 2004; Modesto and Canario, 2003; Mayer et al., 1994). In the

Lusitanian toadfish, 20β -S remains elevated throughout the spawning season, while in damselfish it even exhibits a daily rhythm that mirrors their diurnal mating behaviors. In another study with rainbow trout, it was the progestin that was found to be crucial for the stimulation of spawning behaviors, not 11kT (Mayer et al., 1994).

Exploring the effects of progestins on midshipman fictive calls was a logical conclusion to the investigation of other steroid effects on the vocal motor system and the social vocalizations that are a key component of their reproductive behavior. It also offered a new opportunity to look at central, *neurophysiological* activity of progestins in a fish, since up to now, most or all of the research has focused on endocrine or peripheral gonadal effects.

When the first spring-collected midshipman rolled into the lab in 2006, and systemic progestin injections in type I males at night appeared to magically invoke growls and hums in less than an hour, we concluded with great alacrity that this was new evidence for a rapid steroid effect, acting via a recently cloned membrane receptor, mPR, or through neuromodulatory associations with $GABA_A$ R as long demonstrated in mammals with other reduced progestins (Zhu et al., 2003; Frye and Rhodes, 2007; Biggio et al., 2006). Something indeed was going on, but it was not to be so linearly causal or as black and white as we could have hoped. As time and fish passed, it became increasingly clear that a nocturnal reproductive state was sometimes sufficient to promote induction of these long duration calls, *without* progestin injections. Even worse, there were also fish that didn't or couldn't respond at all to the injections, even during a summer night. Clearly the generation of growls and hums depended upon a more exquisitely delicate regulation of the vocal pattern generator and even upon upstream PAG and hypothalamic inputs. If one crucial, physiological step failed to take place, it was enough to condemn the fish to monosyllabic grunting. We didn't know all the necessary factors and without an mPR antagonist, we couldn't

even determine the importance or not of the progesterin. Did seasonal regulation of gap junctions matter too? Or dopaminergic neuromodulation of the motor nucleus or upstream inhibition by vasotocin triggered by the fish's stress levels? In an *in vivo* preparation, even in one purportedly investigating simple, "stereotyped calls", there is nothing stereotyped in the sum total of the fish's behavior and physiological state. The progesterin experiments were humbling, to say the least and nothing so simple as injecting mass-produced *Xenopus* with gonadotropins to allow the generation of advertisement calls. Radioimmunoassays performed in the laboratory of Peter Thomas did successfully demonstrate a daily rhythm in plasma 20β -S concentrations from wild sampled fish from nest sites in Tomales Bay, CA, but subsequent efforts to use Western blots to compare receptor expression in non-reproductive vs. reproductive fish or reproductive day vs. night were--short of proving the existence of the receptor in the midshipman brain--inconclusive.

There are several possible explanations for this: 1) the subdivided brain compartments: forebrain, midbrain, cerebellum and hindbrain, were too large to discern any finer local rhythms in receptor expression, or 2) so-called "non-reproductive" animals came from trawls in California in the earliest spring when midshipman have already begun gonadal recrudescence and preparation for the spawning season and thus were not truly non-reproductive, or 3) mPR expression is generally static, while it is the hormone that fluctuates. The absence of a daily receptor rhythm is almost believable, but not the lack of any seasonal flux since all of Peter Thomas' work has shown that mPR expression was increased in oocytes and sperm from various fish species collected from their spawning grounds and that progesterins as well as gonadotropins increase mPR expression during gamete maturation (Thomas et al., 2004; Tubbs et al., 2010). One could offer a last caveat that perhaps mPR expression doesn't change in the brain as it does in the gonads, but this would be odd

and rare considering all the evidence for increased central steroid receptor expression in reproductive birds and mammals (Soma et al., 2009; Haywood et al., 1999).

Finally, even the experiments intended to investigate whether 11kT could also promote growls and hums were flawed. Robust growls were easily evoked by 60 min from one out of five tested males at night, casting doubt on the actual mean outcome of the experiments. Instead of injecting 11kT into males at night, males that we had already seen were occasionally capable of growling and humming without exogenous steroids (due possibly to already high central or systemic levels), we should have injected the androgen receptor *blocker*, cyproterone acetate. That would have also been the kind of test the progestin-treated fish needed to help confirm a direct relationship between that steroid and evoked hums, and yet, even then, there were always the reproductive night fish (in all three studies) that simply couldn't or wouldn't hum. We didn't find the master key to more complex, long duration calls because there probably isn't one.

The other proposed mechanism for progestin activity in the midshipman brains was through neuromodulation of the GABA_A receptor, much as allopregnanolone and other reduced progestins upregulate this receptor's function in mammals, and even frogs. Unfortunately, there is no evidence yet that fish even synthesize allopregnanolone or that their teleost-specific progestins have any affinity for GABA_AR. A series of experiments injecting allopregnanolone into reproductive males at night were as inconclusive as the 11kT assays--some hummed, some didn't. So at the end of the day, or night, the only solid conclusion remains that the vocal motor system in type I midshipman males is up-regulated at night during the reproductive season to promote generation of the vocalizations crucial for spawning success. Period. We don't know which of the steroid hormones if any, contributes most, but undoubtedly at least one does and 20 β -S is still a logical and intriguing choice.

The more straightforward success of the GABA study that revealed such an integral role for GABAergic inhibition in the timing and patterning of fictive calls, renews the tantalizing possibility that progestins do indeed interact with the GABA system in the VPG just as mammalian progestins affect GABA_AR activity that underlies diverse behaviors and functions (Lambert et al., 2003; Frye and Rhodes, 2007). The clear evidence for seasonal and daily changes in GABAergic functioning could be supported by natural rhythms in central or peripheral progestin secretion that control subunit expression and thereby synaptic or extrasynaptic densities. Photoperiodic melatonin secretion and its local effects via its own receptors and/or modulation of GABA_AR--would close the circle. That was the hope. Short of putting all those pieces together, the GABA research opened other doors through the discovery of a pattern generator exquisitely controlled by inhibition with striking resemblances to various locomotor CPGs in other species,

Grunt trains and locomotor rhythms--going out on a fin.

In view of the evidence that the outgrowth of vertebrate limbs is determined by a genetic cascade uncannily similar to that controlling insect appendages, one is tempted to posit a deep homology in the regulatory systems that predates even the origin of animal limbs (Shubin et al., 1997). In other words, extant fins, legs and wings develop under the radiated control of an ancient genetic system in basal taxa that originally patterned non-limb outgrowths, such as branchial arches (Gillis et al., 2009). However, it is also possible to conclude that similar, but unrelated, genetic programs were simply convergently drafted to implement the formation of limbs in different taxa, i.e. convergent solutions to similar problems. Determination of whether two structures are homologous may depend upon at which level they are compared (Shubin et al., 1997). A bird and a bat wing may be analogous as wings, but

homologous as forelimbs; and the vertebrate and insect wing may be analogous as appendages but homologous at the level of the genetic mechanisms that pattern them. Evidence for serial homology, or a homology between the repetitive structures of the same organism rather than of different species, can be more straightforward. It is common in arthropods such as the crayfish where variations of a single appendage may subserve swimming, walking, grasping, gas exchange and the transference of sperm. Genetic, embryological and fossil evidence support the idea that the locust flight system was a characteristic that evolved as an adaptation to one set of conditions and has been subsequently co-opted to perform a new and different function under different circumstances. The abdominal origin of some flight interneurons gave rise to the pleural appendage theory that posits that insect wings evolved from serially repeated leg appendages along the thorax and abdomen that previously served a different motor function than flight, such as ventilatory movements (Kukalová-Peck, 1978; Dumont and Robertson, 1986).

If crustacean legs and locust wings are examples of serial homology, and arthropod and vertebrate appendages are even “developmental paralogues” of one another--what does that say about the neural pattern generators that control them? Mustn't they be encoded as well by related genetic sequences resulting in at least serially homologous circuits? Isn't it possible that the motor circuit that controls the motions of pectoral fins was adapted or redeployed to pattern the contractions of the vocal muscle of the toadfish swim bladder? Could the grunt train exist because it is produced by a circuit that was originally adapted to pattern locomotion, while the growl is a derived version of that syncopated output, the hum even more so? Anatomically, the pectoral fin motoneurons and the vocal motoneurons are neighbors in the rostral spinal cord (Bass et al., 2008) and the temporal properties of the grunt train bear resemblances to the locomotor rhythms documented in lamprey and other

fish. In fact, the data collected from extracellular recordings of GABAergic modulated VPG activity even favors the model generated to describe the separation between rhythm and pattern formation in circuits controlling terrestrial locomotion (Rybak et al.). Complementing and evolutionarily predating the proposal that a neuroectodermal compartment originating in a common ancestor of the two major living groups of fishes gave rise to the neural pattern generating circuit that controls the timing of a great variety of vertebrate acoustic behaviors (Bass et al., 2008)--I would propose that this vocal pattern generator is also an example of serial homology, the redeployment of a neighboring locomotor circuit (also see Fig. S1, Bass et al., 2008). If this is true, then it may offer a missing evolutionary link between motor control systems and human speech, an idea that is already hotly debated and well substantiated among scientists studying the origins of human language.

“The traditional theory equating the brain bases of language with Broca’s and Wernicke’s neocortical areas is wrong,” bluntly declared Philip Lieberman in the first line of his abstract (Lieberman, 2002). Broca in 1861, in the phrenological spirit of the age, attributed speech production to an anterior cortical region of the left hemisphere when he found that a patient with lesions in this area was unable to speak more than a single monosyllable. However, he failed to account for the extensive subcortical damage and accompanying non-linguistic motor impairment. The phylogenetically primitive basal ganglia (Grillner et al., 2000; Menard and Grillner, 2008) comprised of the caudate and putamen (or striatum), and the globus pallidus regulates motor control but is also a crucial part of the circuit that supports linguistic ability in human beings. Tracer studies in monkey brains revealed connections between the striatum and cortical areas associated with motor control AND cognition (Alexander et al., 1986; Middleton and Strick, 1994; Graybiel, 1995, 1997), suggesting that the basal ganglia are the functional constituents of not just complete motor acts, but also syntactic

processes. In short, the neural substrates of cortical motor control and “cognitive pattern generation” (Graybiel, 1995) overlap.

The basal ganglia contribute to the learning and expression of patterned motor activity--dancing, typing, picking up an apple. They dictate the order of physical movements and mental operations. Lieberman compared the basal ganglia of normal people to those of patients with Parkinson’s disease and found that the basal ganglia are one of the first brain structures to deteriorate and the hardest hit. Sufferers of neurodegenerative diseases like Parkinson’s exhibit not only motor-based deficits: tremors rigidity, repeated movement patterns, but also struggle with the production and comprehension of syntax, including even behaviors as basic and specific as the correct pronunciation of b’s and p’s. These are stop consonants that depend upon proper voice onset time or VOT, the time that occurs between the abrupt opening and closing of lips to obstruct airflow, and the onset of periodic phonation generated by the larynx. Parkinson’s patients have very isolated issues with parts of speech reliant upon simple motor sequencing, but have no trouble with other aspects such as vowel duration or general tongue and lip movements. Their sentences were also short and simple, lacking complexity and they had trouble comprehending sentences that switched between the active and passive voice. Since Lieberman found that damage to a brain area necessary for motor skills also afflicted syntax, he concluded that there is a biological or neurological relationship between the “syntax of motor control and syntax of language.”

Then there came the Everest study (Kenneally, 2008; Lieberman et al., 2005). Climbers at such high altitude often suffer brain damage from the anoxic conditions, especially to the basal ganglia, and even though the cause is utterly different than that of Parkinson’s disease, they exhibit the same syntactical deficits as the Parkinson’s patients, in speech and motor skills. Just as Parkinson’ patients struggle with the

voicing of b's and p's because of the slight difference in voicing onset, so did the Everest climbers (and not just because their lips were cold). The basal ganglia also permit the interruption of certain motor acts or even thought sequences, and the switch to another. Climbers on Everest are known for becoming increasingly rigid or inflexible in their decision-making and behavior. After the death of one climber, it was found that he had not properly attached a harness that secured him to fixed ropes. For these harnesses to work they must be assembled in the correct sequence of steps. Did deterioration in his basal ganglia cause him to clip and unclip in the wrong order, to bumble his p's and b's? To become confused by syntactically complex sentences? According to Lieberman, whether we string words together or a series of physical motions that compose a complex motor task--we are using the sequencing and timing ability of the basal ganglia. Rats groom with it (UGG--universal grooming grammar) and birds build nests with it, so there is no innately human specialization for language, rather its syntactic nature is simply an adaptation of our motor system, one of our most primitive functions shared by every moving creature. The physical or neurological overlap between motor and cognitive patterns came about because of the way we have evolved--first developing the ability to physically move our bodies in space and then, overlaid upon that, developing the ability to move words in abstract patterns. When Lieberman posited that there was a common ancestor of apes and humans with this adaptable circuit that would only later be co-opted for cognitive and linguistic ability, he failed to see that the first step towards redeployment of a motor circuit might have already been taken in a vocal fish. If you peel off the derived aspects of human language, you uncover the more basic features shared with communication systems in other species, *and even locomotor systems*--the very biological/neurological substrate that may provide a template for a diverse array of seemingly unrelated behaviors that depend upon sequence and timing.

There are several similarities between various locomotor CPGs and the physiological data from the midshipman fictive prep. First, the natural and fictive grunt train repetition rate (1-3 Hz), happens to be exactly that of fictive swimming frequencies recorded from intact lamprey spinal cord (1-3 Hz) and within range of NMDA-induced fictive swimming in adult zebrafish, ~5 Hz (Cangiano and Grillner, 2003; Gabriel et al., 2008). Furthermore, an evaluation of the relationship between fictive burst duration and cycle period in the stimulated and disinhibited grunt train, revealed a statistically significant linear correlation ($p < 0.05$), such that burst duration increased with cycle time, as was also found in goldfish fictive swimming (Fetcho and Svoboda, 1993). Spontaneous fictive grunt trains can be evoked either by stimulation in a properly kindled fish (Rubow and Bass, 2009), or by focal injections of the GABA_AR antagonist, gabazine, into the paired nuclei of VPP. Stimulated grunt trains can maintain a highly regular repetition rate for up to 5 min, while disinhibition initially generated a spontaneous grunt train that was eventually subsumed by long duration, seizure-like buzzes. The physiological properties revealed by manipulation of inhibition levels in VPP, the purported seat of the grunt train rhythm and general call duration control, are the most intriguing of the accumulated data and suggestive of an architectural, physiological and even evolutionary relationship between locomotor CPGs and a vocal pattern generator.

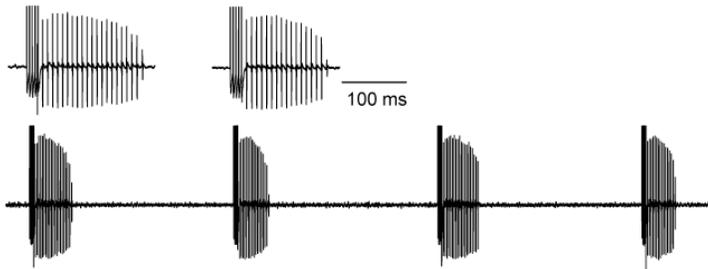
The role of inhibition (glycinergic in locomotor preps) has been thoroughly explored in the lamprey fictive swimming preparation (Cohen and Harris-Warrick, 1984; Cangiano and Grillner, 2007). Variations on the theme of the half-center oscillator, originally thought to be the neurophysiological basis for locomotor rhythms, have developed over the years (Marder and Calabrese, 1996). At this point in time the consensus appears to be that rhythmogenesis does not depend upon reciprocal inhibition, but alternating activity of antagonistic motoneuron pools does, as well as

the slowing of the locomotor rhythm (Roberts et al., 1998; Grillner, 2003; Butt et al., 2002). In one early study, in order to test three models for the organization of the CPG controlling swimming in the lamprey, glycinergic inhibition was blocked with strychnine (Cohen and Harris-Warrick, 1984). In the simplest model, alternation and rhythmic bursting relies upon a single CPG network that depends upon reciprocal inhibition between two half-centers. If this inhibition is removed, all rhythmic firing ceases. In the second model, independently oscillating, unit CPGs are also coupled by reciprocal inhibition, which if blocked would only interfere with regular alternation. Finally, in the third model the unit CPGs are coupled by both inhibition and weak excitation such that removal of inhibition allows the two sides to fire in synchrony. The results of the experiments confirmed the third model: at intermediate concentrations of strychnine between 5 and 45 min, the left and right ventral root bursts of a single segment were synchronized. At lower concentrations, strychnine could also increase burst frequency and burst duration within the cycle. In some cases, seizure-like, synchronized bursts occurred, just as was found upon removal of inhibition from the CPG controlling fictive swimming in the adult zebrafish (Gabriel et al., 2008). In the semi-intact preparations, strychnine initially increased burst frequency while maintaining a degree of alternation, but then degenerated into synchronous, seizure-like bursting.

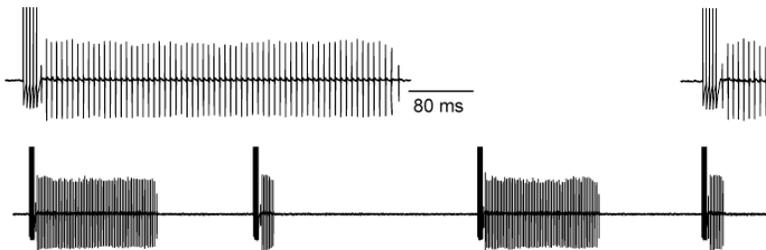
In both the lamprey and the zebrafish preparations, adult or larval, the strychnine was bath applied, thus ensuring complete permeation of the spinal cord. This contrasts with the intact *in vivo* midshipman preparation in which separate focal injections of, in this case, the GABA_AR antagonist, gabazine, were injected into the paired VPP nuclei that connect via a ventral commissural bundle. Sometimes, no matter the care taken, it was possible that the two nuclei did not receive equal injections, resulting in some serendipitous firing patterns that unfortunately cannot be

compared to the lamprey and zebrafish preps in which it would have been impossible to impose only a partial and lopsided blockade of inhibition. In Figure 2A, subtly alternating grunt durations were evoked from a fish that was only receiving episodic stimulation (long grunt, ~ 170 ms; short grunt, ~ 143 ms). In comparison, an animal that purportedly received equal bilateral 1 mM gabazine injections into both VPP nuclei (Fig. 2B), exhibited an utterly regular and dramatically alternating pattern of evoked grunts: long grunt, ~532 ms; short grunt, ~ 48 ms. In Figure 2C, a similar bimodal pattern emerged after simultaneous injections of gabazine into VMN and mPAG that also disinhibit the circuit, though in what manner, remains to be determined. The slow, spontaneous bursting alternated between buzz-like bursts, and even longer duration bursts of similar frequency but with very irregular amplitude. In all these cases it is as if a normal bilateral coupling between VPP nuclei had been skewed enough to allow one side to dominate, or to disrupt reciprocal connections that help to stabilize firing and the unified signal finally received and transmitted by the vocal motor nucleus to the swim bladder muscle. As a reminder, manipulations of inhibition levels in VPN-VMN, never disinhibit the system, nor cause gross changes in rhythm or duration, rather, this region appears to be the locus of fine frequency control as seen in this gabazine induced fictive grunt hum with three discharge frequencies in a single burst (Figure 2D).

A. Stimulation only



B. 1 mM gabazine in VPP



C. Gabazine in VMN + mPAG: disinhibited



D. Gabazine in VMN: 30 min



Figure 5.2 VPP rhythms vs. VPN-VMN frequency control. A. Alternating burst durations spontaneously occur during evoked grunts. B. A presumably bilateral 1 mM gabazine injection in VPP also induces alternating burst durations. C. After disinhibition of the vocal circuit with 1 mM gabazine injections in PAG and VPN-VMN, the patterning of spontaneous bursts alternate. D. A gabazine injection in VPN-VMN alone can evoke multiple frequencies in one evoked vocal burst.

Finally, although the primary source of inhibition in locomotor CPGs appears to be glycinergic, while the inhibitory effects explored thus far in the midshipman VPG have been GABAergic, preliminary studies also revealed a significant and possibly synergistic role for glycine in VPP. The vocal traces in Figure 5.3 show the duration increasing effect of bilateral injections of strychnine. When combined with gabazine, spontaneous firing also erupted, with a characteristic amplitude modulation not seen as often with gabazine alone. These experiments took place with non-reproductive fish and so never released any spontaneous grunt trains, thus it would be interesting to see if glycine supports or even antagonizes the grunt train rhythm in reproductive males.

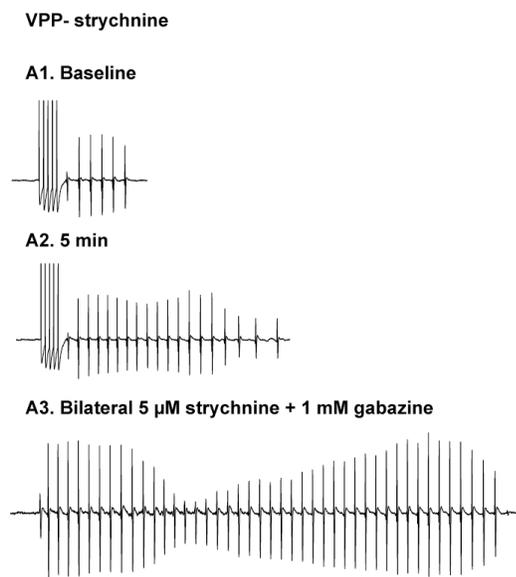


Figure 5.3 Glycinergic inhibition in VPP A1-A2. Strychnine increases evoked grunt duration and amplitude modulation. A3. Bilateral injections of gabazine and strychnine lead to spontaneous bursting.

The ideas put forward here will remain at this point, only that--ideas, the combing of patterns. Future studies would gain nothing by simultaneous left and right occipital root recordings as performed in the fictive locomotion preparations, because unlike the antagonistic motor pools that must fire in alternation for proper locomotion; at the level of the vocal motor nucleus and the swim bladder muscle, firing is synchronous. On the other hand, simultaneous, bilateral, single cell recordings, or even recorded field potentials from the two VPP nuclei might be illuminating. The point is not that the midshipman VPG is identical to the swimming CPG, rather, the question is: if there are enough legitimate, quantifiable, neurophysiological and anatomical similarities between the CPGs, could the circuits be serially homologous? Is it possible to establish the shared evolutionary origins between locomotor CPGs and the midshipman VPG, and thus broaden the pattern-generating, motor system based origins of language proposed by the likes of Philip Lieberman?

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