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Steroid-induced plasticity in the auditory and vocal motor system: Recent studies in a teleost fish

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1. Abstract

*The plainfin midshipman fish, *Porichthys notatus*, is a well-established neuroethological model system that has provided insights into neural and endocrine mechanisms of vocal-acoustic communication that are shared by teleost fishes and tetrapods, including birds and mammals. The reproductive success of midshipman fish is intricately linked to the production and perception of sound. During the breeding season, males emit a*

advertisement call generated by a hindbrain-spinal cord pattern generator, which innervates sonic musculature, and this signal is necessary for females to localize potential mates. Males and females have distinct seasonal patterns of circulating steroids that correspond to changes in gonadal development, and reproductive and vocal behavior. Frequency encoding by the inner ear of females changes seasonally, such that they are better adapted to detect the advertisement call of the male during the breeding season. This plasticity in audition was demonstrated experimentally to depend on elevated plasma levels of either testosterone or estradiol that naturally accompany the seasonal occurrence of reproduction. Furthermore, these same manipulations up-regulate and mimic seasonal changes in expression of the estrogen-producing enzyme, aromatase, throughout the brain. Localization of aromatase and estrogen receptor alpha in the inner ear support the hypothesis that estradiol alone could account for effects of circulating steroids on hearing. Steroid hormones, including androgens and estrogens, can also rapidly modulate the output of the hindbrain-spinal vocal pattern generator. The enzyme aromatase is also localized within vocal circuitry, most prominently surrounding vocal motor neurons and thus is ideally situated to provide a local source of estrogen for rapid modulation of reproductive-related vocalization behaviors. This review will focus on recent studies that elucidate the anatomical sites of action of steroid hormones and their effect on the physiological response properties of both the peripheral auditory and the central vocal motor systems in the midshipman fish.

2. Introduction

Vocal communication in vertebrates is context dependent and therefore influenced by social environment and reproductive state [1, 2]. However, very few studies have identified the neuroanatomical, neurophysiological and neuroendocrine substrates underlying the plasticity of this behavior (reviews: [3, 4]). The vocal control network and auditory system in teleost fishes represent archetypal examples of how a vertebrate nervous system produces and receives social, context-dependent sounds and therefore has proven to be an outstanding model for the investigation of vertebrate vocal-acoustic communication [5].

Teleosts comprise nearly half of all living vertebrate species [6] and many of these species are sonic, that is they produce sound (e.g., see [7, 8]). Since there are several recent reviews of the neural mechanisms of sound communication among teleosts, inclusive of hearing and the production of species- and sex-typical vocalization [5, 9-13], we only highlight the most salient points of those summaries here that are relevant to other sections in this review.

Sound production has independently evolved among several groups of teleosts [12, 13]. Many of the recent investigations of vocal communication among teleosts has been completed in a single family of teleosts, the Batrachoididae

(Order Batrachoidiformes) that include toadfish and midshipman fish. Hence, they are the focus of this commentary. There are three main reasons that lead to the concentration on this teleost group. One is that they have been the subject of numerous behavioral studies over the past three decades documenting the role of vocal signals in intra- and interspecific communication (see [5]). Second, as discussed elsewhere, numerous studies strongly support the hypothesis that the fundamental pattern of organization of the vocal motor and auditory systems of batrachoidids, and likely that of many other teleost groups, share many traits with those of tetrapods [11, 14-16]. This suite of shared traits also likely includes the neuroendocrine mechanisms for modulation of hearing and sound production considered elsewhere in this review. Third, as outlined below, they have an expansive vocal motor network in the brain that has been amenable to a range of experimental studies to reveal the cellular mechanisms of sound production and hearing.

3. Vocal and spawning behaviors

Acoustic communication plays an essential role in the social and reproductive behaviors of batrachoidids [5]. For example purposes, we briefly overview these behaviors in the plainfin midshipman (*Porichthys notatus*). The detection and localization of conspecific vocal signals is essential to the reproductive success of this nocturnal teleost. In the late spring and early summer months, midshipman migrate from deep waters offshore into the intertidal zone where males excavate nests under rocks and defend this territory for the duration of the breeding season [17]. Males produce a long duration (>1 min), multiharmonic advertisement call (“hum”) during the breeding season [18, 19]. Gravid females with mature eggs exhibit a strong phonotactic response to the equivalent male advertisement call played back experimentally, supporting the hypothesis that the hum plays an essential role in the ability of female midshipman to localize males [20, 21] (see [18] for description of natural spawning behavior). Midshipman fish have provided a powerful model system to study the neuroendocrine basis for behavioral variation in spawning and vocal behaviors, in part, because they have two male “morphs” that show distinct reproductive tactics [17]. Territorial, type I males build a nest under a rocky shelter in the intertidal zone and then spawn with several females throughout the breeding season but alone care for the young [18], transitioning from “courting” to “parental” behavior as the reproductive season progresses. Furthermore, males also produce shorter duration agonistic “growls” and “grunts” while defending and interacting with conspecifics [18, 22]. Nonterritorial, type II males represent an alternative reproductive phenotype that reaches sexual maturity earlier than type I’s and invests in gonad size, rather than large body size and sonic muscle, to “sneak” or “satellite” spawn in competition with type I males [17, 18] (also see [23]). Type II males are similar to females but differ from type I males in

a suite of somatic, endocrinological and neurological traits [17]. Type II males, like females, are known to produce only grunts in non-spawning contexts; however, the capacity to vocalize is both male morph and sex-specific as only type I males are known to hum and growl [18, 22]. Thus, vocalization behavior in this species varies seasonally and with reproductive state, is rapidly modulated during social interactions, and divergent both between and within the sexes. Since steroid hormones are potent modulators of brain function and reproductive-related behavior in all vertebrates, they are also likely candidates which may influence the development, maintenance and seasonal changes of sex- and male morph- specific vocal-acoustic traits in midshipman as well as all vocal tetrapods.

As described in the next few sections, we have taken a multidisciplinary, neuroethological approach to investigate the neural basis of vocal-acoustic communication in the plainfin midshipman fish, *Porichthys notatus* (see [24]). These behavioral, neurophysiological, neuroanatomical and molecular studies have identified mechanisms of auditory reception, neural encoding, and vocal production shared by all vertebrates [5, 11, 14]. Delineation of the neural circuitry comprising the ascending auditory system [25, 26], the descending vocal motor system and vocal-acoustic integration centers [15, 16, 27] in midshipman fish has laid the neuroanatomical foundation for investigating where and how the endocrine system may interact with acoustic and vocal pathways. This review summarizes recent studies that have elucidated the role of steroid hormones as important modulators of both auditory and vocal motor systems.

4. Vocal-auditory network

A combination of neuroanatomical and neurophysiological studies in both midshipman and toadfish have delineated a vocal motor network in the central nervous system and identified sites where vocal information is integrated with central inputs from the auditory and lateral line systems that encode the temporal and spectral attributes of underwater sounds. These studies demonstrate an expansive sonic motor circuit that extends between the caudal hindbrain and rostral spinal cord (Fig. 1). A combination of intracellular recording and staining (horseradish peroxidase) studies together with transneuronal transport of biotin compounds first identified three main populations of neurons in this circuit [27-29]. First, there is a midline sonic motor nucleus (SMN) that innervates the ipsilateral sonic muscle attached to the lateral wall of the gas-filled swim bladder via paired occipital nerve roots that form a sonic nerve. Second, a column of pacemaker-like neurons (PN) extends ventrolaterally along the rostral-caudal extent of each SMN and provides bilateral input to the midline pair of SMN. Third, a ventral medullary nucleus (VM) located immediately rostral to the PN-SMN complex provides both extensive bilateral coupling of the PN-SMN across the midline and links the PN-SMN circuit to more rostral hindbrain and midbrain nuclei.

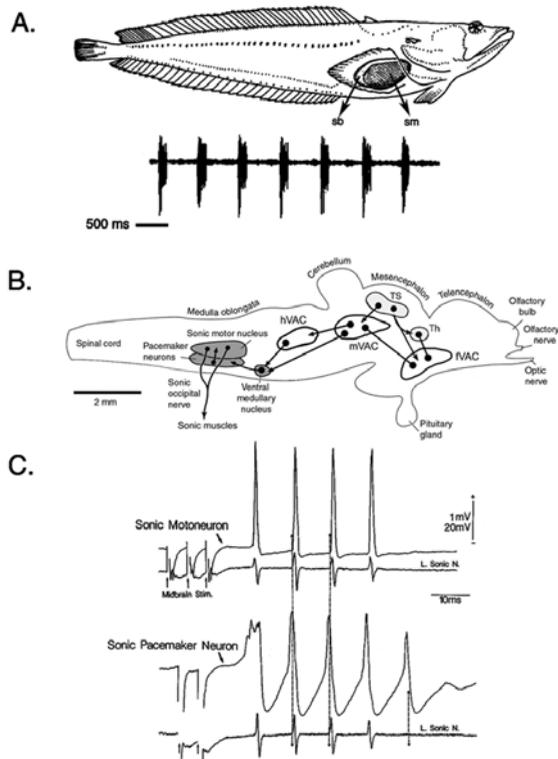


Figure 1. Central control of vocalization in batrachoidid teleosts, midshipman and toadfish. (A) Line drawing of a type I male midshipman fish showing the position of one of the pair of sonic muscles (sm) attached to the lateral wall of the swimbladder (sb). Midshipman have several classes of vocalizations including a “grunt train” as shown here which is a repetitive series of broadband, brief duration “grunts” that type I males produce during nest defense (see [18]). (B) Schematic, sagittal view of the brain and rostral spinal cord showing the relative positions of the forebrain (fVAC), midbrain (mVAC) and hindbrain (hVAC) vocal – acoustic complexes in batrachoidid fish. Solid dots represent somata, and lines represent axonal projection pathways. Two connected dots indicate reciprocal connections. The midbrain torus semicircularis (TS) and thalamic central posterior (Th) auditory nuclei are the central source of auditory inputs to the fVAC and mVAC, while the VIIIth nerve is the major source of auditory input to the hVAC. The vocal pattern generator is indicated in dark shading and includes a column of pacemaker neurons positioned ventrolateral to the sonic motor nucleus that innervates the sonic muscles via ventral occipital nerve roots that are homologous to the hypoglossal nerve of tetrapods [14]. A ventral medullary nucleus provides for extensive coupling of the pacemaker–sonic circuit across the midline. Based on neurophysiological

Figure 1. Legend continued

and anatomical studies by Bass and Baker [28], Bass et al. [25-27] and Goodson and Bass [15]. (C) Intracellular records from anatomically identified (intracellular horseradish peroxidase injections) sonic motor (top) and pacemaker (bottom) neurons from a type I male midshipman fish (adapted from [28]). A rhythmic sonic motor volley is evoked following stimulation at vocally active forebrain and midbrain sites including the midbrain site used here (see [15]). Each trace is the average of four records. Top trace for each neuron is DC-coupled low-gain intracellular record, while bottom trace is an intracranial extracellular recording from the left (L.) sonic occipital nerve (N.) root. Midbrain stimulation evokes a rhythmic response in sonic motor and pacemaker neurons; small arrows at the beginning of each of the lower traces indicate the onset of each stimulus. The nerve recordings reflect the highly synchronous firing of the sonic motor neurons. Each sonic nerve potential could be aligned (hatched vertical lines) with the firing of the pacemaker and motor neuron to indicate their relative timing; pacemaker neurons fire just prior to motor neurons that are nearly coincident with nerve record. Firing rates are independent of either stimulus number or frequency (three vs two stimuli for motor and pacemaker neurons, respectively). The motor volley recorded from the nerve is referred to as a fictive vocalization because its duration and repetition rate predict, respectively, the duration and either the fundamental frequency or the pulse repetition rate of natural calls. Time scale and direction of polarity for all records are indicated in top trace. Amplitude bar represents 20 mV and 1 mV for intracellular and nerve records, respectively. Modified from [36].

An essential component of the strength of this preparation as a model system for identifying mechanisms of vocal communication was the demonstration that the temporal firing properties of the PN-SMN circuit predict the temporal properties of natural calls. Thus, intracellular recording and staining showed early on that PNs set the firing rate of individual sonic motor neurons [28]. The rhythmic, oscillatory-like output of the SMN establishes the firing rate of the sonic motor volley (recorded from occipital nerve roots) that sets the contraction rate of the sonic muscles that, in turn, determines the fundamental frequency of natural sounds [30]. The output of the PN-SMN circuit is reflected in the temporal firing properties of the sonic motor volley, which is recorded from occipital nerve roots. We designate this motor volley as a “fictive vocalization” because it mimics the basic temporal features of natural vocalizations, namely fundamental frequency and duration [28, 31]. Surgical isolation of the hindbrain-spinal region shows that all of the “neural machinery” both necessary and sufficient to generate a rhythmic fictive vocalization is contained in this region [32, 33]. We later refer to this region that contains the VM-PN-SMN circuit as the vocal pattern generator or VPG.

Since the initial studies reviewed above, a combination of neuroanatomical tract tracing methods with brain microstimulation and electrophysiological recordings of fictive calls and single central neurons have revealed a descending

vocal motor pathway that interfaces with auditory-recipient nuclei in forebrain, midbrain and hindbrain vocal-acoustic centers (VAC) [15, 16, 34]. Each VAC includes several interconnected nuclei that receive auditory input either from the inner ear (see [25, 26]) or centrally from the main auditory-encoding nucleus in the midbrain's torus semicircularis (TS, see [35]) and dorsal thalamus (Th, see [25, 26]). The midbrain VAC includes the periaqueductal gray, a compact cell layer that lines the medial aspect of the ventricular space separating the tectum from the underlying tegmentum and torus semicircularis [15, 16]. Brain microstimulation together with tract tracing first showed the connectivity of a midbrain region inclusive of the PAG to VM [15]. Most recently, a single neuron recording study that included a study of the connectivity of the PAG clearly showed that the PAG projects to VM via the medial longitudinal fasciculus (MLF) [16]. This same study used a combination of methods to show that PAG neurons are essential to the initiation of fictive, and hence natural, vocalizations. Additional evidence suggests that the PAG may also participate in shaping the duration of natural calls, while it has no influence on fundamental frequency, consistent with the hypothesis that fundamental frequency is set by the PN-SMN circuit [28] (see also [32, 33]).

While our anatomical studies clearly show that the vocal motor system interfaces with the auditory system at several sites (see above), a recent neurophysiological study shows how the vocal system directly influences auditory encoding mechanisms [37]. The hindbrain VAC includes a midline positioned efferent nucleus that provides direct inputs to the hair cell epithelium and eighth nerve afferents of the sacculus, the main auditory end organ in batrachoidids and other teleosts [10] (also see [38]). Early on, transneuronal biocytin transport studies of the vocal motor system showed that it provides afferent inputs to the efferent nucleus [27]. Weeg et al. [37] now provide the essential electrophysiological evidence that supports an influence of the vocal system on the electrophysiological response properties of the auditory system. Studies in midshipman show that efferent neurons exhibit increases in firing rate that are temporally correlated with the onset and offset of each fictive pulse of the fictive vocalization. Earlier studies had shown that efferents provide an inhibitory input to the sacculus (see references in [37]). Thus, the rhythmic vocal motor volley apparently decreases the sensitivity of the sacculus, via the inhibitory actions of efferent neurons, to each sound pulse of the fish's own call. These same studies also provide evidence showing that the vocal motor system also likely modulates the sensitivity of the peripheral lateral line system [37], consistent with the sensitivity of this system to the frequency content of natural calls [39]. These studies now set the stage for future studies of vocal-auditory/lateral line integration in each VAC, including the possible influence of steroid hormones on those mechanisms.

5. Steroid effects on vocal-acoustic circuitry: Neuroanatomical evidence

Like other seasonally breeding vertebrates, natural fluctuations of circulating gonad-derived steroid hormones occur during reproductive cycling in midshipman fish, and variation in steroid levels are correlated with reproductive-related behaviors [40-42] (Fig. 2). Certainly, gonad-derived hormones

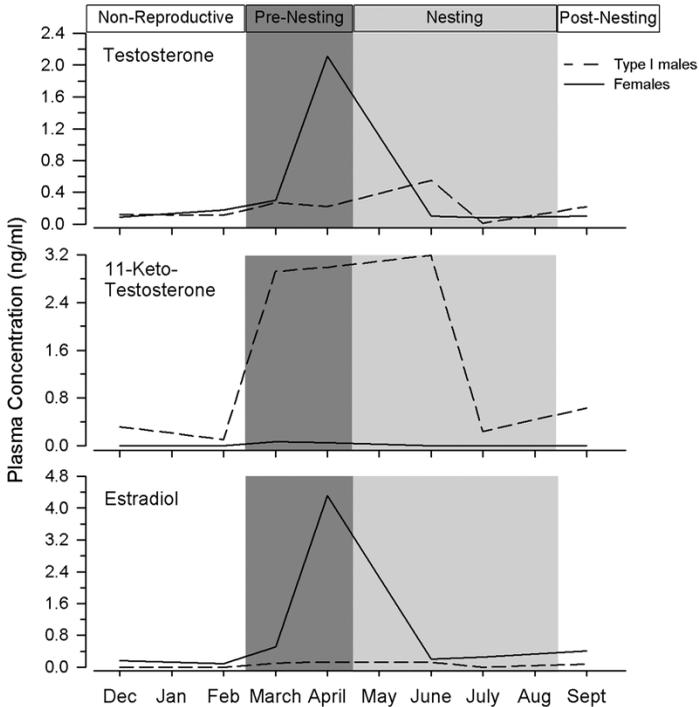


Figure 2. Summary diagram of the plasma sex steroid levels for wild-caught type I male and female plainfin midshipman collected from Monterey Bay and Tomales Bay (CA - USA) during the non-reproductive, pre-nesting, nesting, and post-nesting periods. Median steroid concentrations are plotted for both type I males and females. Note that the temporal difference in seasonal changes in major circulating steroid levels between males and females parallel sex differences in reproductive behavior. The maintenance of elevated levels of androgens in males into the spawning period reflects their continued courtship and spawning activity throughout the summer, while a single peak of E_2 and T prior to spawning in females reflects the behavior of a single spawning event. Adapted from [42].

provide one source for steroid effects on the nervous system and may function to link changes in reproductive state with changes in behavior. Neurosteroids are defined as steroids manufactured wholly in nervous tissue or derived in the brain from circulating precursors (review: [43]). Aromatase, the enzyme which converts androgens to estrogens, functions as the terminal step in steroid synthesis, and teleost fishes show the highest levels of aromatase in the brain compared to other vertebrate groups [44]. Thus, neuroanatomical expression of this enzyme can function to provide estrogen and/or regulate how much androgen (testosterone) reaches specific populations of neurons in either the central or peripheral nervous system. Here we will review the anatomical distribution of aromatase expression in the midshipman brain in relation to one of its potential targets, estrogen receptor alpha ($ER\alpha$), as well as androgen receptor (AR) expression in the context of defined vocal-acoustic circuitry (described above). Cloning of partial cDNAs from conserved regions for all three genes combined with *in situ* hybridization technique and a novel teleost-specific aromatase antibody allowed for precise mapping of expression throughout the midshipman CNS.

5.1 Vocal-acoustic integration centers (VAC) and descending vocal pathways

I. Forebrain

Aromatase is expressed in glial cells along ventricular surfaces throughout the brain in midshipman as well as other teleosts [45-47]. In midshipman, the forebrain contains the highest aromatase activity, number of aromatase-immunoreactive (-ir) cells and mRNA expression [45, 48]. Aromatase-containing radial glial cell bodies line the entire telencephalon ventricular wall with processes covering and potentially providing virtually the entire telencephalon with a local source of estrogen [45] (Fig. 4E). In contrast to widespread aromatase expression, both $ER\alpha$ and AR are concentrated in discrete nuclei, most prominently in preoptic areas and ventral telencephalon [49, 50]. All nuclei which comprise the forebrain vocal-acoustic integration centers (VAC): anterior and posterior parvocellular preoptic areas (PPa, Ppp) and ventral and anterior tuberal hypothalamus (vT, AT) contain high aromatase mRNA and protein levels (Fig. 3B). AT, an important vocal-acoustic integration center, not only is part of the descending vocal motor pathway (downstream of vT, [15]; Fig. 3A), but also has strong connections with ascending auditory circuitry in the thalamus [25]. High levels of $ER\alpha$ mRNA are found in the PPa with relatively lower abundance in vT and AT (Fig. 3C). Expression of AR mRNA is quite robust in all these areas. The neuropeptides arginine vasotocin (AVT) and isotocin also densely innervate AT and modulate vocal patterning in midshipman [51-53], and thus may themselves be regulated via local steroid interactions [49].

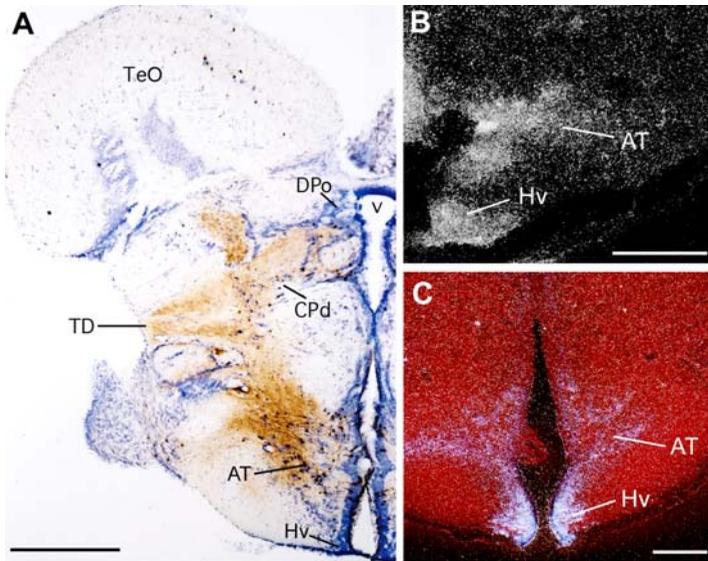


Figure 3. Vocal-acoustic-steroid hormone integration center at the anterior tuberal nucleus (AT) of the hypothalamus in the plainfin midshipman fish. (A) Low-power montage showing labeled cells and fibers in AT after a neurobiotin injection into the ventral tuberal nucleus which indicates connectivity (brown reaction product) between both vocally-active areas. Also note projections into the diffuse portion of the central posterior (CPd) nucleus, a part of the auditory thalamus (adapted from [15]). (B) Dark-field visualization of abundant aromatase mRNA expression in AT. (C) Robust ER α mRNA in AT in a pre-nesting female. Scale bars = 500 μ m in A and B; 200 μ m C. A and C adapted from [49].

II. Midbrain

Unlike non-vocal species of teleosts, midshipman also have high aromatase expression in the midbrain, especially in areas corresponding to mVAC and descending vocal motor nuclei. In the rostral midbrain, aromatase-ir cell bodies line the periaqueductal gray (PAG) and dense tracts of fibers extend ventrolaterally to cover much of the tegmentum, including the paratoral tegmentum (PTT) and paralemniscal (PL) nuclei [45] (Fig. 4C,D). All three of these nuclei are robust vocal-stimulation sites [15, 16]. In more caudal areas of the midbrain, aromatase mRNA and protein is dense around the cerebral aqueduct, fourth ventricle and PAG, while aromatase-ir projections extend ventrolaterally along the reticular formation (RF). Interestingly, ER α mRNA is sparse in the tegmentum, weakly expressed in PAG and in the nucleus of the medial longitudinal fasciculus (nMLF) just ventromedial to the PAG [49]. Like ER α ,

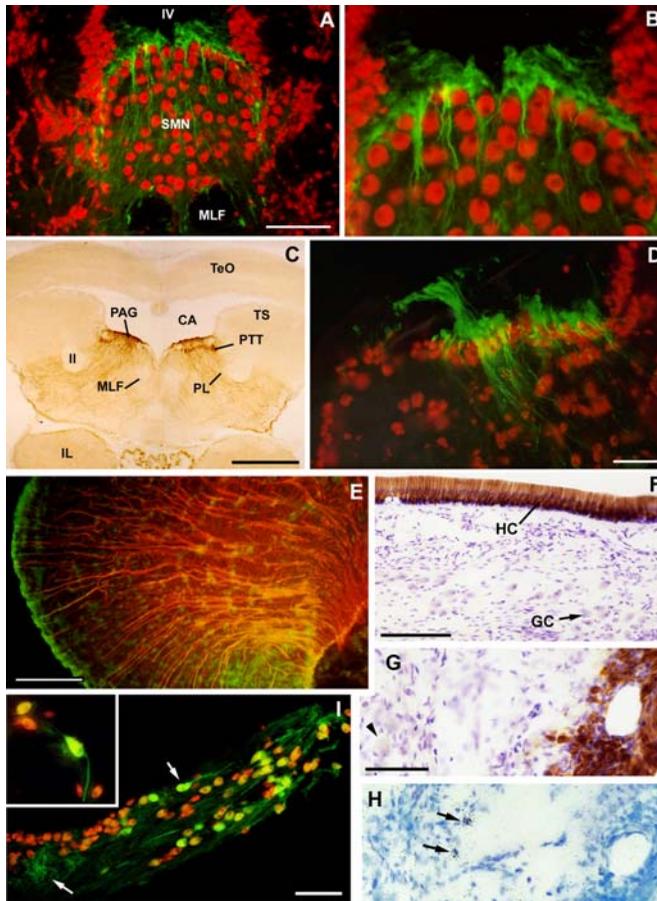


Figure 4. Cellular identification of aromatase in the brain and inner ear of midshipman. (A) Photomicrograph of the sonic motor nucleus fluorescently double-labeled with teleost specific anti-aromatase (green) and neuronal specific anti-Hu (red). Scale bar = 240 μm . (B) Higher magnification of (A). Aromatase-immunoreactivity (-ir) cells are concentrated at the dorsal periphery and fibers course around motor neurons throughout the nucleus. Scale bar = 160 μm . (C) Dense aromatase-ir (brown reaction product) in midbrain vocal motor sites: cells line the periaqueductal gray (PAG) and paratotal tegmental (PTT) nucleus and fibers extend around the lateral lemniscus (II) through the paralemniscal nucleus and medial longitudinal fasciculus (MLF). Note absence of aromatase-ir in the torus semicircularis (TS) and optic tectum (TeO). CA = cerebral aqueduct; IL = inferior lobe of hypothalamus. Scale bar = 500 μm . (D) High magnification of the midbrain PAG double-labeled with teleost anti-aromatase (green)

Figure 4. Legend continued

and anti-Hu (red). Scale bar = 80 μm . (E) Co-localization of aromatase in radial glial cells in midshipman telencephalon. Cells that line the periphery of the telencephalic lobes are double-labeled with anti-aromatase (green cell bodies) and anti-GFAP (yellow-orange fiber projections). Scale bar = 200 μm . (F) Sagittal section through the saccular epithelium of the inner ear shows the hair cell layer (HC), revealed by a hair-cell specific antibody, relative to ganglion cells (GC) that are positioned within the saccular branch of the eighth nerve that innervates the HC (brown reaction product, Nissl counterstain). (G,H) Adjacent sections which show ER α mRNA by *in situ* hybridization label (H; arrows) in relation to the hair cell layer (brown) in G in a female midshipman. Arrowhead in G indicates relative position of a ganglion cell. Scale bar = 50 μm . (I) Double-label immunofluorescence using aromatase (green) and neuronal (soma) specific Hu (red) antibodies reveals aromatase in ganglion cell somata (bright yellow) and their processes (green) in the eighth nerve in a female midshipman. (I inset) high magnification of aromatase expression in bipolar ganglion cells (yellow) and processes (green) (center and upper left). Scale bar = 100 μm ; 50 μm for inset. Photomicrographs adapted and modified from [45, 54].

AR mRNA is found in the PAG and nMLF but also more broadly expressed along ventricular surfaces in this area, more robustly than ER α , but much less relatively compared to forebrain levels [50].

5.2 Vocal pattern generator (VPG)

Throughout the “sonic” hindbrain (see previous section), aromatase-ir cells are located around the fourth ventricle and the MLF with processes coursing ventrolaterally through the reticular formation, including the ventral medullary nucleus (VM). The extensive SMN is surrounded by aromatase-ir glial somata along its dorso-lateral borders and fibers weave prominently between the motor neurons throughout the nucleus [45] (Fig. 4A,B). In contrast to aromatase, which lies adjacent to motor neurons in glial cells, ER α mRNA appears to be expressed in sonic neurons themselves [49]. Thus, aromatase is ideally located to provide a local source of estrogen throughout the vocal pattern generator (VPG). Interestingly, AR mRNA is expressed in a similar pattern to aromatase, primarily along the dorsal periphery of the SMN. Whether or not AR is localized in glial cells in the SMN is not known; however, robust AR mRNA is found specifically over VM neurons [50].

In general, aromatase mRNA and protein are more widespread and in higher abundance than ER α throughout the brain, especially in the midbrain and hindbrain. Thus, aromatase likely provides estrogen in a paracrine fashion to discrete nuclei where ERs are located. In areas where aromatase and ER α are not co-regionalized, other nuclear ERs [55, 56] (i.e. ER β 1, ER β 2) could be present, ERs may be present but undetectable by conventional means (above),

E_2 signaling could occur through diffuse membrane receptors or by activation of neurotransmitter receptor subunits, or aromatase could act as a “sink” or buffer to regulate the amount of androgens affecting local brain areas [49].

I. Sex differences in the sonic motor nucleus (SMN)

As mentioned above, only type I males have the capacity to make long duration vocalizations, and the temporal properties of calling behavior are governed by the output of the sonic motor nucleus and pacemaker neurons (see earlier section). Previous studies have demonstrated inter and intra-sexual dimorphisms in SMN morphology (individual neurons and nuclear volume are larger in type I males compared to type II males and females), firing properties, as well as the sonic muscle which it directly innervates [28, 29, 57-59]. Androgens are known to masculinize dimorphic vocal motor circuitry and muscle [60-62] and aromatase activity is higher in hindbrain homogenates of type IIs and females compared to type I males [48]. Aromatase expression within and around the SMN probably accounts for the majority of its activity levels in the hindbrain [45]. Quantitative *in situ* hybridization revealed that indeed, type II males and females have significantly higher aromatase mRNA in the SMN compared to type I males [61, 63]. Since both type II males and females have relatively high circulating testosterone levels, we hypothesized that up-regulation of aromatase in and around the SMN may function to prevent its masculinization by androgens toward a type I phenotype and therefore may play a key role in the development and maintenance of alternative male reproductive morphs and sexual brain dimorphism [48, 54]. Whether there are sex and/or morph differences in ER α and AR mRNA in specific brain areas is not currently known.

II. Seasonal plasticity and steroid regulation of aromatase in the SMN

Changes in aromatase mRNA expression in the SMN are correlated with seasonal fluctuations in circulating steroid hormones and reproductive behavior in females and type I males. Quantitative *in situ* hybridization has shown the highest expression levels in the female SMN during the pre-nesting period [63] when circulating testosterone (T) and 17 β -estradiol (E_2) peak during gonadal recrudescence, just prior to the mating season (Fig. 2). Similarly, aromatase mRNA in the SMN in type I males is greatest when circulating androgens (11-ketotestosterone, 11-kT; testosterone) and estrogen levels are elevated at the start of the nesting period (Fig. 2) when territories are obtained and vocal courtship occurs at night [63]. Notably, although not quantified, seasonal changes in aromatase mRNA in the PAG, a midbrain vocal control center [16], appeared to parallel those seen in the SMN, while forebrain nuclei (preoptic) did not [63]. To test whether circulating steroids directly affect brain aromatase mRNA, females were ovariectomized and given T, E_2 or blank implants. Both T and E_2 implanted

females showed very similar mRNA expression levels in SMN to natural levels found in pre-nesting, recrudescing females, while control implanted females resembled SMN levels found in non-reproductive females when both circulating steroids and aromatase expression is lowest [64].

Paradoxically, although E_2 is well documented to up-regulate brain aromatase and estrogen responsive elements (ERE) are found in the promoter region of the brain aromatase gene in teleosts (see [54, 65] for review), ERs and aromatase do not appear to be colocalized by conventional anatomical techniques. However, *in vitro* experimentation has identified ER α transcripts by RT-PCR in glial cell culture from trout brain and very low levels of E_2 can induce the brain aromatase gene in a glial cell context in zebrafish [46, 47]. In contrast, AR mRNA, at least in the SMN in midshipman, appears to be expressed in the same pattern as aromatase, which supports androgenic regulation of aromatase in glial cells [50]. The rise in circulating steroids (both T and E_2) associated with gonadal recrudescence prior to the reproductive period appears to up-regulate brain aromatase. This local source of E_2 may then exert a positive feedback on expression of the enzyme to drive precise steroid-dependent brain functions independent of gonadal state [54]. The significance of seasonal changes in aromatase expression in the SMN is exemplified by the importance of local estrogen in modulating vocal output from the hindbrain-spinal vocal pattern generator, which directly affects reproductive-related social behaviors (see below). Thus, an intricate neuroendocrine coupling exists between reproduction and vocal-acoustic function in midshipman.

5.3 Central and peripheral auditory system

While forebrain auditory centers in the thalamus and telencephalon contain an abundance of aromatase mRNA and protein, the torus semicircularis (TS), the main auditory and lateral line processing area in the midbrain [5, 25, 26, 34] is devoid of aromatase expression [45] (Fig. 4C). ER α mRNA is only sparsely expressed in the TS and in low abundance in the auditory thalamus, but highly expressed in the dorsal medial telencephalon, which is likely an auditory processing center (see [15]). Several subdivisions of the ventral telencephalon receive inputs from the auditory thalamus (see [15]) and ER α mRNA expression in this region is variable but most robust in recrudescing females [49]. AR mRNA is consistently expressed in the periventricular layer of TS as well as in the auditory thalamus and highly expressed in the ventral and dorsal medial telencephalon [50]. Neurons, which express ER α in the TS, likely receive E_2 from a peripheral source (gonad) or via a paracrine source from an adjacent brain area such as PAG. At least in midshipman, the brain appears to be the primary source of estrogen in males [42]. In the peripheral auditory system, aromatase-ir was identified in ganglion nerve cells in the branch

of the eighth nerve adjacent to the sensory epithelium of the main auditory end organ (sacculle) of the inner ear of females (Fig 4I). Furthermore, ER α mRNA expression was found in unidentified cells just outside the saccular hair cell layer [49] (Fig. 4F-H). Thus, the ear itself may provide a local source of estrogen independent of the gonad to modulate plasticity of hearing (see section below).

6. Steroids and hearing

As discussed earlier, nesting type I males produce trains of grunts and growls during agonistic behaviors and long duration multiharmonic advertisement calls or “hums” to attract reproductively active females to their nests. Females use the auditory sense to detect and locate calling type I males that produce the multiharmonic hums during the breeding season. Underwater playback experiments of natural and synthetic hums evoke strong phonotactic responses in reproductively gravid females but not in females that have already released their eggs [20, 21].

Recent work shows that in a wild population of plainfin midshipman the frequency sensitivity of auditory primary afferents that innervate the sacculle, the main endorgan of hearing in midshipman and most other teleost fish (see [10]), changes seasonally with female reproductive state such that summer gravid females become better suited than winter non-gravid females to detect higher harmonic components of the male hum [66]. Gravid females exhibit a dramatic increase in best frequency sensitivity and in the phase-locking accuracy of auditory saccular afferents to a broad range of frequencies > 100 Hz compared to that of non-gravid females. The improvement in the precision of temporal encoding by the auditory saccular afferents to the dominant frequencies of the male hum should improve conspecific detection and localization in shallow water environments where midshipman breed, in part, because higher harmonics propagate farther in shallow water as a result of the inverse relationship between water depth and the cutoff frequency of sound transmission [67, 68].

Wild populations of *P. notatus* exhibit an annual reproductive cycle containing four time periods (non-reproductive, pre-nesting, nesting, and post-nesting) that corresponds to seasonal fluctuations in midshipman reproductive biology and sexual behavior [42]. During the pre-nesting period of the annual reproductive cycle (Fig. 2), female midshipman exhibit peaks in circulating blood plasma levels of T and E₂ about one month prior to the beginning of the summer spawning season. Experimental implants of T and E₂ in ovariectomized females collected during the non-reproductive period induce an increase in the phase-locking precision of the saccular afferents at higher frequencies that correspond to the second (~ 200 Hz) and third (~ 300 Hz) harmonics of the male’s hum, which often contains either as much or more energy as the fundamental

frequency (~ 100 Hz). Thus, the winter non-reproductive female midshipman fish treated with either T or E_2 exhibit an increase in the degree of temporal encoding of the frequency content of male vocalizations by the inner ear saccule that mimics the summer reproductive female's auditory phenotype (Fig. 5) [69]. It is not currently known whether reproductive state and/or steroid-dependent auditory plasticity also extend to males, which would also be adaptive for the detection of conspecifics during male competition for the establishment of nest sites and the access to females. The steroid-dependent plasticity of peripheral auditory frequency sensitivity in female midshipman fish may represent an adaptable mechanism that seasonally enhances the probability of conspecific detection and localization by the frequency coupling between sender and receiver in this vocal communication system.

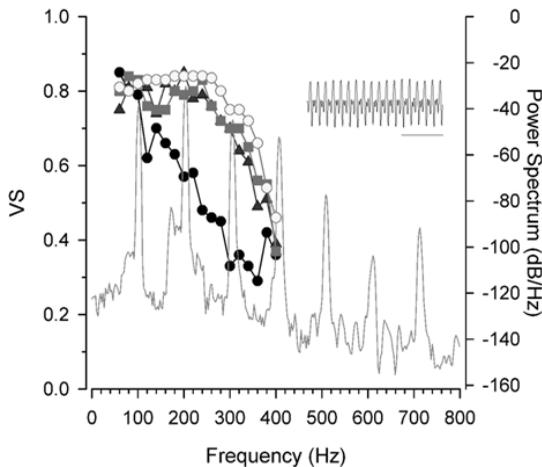


Figure 5. Match between vocal characteristics and the degree of frequency encoding of eighth nerve saccular afferents. Shown here is a combined plot of the phase-locking precision of saccular afferents as a function of the vector strength of synchronization (VS, Y-axis to the left) and the power (amplitude) spectrum of a hum advertisement call from a nesting male midshipman fish (Y-axis to the right in relative dB values); insert shows the temporal waveform of this call recorded at 16°C at the nest site (bar scale = 50 ms). Frequency is plotted along the X-axis for both sets of measures. Shown here are median VS values of afferents emphasizing the overlap in frequency sensitivity between testosterone (\blacktriangle) and 17β -estradiol (\blacksquare) treated, nonreproductive females and wild caught reproductive females (\bullet). While all of these females show robust encoding of the fundamental frequency (F_0) and the second and third harmonics (F_1 , F_2) of the male advertisement call, the saccular afferents of nonreproductive females (\bullet) show comparable encoding only for frequencies close to F_0 . Adapted from [69].

The mechanism(s) by which T and estrogen modulate the frequency sensitivity in the midshipman peripheral auditory system still remain to be demonstrated. Adaptive shifts in the sensory sensitivity to reproductive-state or sex steroid levels are well established for another hair cell based sensory system, the electroreceptor systems of weakly electric fishes and elasmobranchs. Previous studies indicate that steroids can alter the tuning of the tuberous electroreceptors of weakly electric fishes [70-72] and the ampullary electroreceptors of elasmobranchs [73]. Androgen treatment is known to lower both the best frequency sensitivity of tuberous electroreceptors and the electric organ discharge frequency in tandem so that the electrosensory and electromotor systems of weak electric fishes remain matched or “frequency coupled” for social communication and electrolocation [70, 72]. Dihydrotestosterone is known to affect the activation and inactivation kinetics of the Na⁺ current in the electrocytes of the electric organ of weakly electric fishes that results in an increased pulse duration of the electric organ discharge [74]. These changes in current kinetics of the electrocytes are hypothesized to result from the differential expression of multiple ion channels (e.g., Na⁺ and K⁺) genomically regulated by gonadal sex steroids [36, 75, 76]. Similar steroid induced changes to Ca⁺ dependent and voltage sensitive K⁺ channels may also affect the current kinetics and frequency tuning of the electroreceptors and the auditory hair cell receptors of other vertebrates, including the midshipman fish. A recent study shows that midshipman-specific ER α mRNA is present in the auditory saccular nerve branches adjacent to the hair cell layer in the saccule (Fig. 4H) [49]. Future studies that examine the expression of androgen receptors in the midshipman peripheral auditory system and detail the possible genomic effects of T and E₂ on hair cell ionic membrane properties will provide important insight into the mechanism(s) responsible for steroid-dependent neurophysiological changes seen in the midshipman’s auditory periphery.

7. Steroids and vocal patterning

7.1 Steroids and rapid modulation of a vocal pattern generator

In vertebrates and invertebrates, overt behaviors are encoded by motor commands that are in turn governed by hindbrain or spinal pattern generators. The current detailed understanding of pattern generators is the result of over two decades of experimentation on: 1) motor networks that pattern behavior such as those involved in locomotion [77, 78], and 2) the role that neuromodulators such as peptides and monoamines play in modulating pattern generators to coordinate behavioral transitions [79, 80]. The studies described below in midshipman fish broaden this understanding to include another class of neuromodulators, steroid hormones. This work formally tests the hypothesis that rapid behavioral effects of steroids occur via their actions on central pattern generators.

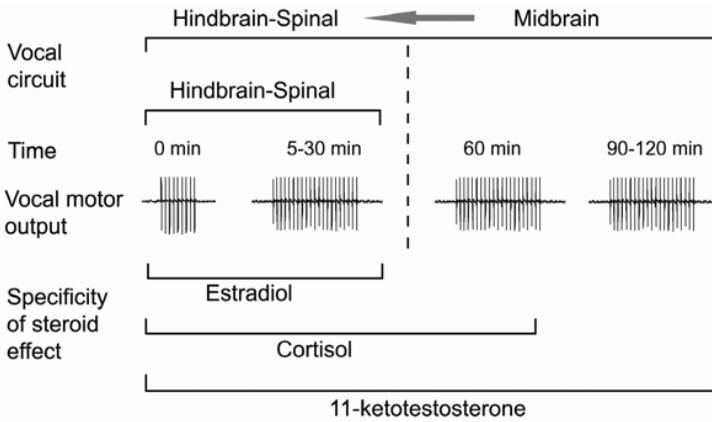


Figure 6. Steroid-dependent modulation of fictive vocalizations (from [32]). Intramuscular injections of 17β -estradiol, cortisol, and the teleost specific androgen 11-ketotestosterone (11KT) all induce similar increases in the duration of the fictive vocalization in type I males as represented by neurophysiological recordings of the occipital nerve motor volley in the center of the figure (see Fig. 1C). However, each steroid has a specific effect on the longevity of the duration increase; 17β -estradiol has the briefest and 11KT the longest lasting effect. The region containing the hindbrain-spinal pattern generator circuit can be surgically isolated *in vivo* by making complete transections just rostral to hVAC and just caudal to the sonic motor nucleus (see Fig. 1). Studies of the isolated vocal pattern generating circuitry show that it is both necessary and sufficient for the rapid steroid effects lasting up to 30 min, while a descending input from the midbrain is necessary for sustained effects beyond 30 min (see [32]).

Table 1. The rapid effects of steroid hormones on vocal motor patterning in plainfin midshipman is convergent between type II males and females, which are divergent from type I males. Up-arrows indicate significant rapid elevation, down-arrows indicate significant rapid suppression, even-arrows indicate no significant changes following steroid injection. Data for type I males are adapted from [32] and data for type II males and females are adapted from [92].

<i>Sex / Steroid</i>	<i>11kT</i>	<i>Testosterone</i>	<i>Estradiol</i>	<i>Cortisol</i>
Type I male	↑	↔	↑	↑
Type II male	↔	↑	↑	↓
Female	↔	↑	↑	↓

Since the VPG of midshipman and toadfish has a well-defined behavioral output (social vocalizations), electrophysiology experiments can assess the influence of neuromodulators, including neuropeptides (see [51]) and steroids (see below) on the activity of the VPG, and infer direct effects on social behavior. An example of the effects of intramuscular injection (trunk muscle) of steroids on vocal patterning in adult type I male midshipman are shown in Figure 6. A summary of results for all morphs is presented in Table 1.

I. 17 β -estradiol (E₂)

E₂ is one of the primary circulating steroids in teleosts, and its plasma levels are elevated during the breeding season in both midshipman and toadfish [42, 81, 82]. In addition, the midshipman brain exhibits robust expression of the enzyme aromatase in the VPG (Fig. 4A,B) [45, 48, 63], raising the possibility that circulating androgens are converted locally into estrogens to regulate VPG activity. Systemic injections of E₂ cause rapid increases in the duration of VPG activity within 5 min, and this effect is sustained up to 30-45 min in type I males, type II males, and females [32] [92]. Interestingly, in contrast to all other rapid steroid treatments in midshipman (see below), the effects of E₂ on fictive call duration are similar in direction (facilitatory), magnitude (~140% elevation above baseline), and time-course (sustained 30-45 min after injection) in all three adult midshipman morphs (Table 1). This collection of common results likely reflects the primacy of E₂ in regulating events within the central nervous system, since E₂'s effects on neuronal activity are widespread among vertebrates [83].

II. 11-ketotestosterone (11-kT) and testosterone (T)

The predominant circulating androgen in type I male midshipman is 11-kT [40, 42] a hormone that is similar to dihydrotestosterone in mammals as a potent, non-aromatizable androgen [41, 84, 85]. Territorial male toadfish and midshipman have elevated 11-kT during vocal advertisement compared to non-calling cases [86, 87], suggesting that this steroid may regulate reproductive advertisement calling in a rapid manner. In type I male midshipman, systemic injections of 11-kT exert pronounced and rapid increases in fictive call duration within 5 min, and this effect is sustained up to 120 [32] (Fig. 6). However, 11-kT has no significant effects on fictive call duration in type II males or females. Instead, T is potently neuromodulatory in type II males and females, which directly reflects the observation that T is the dominant androgen in both sexes of type II (Table 1) [92].

III. Cortisol

The predominant stress hormone (glucocorticoid) in teleost fish is cortisol (see references above for 11-kT). Among teleosts, acute stress causes rapid increases

in plasma cortisol, and this phenomenon has been documented in the closely-related Gulf toadfish [88] and likely occurs in adult midshipman (see [42] for discussion). These rapid changes in plasma cortisol could exert similarly fast effects on the midshipman VPG, implying a role in modulating vocalizations during acute stress. In type I male midshipman, systemic injections of cortisol exert rapid increases in fictive call duration within 5 min, and this effect is sustained up to 60 min (Fig. 6) [32]. Conversely, in type II males and females cortisol causes rapid decreases in fictive call duration within 5 min, and these effects are sustained up to 60 min (Table 1) [92]. Together, these results demonstrate an unanticipated role for glucocorticoids in the rapid regulation of vocal behavior in vertebrates, suggesting that glucocorticoids regulate vocalization during aggressive encounters with conspecifics and/or predators [33].

7.2 Steroids act through receptor-like binding sites

Experiments with nuclear hormone receptor antagonists indicate that the rapid actions of steroids occur through receptors or membrane binding sites that resemble nuclear hormone receptors. In type I male midshipman, the anti-androgen cyproterone acetate (CA) eliminates the rapid actions of 11-kT [32]. Similarly, in type II male midshipman, the rapid actions of T are eliminated in the presence of CA [92]. In females, however, CA is not effective at blocking the rapid effects of

Table 2. The mechanisms of rapid steroid action appear to be dependent on gonadal phenotype, and likely reflect early organizational influences of a male vs. female gonad. The androgen receptor antagonist cyproterone acetate (CA) eliminates the rapid effects of androgens in type I and type II males but not females, while the aromatase inhibitor fadrozole (FAD) blocks the rapid effects of androgens in females but not type I and type II males. The glucocorticoid receptor antagonist RU486 eliminates the rapid effects of cortisol in all three adult midshipman morphs. Data for type I males are adapted from [32] and data for type II males and females are adapted from [92].

<i>Sex / Steroid action</i>	<i>Androgen action blocked by CA?</i>	<i>Androgen action blocked by FAD?</i>	<i>Cortisol action blocked by RU486?</i>
Type I male	Yes	No	Yes
Type II male	Yes	No	Yes
Female	No	Yes	Yes

T, suggesting that the rapid actions of T may be mediated by downstream metabolites, such as E₂ (see below). In all three adult morphs, the rapid actions of cortisol (both increasing fictive call duration in type I males and decreasing fictive call duration in type II males and females) are eliminated in the presence of mifepristone (RU486). Together these results for RU486 suggest that cortisol's rapid actions occur through a similar receptor-mechanism (sensitive to RU486) in all three morphs, but that the downstream effects on the electroresponsive properties of vocal neurons are divergent in type I males vs. type II males and females (Table 2).

7.3 T is rapidly aromatized in females but not males

The potent aromatase inhibitor fadrozole can inhibit the rapid production of estrogens from androgens in midshipman brain homogenates [48]. We therefore tested whether fadrozole treatment would interfere with the rapid actions of androgens on the VPG in the three morphs of adult midshipman. Fadrozole pre-treatment did not interfere with the rapid actions of 11-kT in type I males [92], consistent with the fact that 11-kT is a non-aromatizable androgen. Fadrozole pre-treatment also did not interfere with rapid actions of T in type II males [92], consistent with rapid actions of T occurring through an androgen-receptor-like mechanism, sensitive to androgen receptor blockade. However, fadrozole pre-treatment completely eliminated the rapid actions of T in female midshipman [92] (Table 2). This observation together with the above results for CA in females, indicate that the rapid actions of T in females occur primarily through an estrogen-receptor-like mechanism.

7.4 Rapid steroid actions are localized to the VPG region

Experiments with the isolated hindbrain-spinal region that contains the VPG have tested the hypothesis that steroids can interact with this region alone to produce rapid modulation of fictive vocalizations. Using surgical isolation,

the VPG region can be separated from descending midbrain inputs as well as ascending spinal cord inputs. In type I males the rapid actions of 11-kT and cortisol up to 30 min are still evident in this isolated VPG preparation, showing that this region is both necessary and sufficient for rapid steroid actions. In addition, when VPG is electrically stimulated directly (hindbrain vs. midbrain stimulation) in an intact preparation, rapid E_2 actions are observed up to 30 min following injection (Fig. 6) [32].

8. Steroids and vocal dimorphisms

8.1 Morph differences in burst duration at baseline

Call duration is a major trait that distinguishes brief (~50-100 msec) agonistic grunts from long duration (mins - > 1h) advertisement hums [18] (also

see [87] for toadfish). Since only type I males produce hums, we compared baseline fictive call duration across all three morphs (data were collected for [32] and [92]). One-way ANOVA revealed that fictive call duration at baseline, prior to steroid treatment, was significantly different among the three adult midshipman morphs ($F = 29.86$; $df = 2, 101$; $p < 0.0001$). *Post-hoc* tests revealed that mean fictive call duration at baseline was significantly longer in type I males (mean = 70.85 ms; SE = 6.83; $n = 41$) vs. type II males (mean = 28.96 ms; SE = 2.30; $n = 30$; $p < 0.0001$ compared to type I's) and females (mean = 23.21 ms; SE = 2.03; $n = 33$; $p < 0.0001$ compared to type I's). There were no significant differences between type II males and females in mean baseline fictive call duration ($p = 0.44$).

We can now identify at least five classes of vocal dimorphisms in midshipman. First, there is a dimorphism in vocal behavior - only type I males are known to generate hums [18]. Second, the size of the sonic muscle (including ultrastructural features of individual myofibrils) and the neuromuscular junction are greater in magnitude in type I males compared to type II males and females (reviewed in [89]; also see [90] for additional dimorphisms in metabolic enzymes). Third, vocal neurons in the hindbrain-spinal pacemaker circuitry are larger in type I males vs. type II males and females [17, 28]. Fourth, the temporal properties of the vocal pattern generator are fundamentally different: baseline discharge frequency is 15-20% higher [28] and baseline fictive burst duration is >200% longer (see above) in type I males vs type II males and females. Fifth, there are dimorphisms in the rapid neuroendocrine modulation of the vocal pattern generator. Rapid neuropeptide modulation of fictive calling is divergent among morphs, in which arginine vasotocin and isotocin are neuroactive in, respectively, type I males vs. type II males and females [51]. This intra- and intersexual divergence in rapid hormone action is now complemented by a divergence in rapid androgen modulation of the neural patterning of vocalizations: 11-kT and T are neuroactive in, respectively, type I males vs. type II males and females [32] [92]. Given the known influence of

steroids on neuropeptide expression [91], steroids and neuropeptides likely interact to rapidly modulate seasonal changes in vocal behaviors. Steroids may also have more long term, seasonally-dependent effects on the maintenance of neuropeptide expression, as shown for aromatase within the VPG and forebrain nuclei [61, 63, 64] and for the sonic muscle and VPG neurons [60-62].

9. Concluding remarks

As briefly reviewed here, a multidisciplinary, neuroethological approach (behavioral, neurophysiological and neuroanatomical) for investigating the neural basis of auditory communication in teleost fish has yielded strong evidence for steroid hormone modulation of the auditory and vocal motor systems through long (auditory, vocal) and short (vocal) term processes whose

unknown mechanisms offer exciting new avenues of future research. Thus, we have demonstrated seasonal plasticity in the auditory system of midshipman fish showing that frequency encoding among females is broadened during the breeding season to better detect the advertisement call of males. Moreover, this plasticity in the sense of hearing is dependent on elevated levels of circulating steroid hormones (T and E₂) that peak just prior to and during the reproductive period. Steroid hormones can also rapidly modulate output of the central vocal pattern generator (VPG) and mimic natural changes in vocalizations of this species. Still other studies show the presence of estrogen receptor, androgen receptor and aromatase mRNA within the vocal and auditory systems, supporting the hypothesis that regional steroid action shapes steroid-induced vocal and auditory plasticity. Given the many functional traits shared by the auditory and vocal systems of teleosts and other vertebrates, we propose that these findings elucidate general mechanisms of steroid modulation of vocal-acoustic communication in all vertebrates.

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