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Luke Remage_Healey, *University of Massachusetts - Amherst*
Andrew H Bass



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Plasticity in Brain Sexuality Is Revealed by the Rapid Actions of Steroid Hormones

Luke Ramage-Healey and Andrew H. Bass

Department of Neurobiology and Behavior, Cornell University, Ithaca, New York 14853

Divergent steroid hormone profiles can shape the development of male versus female neural phenotypes, but whether they also determine differences in the short-term, neurophysiological patterning of behavior is unknown. We now show that steroid hormone-specific modulation of a vocal pattern generator (VPG) diverges between reproductive morphs in a teleost fish. Only type I male midshipman acoustically court females, whereas type II males steal fertilizations from type I males and, like females, generate only agonistic calls. The androgen 11-ketotestosterone (11kT), but not testosterone (T), rapidly (within 5 min) increases type I VPG output. As now shown, T, but not 11kT, rapidly increases VPG output in type II males and females, consistent with the predominant circulating androgen in type II males and females (T) versus type Is (11kT). Receptor and enzyme antagonists reveal an unexpected divergence in androgen- versus estrogen-dependent mechanisms in, respectively, type II males versus females. Cortisol, the main circulating glucocorticoid, also has divergent actions: suppressing versus increasing VPG output in, respectively, type II males and females versus type Is. In summary, rapid steroid action on VPG activity is uncoupled from gonadal phenotype (convergent between type II males and females), whereas the receptor-mediated mechanisms of androgen action are predicted by gonadal phenotype (both male morphs are sensitive to androgen receptor blockade, whereas females are not). A comparable mix of neuroendocrine traits may explain the widespread distribution of intrasexual behavioral phenotypes among teleosts and vertebrates in general. Moreover, the fundamental organization/activation principles that predict the steroid-dependent expression of “maleness” and “femaleness” may now include rapid steroid actions on the neurophysiological patterning of behavior.

Key words: nongenomic; extranuclear; membrane; neuromodulation; central pattern generator; estrogen

Introduction

Neuroendocrine mechanisms are proposed to underlie variation in behavioral phenotypes, including human personality types and sexual preferences (de Vries and McCarthy, 2006). An opportunity to examine discrete rather than continuous variation in such mechanisms is provided by animals that exhibit alternative reproductive tactics (ARTs) (Rhen and Crews, 2002; Knapp, 2003). Teleost fishes, which comprise nearly half of all living vertebrates (Nelson, 1994), present many examples of male ARTs (Mank and Avise, 2006). Typically, territorial males exhibit traits adapted to nest and egg defense (e.g., large body size) and mate attraction (e.g., hypertrophied neural circuitry for advertisement calling). In contrast, noncourting “sneaker” males steal fertilizations from territorial males, achieved in part by mimicking female behavior, and exhibit a combination of traits typical of

males (e.g., sperm production) and females (e.g., nonhypertrophied calling circuitry) (Bass, 1996; Oliveira et al., 2005).

An intraspecific divergence in circulating steroid hormone levels occurs in vertebrates with ARTs (Rhen and Crews, 2002), although the neural consequences of such differences are unclear. In teleosts, circulating levels of the androgen 11-ketotestosterone (11kT) [non-aromatizable derivative of testosterone like 5- α -dihydrotestosterone (DHT)] is often several fold higher in territorial males (Brantley et al., 1993; Oliveira, 2004). In sneakers, long-term 11kT treatment can induce hypertrophied morphological traits that partially mimic those of territorials, yet 11kT does not activate territorial behavior and can intensify sneaking (Lee and Bass, 2005; Oliveira et al., 2005). This discrepancy suggests that each morph has unique steroid-dependent mechanisms that modulate the neural patterning of reproductive behavior, yet neurophysiological evidence for such mechanisms has not been forthcoming.

The midshipman fish provides a model for the direct translation of neural activity into context-dependent vocal behavior. The rhythmic firing properties of a vocal pattern generator (VPG) directly establish the temporal properties of natural calls (Bass and Baker, 1990). Territorial, nesting type I males acoustically court females with advertisement “hums,” whereas type II males neither build nests nor hum but rather steal fertilizations from type I males (all adults emit agonistic “grunts”) (Brantley and Bass, 1994). The dominant circulating androgen is 11kT in

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Correspondence should be addressed to Luke Ramage-Healey, Laboratory of Neuroendocrinology and Brain Research Institute, University of California, Los Angeles, 621 Charles Young Drive South, P.O. Box 951606, Los Angeles, CA 90095-1606. E-mail: healey@ucla.edu.

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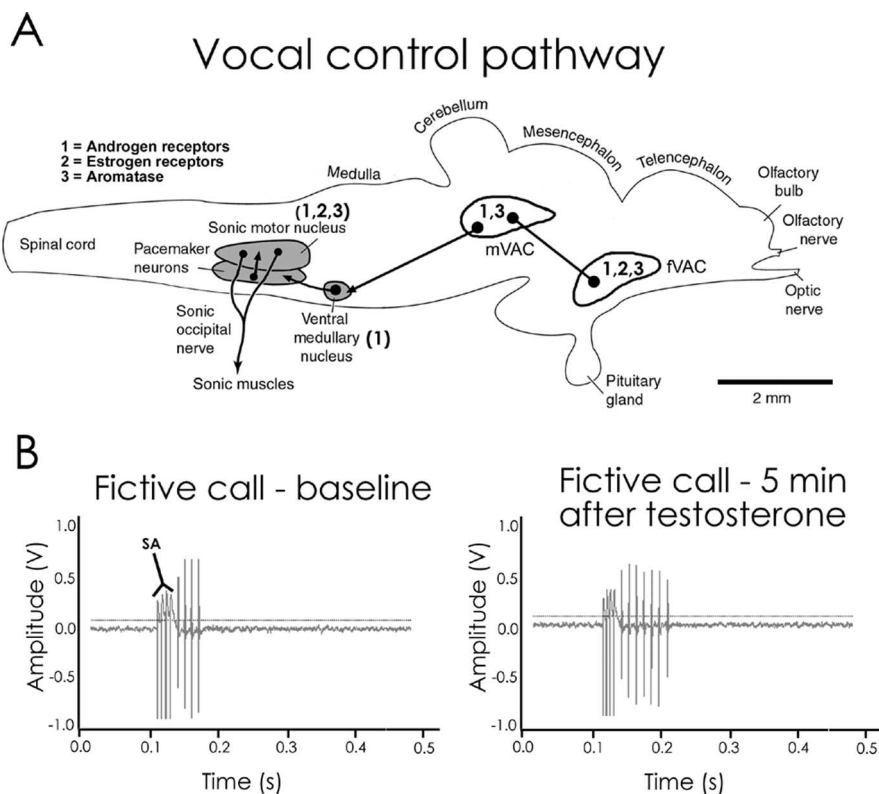


Figure 1. *A*, Vocal control pathway of midshipman fish (adopted from Goodson and Bass, 2002). Sagittal view of the brain showing pattern of connectivity of forebrain (f) and midbrain (m) vocal acoustic centers (VAC) and a hindbrain–spinal VPG (shaded region), which determines the temporal properties of natural vocalizations. The VPG includes (1) paired midline sonic motor nuclei that innervate the sonic muscles via occipital nerve roots, (2) adjacent columns of pacemaker neurons that directly establish the firing rate of the motor neurons, and (3) ventral medullary nuclei that bilaterally couple the motor neuron–pacemaker circuit and link it to the mVAC and fVAC (Bass and Baker, 1990) (also see Goodson and Bass, 2002; Kittelberger et al., 2006). The expression patterns of androgen receptors, estrogen receptors, and aromatase in the vocal control pathway of all three adult morphs are as indicated by numbers (data from Forlano and Bass, 2005; Forlano et al., 2005a,b). *B*, Oscillograms of fictive calls evoked by mVAC stimulation from a type II male; stimulus artifact (SA) is indicated. Each fictive call consists of a series of compound action potentials (rhythmic VPG activity) that predicts the fundamental frequency and duration of natural calls (see Materials and Methods). At left is a fictive call recording taken at baseline, before hormone treatment. At right is a recording taken 5 min after intramuscular injection of testosterone.

type I males, whereas it is testosterone (T) in type II males and females (Brantley et al., 1993; Knapp et al., 1999; Sisneros et al., 2004). Females have up to 20-fold higher concentrations of 17- β -estradiol (E2) than either male morph.

Although steroids such as T and E2 can shape sex differences in neural function and behavior via long-term (days to weeks) mechanisms (for review, see Arnold and Breedlove, 1985), it remains unknown whether divergent circulating steroid profiles have consequences for the rapid neurophysiological patterning of behavior in any vertebrate. Taking advantage of recent findings that steroids modulate VPG activity within minutes in type I midshipman (Remage-Healey and Bass, 2004), we show that rapid androgen actions on VPG output are predicted by the predominant circulating androgen in type II males and females (T) versus type I males (11kT). Receptor and enzyme antagonists then reveal predominantly androgen- versus estrogen-dependent mechanisms in, respectively, type II and I males versus females. Surprisingly, cortisol, the principle glucocorticoid, suppresses VPG output in type II males and females but facilitates it in type I males. Comparable, divergent neuroendocrine mechanisms may explain intrasexual behavioral variation in other vertebrates (Rhen and Crews 2002; Roselli et al., 2004), including that asso-

ciated with divergent endocrine profiles in primates (Maggioncalda et al., 2002; Morris et al., 2004).

Materials and Methods

Subjects. Females (11.4–17.1 cm) and type II males (9.3–11.2 cm) were collected from either offshore trawls or nest sites in northern California during summer and fall of 2003–2005 (Sisneros et al., 2004). Type I males (12.4–19.2 cm), collected under similar conditions, were used in a subset of experiments. Fish were maintained in artificial seawater tanks at 15–16°C on a diet of goldfish for no more than 35 d until experimentation.

Neurophysiology. All procedures, including surgery and extracellular recordings, were conducted *in vivo* and followed those presented previously (Bass and Baker, 1990; Goodson and Bass, 2000; Remage-Healey and Bass, 2004). The brain and rostral spinal cord were exposed by dorsal craniotomy under general anesthesia (0.025% benzocaine; Sigma, St. Louis, MO) and long-lasting local anesthesia [surgical-site subdermal injection of 0.25% bupivacaine (Abbott Laboratories, Chicago, IL) with 0.01 mg/ml epinephrine (International Medication Systems, El Monte, CA)]. After surgery, fish were stabilized in a Plexiglas tank and perfused through the mouth with fresh saltwater maintained at 16–17°C with a peltier device. During *in vivo* recording, exposed brain areas were covered with Fluorinert (3M, St. Paul, MN), and intramuscular injections of pancuronium bromide were used for immobilization (0.5 mg/kg; Astra Pharmaceutical Products, Westborough, MA). All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee.

Fictive calls that reflect the rhythmic activity of a hindbrain–spinal VPG were evoked by brief (25–30 ms) trains of stimuli (0.1 ms duration, 300 Hz) delivered to midbrain sites through insulated tungsten electrodes (125 μ m diameter; 5 μ m exposed tips; A-M Systems,

Calsburg, WA). The midbrain site used here and previously (Bass and Baker, 1990; Remage-Healey and Bass, 2004) projects directly to the VPG (Fig. 1*A*) (Goodson and Bass, 2002; Kittelberger et al., 2006). Fictive calls were recorded with an extracellular electrode (Teflon-coated silver wire with exposed ball tip 50–100 μ m in diameter) placed on a ventral occipital nerve root that innervates the ipsilateral sonic muscle (Fig. 1*A,B*).

Each fictive call recorded from an occipital nerve root consisted of a burst of compound action potentials, with each spike-like potential representing the synchronous activity of the ipsilateral sonic motor neurons; both nerves fire in-phase (Fig. 1*B*) (Bass and Baker, 1990). Recordings consisted of 15 fictive call responses to 15 trains of stimuli at 1 s intervals and were digitized using a Power Macintosh 8100 (Apple Computers, Cupertino, CA) with IGOR Pro software (WaveMetrics, Lake Oswego, OR). The total duration of each fictive call that is predictive of the duration of natural calls (Bass and Baker, 1990) was measured in all experiments (Fig. 1*B*). The fictive call discharge frequency (number of fictive pulses per burst duration), which is predictive of the fundamental frequency of natural calls (Bass and Baker, 1990), and burst latency, measured as the time interval between stimulus end and burst onset, were also measured for the four main steroid treatment groups (T, 11kT, E2, and cortisol).

For hormone delivery, a 23 gauge butterfly needle (infusion set SV23BLK; Terumo, Tokyo, Japan) attached to a 1 cc syringe prefilled

with hormone or drug solution (doses outlined below) was inserted into the dorsal epaxial muscle before the start of recordings. For experiments when a receptor antagonist or enzyme inhibitor was delivered before a hormone (see below), one butterfly/syringe each (antagonist/inhibitor, hormone) was inserted into the left and right dorsal epaxial muscles. For each experiment, two baseline recordings were obtained (separated by a 5 min interval) before hormone treatment. These baseline recordings were used to standardize all subsequent recordings for that experiment (relative to baseline output = 100%). For all experiments described below, records were subsequently taken at 5, 15, 30, 45, 60, 90, and 120 min after steroid injection.

Rapid steroid hormone effects. We studied the influence of the naturally occurring androgenic and estrogenic steroids T, 11kT, and E2 (see Introduction) on vocal patterning in type II males and females. Cortisol, the primary glucocorticoid in midshipman (Sisneros et al., 2004), was also studied for two main reasons. First, field studies of the closely related toadfish (*Opsanus beta*) show fourfold increases in cortisol levels during the shift from a non-calling to a calling state (Remage-Healey and Bass, 2005). Second, previous studies of type I male midshipman and toadfish identify a rapid cortisol effect on fictive call duration (Remage-Healey and Bass, 2004, 2006).

Only one steroid was delivered per experiment at doses based on the same method used previously (Remage-Healey and Bass, 2004, 2006) to achieve rapid changes in circulating steroids at physiological levels (T, 0.002 mg/kg; 11kT, 0.04 mg/kg; E2, 0.02 and 0.002 mg/kg; or cortisol, 0.05 mg/kg; all steroids from Sigma). All doses for T, cortisol, and 11kT were the lowest one used in previous studies with type I males (Remage-Healey and Bass, 2004). The two E2 doses used here were (1) the low dose used previously with type I males (0.02 mg/kg), and (2) a dose reduced by an order of magnitude (0.002 mg/kg) to further test for E2 sensitivity. Blood samples were obtained after completion of an experiment. Whole blood was separated under centrifugation, and plasma was frozen until analysis.

Receptor antagonist experiments. Previous experiments with type I males showed that the rapid actions of cortisol and 11kT were each separately blocked by specific steroid receptor antagonists (Remage-Healey and Bass, 2004). Cyproterone acetate (CA) selectively interferes with the rapid actions of androgens but not glucocorticoids, whereas the anti-glucocorticoid mifepristone [RU486 (11 β -[(4-dimethylamino)phenyl]-17 β -hydroxy-17-(1-propynyl)estra-4,9-dien-3-one)] specifically blocks the rapid actions of glucocorticoids but not androgens. Importantly, the two forms of teleost glucocorticoid receptor are antagonized by RU486 (Bury et al., 2003), and RU486 does not compete for either the nuclear or the membrane progesterone receptor in sea trout (Thomas et al., 2007). Equally important, CA is a more effective androgen antagonist than flutamide in teleost brain (Wells and Van Der Kraak, 2000). As with type I males (Remage-Healey and Bass, 2004), type II males and females were pretreated with an intramuscular injection of either CA or RU486 (0.25 mg/kg; $n = 6$ for each morph for each drug) for 30 min, and then baseline recordings were obtained as above before intramuscular injection of either (1) the steroid (T at 0.002 mg/kg after CA; cortisol at 0.05 mg/kg after RU486) or (2) nothing (a no-injection control for CA or RU486 alone). After steroid injection, a series of fictive calls were obtained as above. The most widely used estrogen receptor antagonist (ICI 182,780) was not tested here because it is not an effective blocker of rapid E2 effects in type I males (our unpublished observations).

Aromatase inhibition experiment. Results of the above experiments indicated that T and E2 exert rapid actions on fictive call duration in type II males and females. Because the enzyme aromatase is expressed in the VPG region of all adult morphs, although at much higher levels in type II males and females (Schlinger et al., 1999; Forlano and Bass, 2005), we tested whether the actions of T were attributable to its rapid conversion to E2 (Balthazart et al., 2003). The aromatase inhibitor fadrozole (FAD) blocks the rapid (5 min) conversion of T into E2 by aromatase in midshipman brain homogenates (Schlinger et al., 1999). Here, type II males ($n = 5$) and females ($n = 4$) were pretreated with an intramuscular injection of FAD (8 mg/kg) for 30 min, and then baseline measurements were obtained as above before intramuscular injection of T (0.002 mg/kg). After T injection, a subsequent series of fictive call recordings were

obtained as above. The results from the 8 mg/kg dose suggested a possible effect on the magnitude of rapid T effects in type II males (see Results), and so the dose of FAD was increased twofold (to 16 mg/kg) in a separate group of type II males ($n = 3$), and T injections and vocal motor recordings were performed as above. To fully test the androgen and morph specificity of FAD effects, an additional series of experiments were performed with type I males ($n = 3$). Males were pretreated with intramuscular injections of FAD (8 mg/kg; dose as above) for 30 min, and then baseline measurements were obtained as above before intramuscular injection of 11kT (0.04 mg/kg) and a subsequent series of fictive call recordings (see above).

Androgen specificity experiment. 11-Ketotestosterone is comparable with DHT in other vertebrates because both androgens are not converted to estrogens by aromatization. DHT can act as a potent agonist for the androgen receptor in teleosts (Olsson et al., 2005). To further test the specificity of the rapid 11kT (type I males) and T (type II males, females) effects on fictive calls, DHT was injected intramuscularly, and recordings were obtained similar to the steroid treatments outlined above ($n = 3$ for each of the three morphs). The DHT dose used (0.04 mg/kg) was equivalent to the 11kT dose used in previous experiments (see above).

Hormone assays. Plasma was analyzed for steroid hormones using radioimmunoassay (RIA) and enzyme immunoassay (EIA). Cortisol and T were analyzed with RIA at Cornell's Diagnostic Laboratory, College of Veterinary Medicine. 17- β -Estradiol and 11kT were analyzed with EIA (Cayman Chemical, Ann Arbor, MI), using techniques previously optimized for batrachoidids (Remage-Healey and Bass, 2004, 2005, 2006). All hormone doses (see above) produced levels that were within the physiological range for adult midshipman (data not shown).

Analysis. Results for hormone treatments were analyzed using Statview version 4.57 and SAS version 8 (both from SAS, Cary, NC) on within- and between-subject bases using repeated-measures ANOVA, followed by Tukey's *post hoc* tests for differences among sampling times. Baseline differences in vocal burst duration were analyzed on a between-morph basis using one-way ANOVA, and Bonferroni's *post hoc* comparisons were used to test differences in baseline durations among type I and II males and females.

Results

Rapid steroid effects on vocal patterning

In type II males and females, steroids modulated fictive call duration, and these effects varied by steroid treatment and time after injection. Repeated-measures ANOVA revealed an overall significant effect of steroid treatment ($F = 14.05$; $df = 3,196$; $p < 0.0001$), and an interaction of steroid treatment $\times$ time after injection for fictive call duration ($F = 5.82$; $df = 3,196$; $p < 0.0001$). There was no significant effect of steroid treatment ($p > 0.05$ for all effects) on burst latency or discharge frequency for either type II males (latency mean $\pm$ SEM at 30 min after injection: E2, 28.17 ± 19.08 ms; cortisol, 11.65 ± 0.59 ms; 11kT, 18.71 ± 0.71 ms; T, 13.78 ± 2.68 ms; frequency mean $\pm$ SEM at 30 min after injection: E2, 116.31 ± 6.02 Hz; cortisol, 155.89 ± 10.59 Hz; 11kT, 108.86 ± 3.87 Hz; T, 114.23 ± 7.48 Hz) or females (latency mean $\pm$ SEM at 30 min after injection: E2, 11.60 ± 0.31 ms; cortisol, 11.24 ± 1.67 ms; 11kT, 14.38 ± 1.05 ms; T, 27.20 ± 6.18 ms; frequency mean $\pm$ SEM at 30 min after injection: E2, 131.68 ± 10.87 Hz; cortisol, 175.71 ± 16.29 Hz; 11kT, 126.59 ± 13.61 Hz; T, 112.05 ± 6.03 Hz).

Testosterone and 11-ketotestosterone

Rapid increases in fictive call duration occurred after T, but not 11kT, treatment in both type II males and females.

Within-group effects

Testosterone produced significant increases in fictive call duration over time in both type II males (Fig. 2A) ($F = 5.20$; $df = 7,14$; $p = 0.004$) and females (Fig. 2B) ($F = 2.46$; $df = 7,42$; $p = 0.03$). *Post hoc* tests revealed that T produced significant ($p < 0.05$)

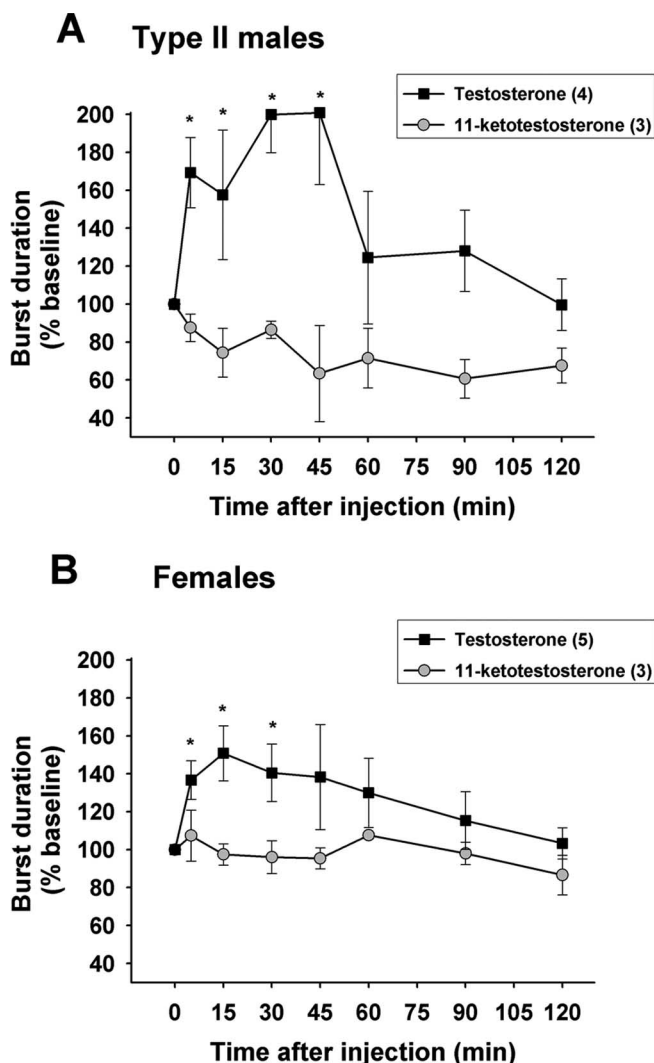


Figure 2. Rapid actions of androgens on vocal patterning in type II male (**A**) and female (**B**) midshipman fish. Testosterone (0.002 mg/kg) produces significant increases in vocal burst duration (expressed as percentage of a baseline value; see Materials and Methods) in both morphs, whereas 11-ketotestosterone (0.04 mg/kg) has no effect in either morph. * $p < 0.05$ for significant within-group differences; sample sizes are indicated in parentheses; values are mean $\pm$ SEM. Upper bound of SEM of testosterone response in type II males does not exceed 238% (data not shown).

elevations in fictive call duration in type II males and females, respectively, at 5, 15, 30, and 45 min, and at 5, 15, and 30 min after injection. There were no significant changes in fictive call duration after 11kT injection in either type II males (Fig. 2A) ($F = 2.44$; $df = 7,14$; $p = 0.07$) or females (Fig. 2B) ($F = 1.83$; $df = 7,14$; $p = 0.16$).

Between-group effects

There were significant treatment effects on fictive calling when comparing T versus 11kT in type II males (Fig. 2A) (main effect: $F = 10.58$; $df = 1,28$; $p = 0.03$; interaction over time: $F = 4.92$; $df = 7,28$; $p = 0.001$). *Post hoc* tests revealed significant ($p < 0.05$) differences between T and 11kT at 5, 15, 30, and 45 min in type II males. There was also a significant treatment effect when comparing T versus 11kT in females (Fig. 2B) (main effect: $F = 5.97$; $df = 1,35$; $p = 0.05$; interaction over time: $F = 0.91$; $df = 7,35$; $p = 0.51$). *Post hoc* tests revealed significant ($p < 0.05$)

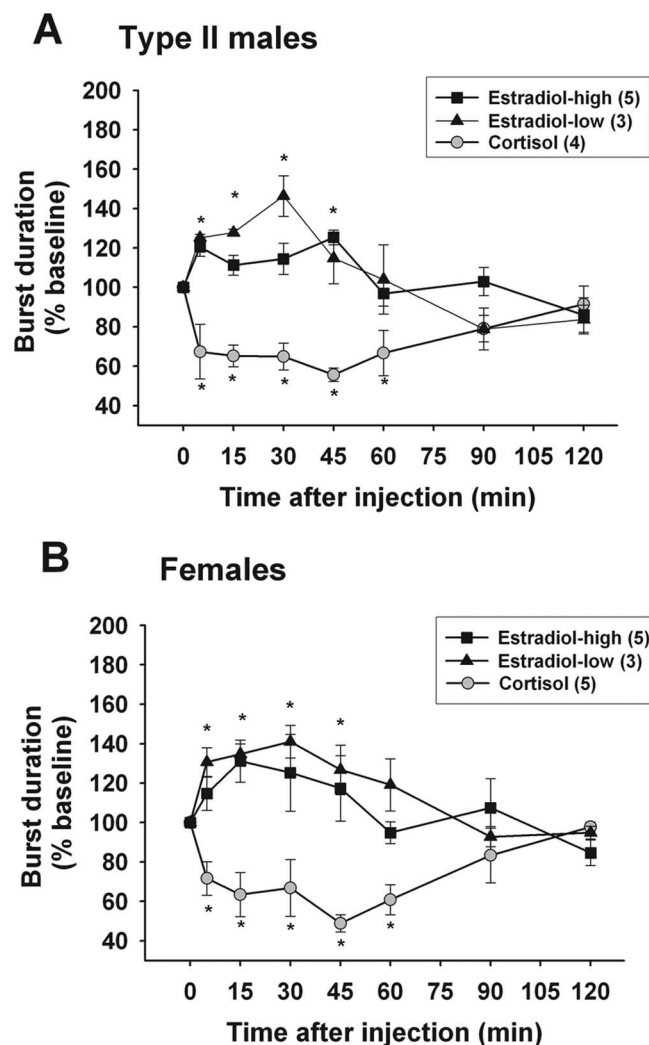


Figure 3. Rapid actions of 17- β -estradiol and cortisol on vocal patterning in type II male (**A**) and female (**B**) midshipman fish. Estradiol at both low (0.002 mg/kg) and high (0.02 mg/kg) doses produces significant increases in vocal burst duration in both morphs, and cortisol (0.05 mg/kg) produces significant decreases in vocal burst duration in both morphs. * $p < 0.05$ for significant within-group differences; sample sizes are indicated in parentheses; values are mean $\pm$ SEM.

differences between T and 11kT treatments at 5, 15, and 30 min in females.

17- β -Estradiol

Rapid increases in call duration occurred after E2 treatment in both type II males and females.

Within-group effects

Both low and high doses of E2 produced significant increases in fictive calling over time in both type II males (Fig. 3A) ($F = 11.80$; $df = 7,42$; $p < 0.0001$) and females (Fig. 3B) ($F = 6.48$; $df = 7,42$; $p < 0.0001$). For type II males, *post hoc* tests revealed that the 0.02 and 0.002 mg/kg doses produced significant ($p < 0.05$) elevations in fictive call duration, respectively, at 5 and 45 min, and at 5, 15, and 30 min after injection. For females, *post hoc* tests revealed that both doses produced significant ($p < 0.05$) increases, respectively, at 5 and 15 min, and at 5, 15, 30, and 45 min after injection. There were no significant interactions of dose $\times$ time for either type II males or females ($p > 0.05$ in each case), indicating that both E2 doses had broadly similar effects over time.

Cortisol

Rapid decreases in call duration occurred after cortisol treatment in both type II males and females.

Within-group effects

Cortisol produced significant decreases in fictive calling over time in both type II males (Fig. 3A) ($F = 3.55$; $df = 7,21$; $p = 0.01$) and females (Fig. 3B) ($F = 4.73$; $df = 7,28$; $p = 0.001$). *Post hoc* tests revealed that cortisol produced significant ($p < 0.05$) suppression of fictive call duration in both type II males and females at 5, 15, 30, 45, and 60 min after injection.

Cyproterone acetate

The rapid actions of T in type II males, but not females, were inhibited by the androgen receptor antagonist CA.

Type II males, within-group effects

In the presence of CA, T produced no significant changes in fictive call duration over time (Fig. 4A) (T + CA, $F = 1.01$; $df = 7,14$; $p = 0.46$). There was also no significant change in response to CA alone (data not shown) ($F = 1.02$; $df = 3,6$; $p = 0.49$).

Type II males, between-group effects

Cyproterone acetate significantly altered the response to T (Fig. 4A). Both the main effect of treatment (T + CA vs T alone, $F = 6.53$; $df = 1,35$; $p = 0.05$) and the interaction of treatment over time ($F = 3.6$; $df = 7,35$; $p = 0.005$) were significantly different between T + CA versus T alone. *Post hoc* tests revealed that CA significantly ($p < 0.05$) attenuated the rapid actions of T at 5, 15, 30, and 45 min after injection.

Females, within-group effects

In the presence of CA, T still produced significant changes in fictive call duration over time (Fig. 4B) (T + CA, $F = 3.56$; $df = 7,14$; $p = 0.02$). *Post hoc* tests revealed that T + CA produced significant ($p < 0.05$) elevation in vocal motor activity at 5, 15, 30, and 45 min after injection. There was no significant change in response to CA alone (data not shown) ($F = 2.55$; $df = 3,6$; $p = 0.15$).

Females, between-group effects

Cyproterone acetate did not significantly alter the response to T (Fig. 4B). Both the main effect of treatment (T + CA vs T alone, $F = 0.10$; $df = 1,35$; $p = 0.76$) and the interaction of treatment over time ($F = 1.10$; $df = 7,35$; $p = 0.38$) were not significantly different between T + CA versus T alone.

Fadrozole

Inhibition of the conversion of T to E2 by the aromatase inhibitor FAD eliminated the rapid actions of T in females but not in type II males.

Type II males, within-group effects

In the presence of the aromatase inhibitor FAD, T produced significant increases in fictive call duration over time (Fig. 4A) ($F = 7.24$; $df = 7,14$; $p = 0.0009$). *Post hoc* tests revealed that T + FAD produced significant ($p < 0.05$) elevations at 5, 15, 30, and 45 min after injection.

Type II males, between-group effects

Fadrozole did not significantly alter the response to T (Fig. 4A). Both the main effect of treatment (T + FAD vs T alone, $F = 1.06$; $df = 1,35$; $p = 0.35$) and the interaction of treatment over time ($F = 0.99$; $df = 7,35$; $p = 0.45$) were not significantly different between T + FAD low dose versus T alone. Despite no statistical differences, the mean responses in the T + FAD low-dose group

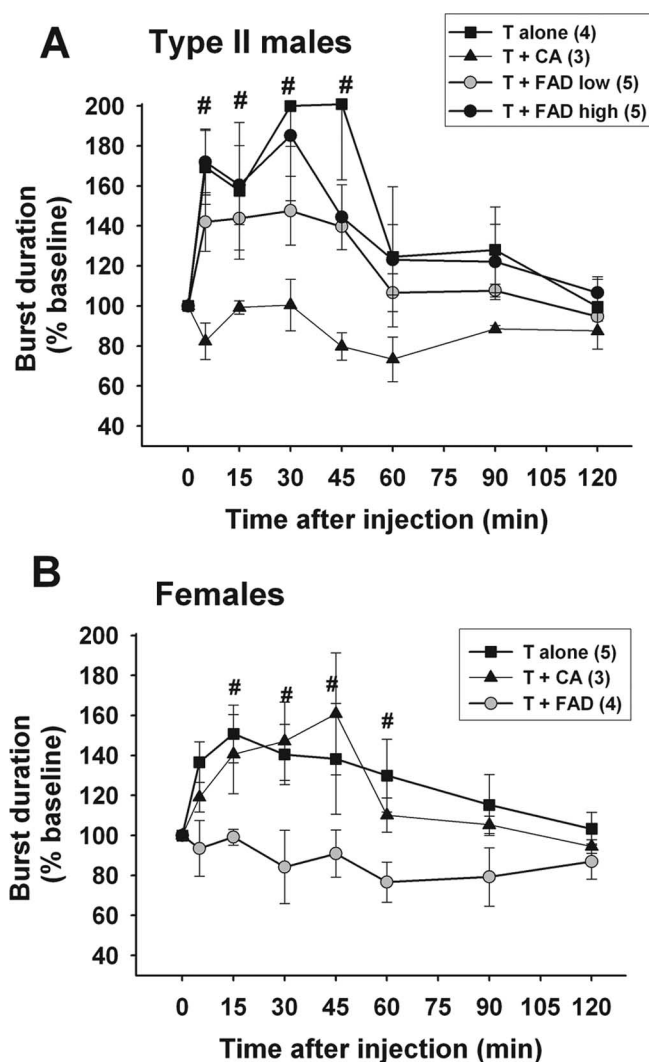


Figure 4. Rapid actions of testosterone (steroid dose as in Fig. 2) occur via distinct mechanisms in type II males (A) and females (B). The androgen receptor antagonist CA (0.25 mg/kg) blocks the rapid actions of testosterone in type II males (A), but CA has no significant effect on the response to testosterone in females (B). This indicates that the actions of testosterone occur via an androgen-receptor-like mechanism in type II males but not in females. A low dose (8 mg/kg) of the aromatase inhibitor FAD blocks the rapid actions of testosterone in females (B), but neither low nor high (16 mg/kg) doses of FAD significantly affect the response in type II males (A). Thus, the actions of testosterone occur via rapid conversion to estradiol in females but not in type II males. Testosterone data shown here are the same as in Figure 2 for each morph. # $p < 0.05$ for significant between-group differences (T alone vs T + CA) in type II males (A) and for significant between-group differences (T alone vs T + FAD) in females (B); sample sizes are indicated in parentheses; values are mean $\pm$ SEM. Upper bound of SEM of testosterone response in type II males does not exceed 238% (data not shown).

were slightly lower than in the T-alone group (see Fig. 4A). To test whether there was a dosage effect, we doubled the dose of FAD (to 16 mg/kg) for a separate set of type II males ($n = 3$). The responses were not significantly different in this T + FAD high-dose treatment from the T + FAD low-dose or T-alone groups (Fig. 4A).

Females, within-group effects

In the presence of FAD, T did not produce significant changes in fictive calling over time (Fig. 4B) ($F = 0.97$; $df = 7,21$; $p = 0.47$).

Females, between-group effects

The low dose of FAD significantly reduced the response to T (Fig. 4B). There was a main treatment effect (T + FAD vs T alone,

$F = 6.66$; $df = 1,42$; $p < 0.05$) but no interaction of treatment over time ($F = 1.01$; $df = 7,42$; $p = 0.43$). *Post hoc* tests revealed that the response to T + FAD was significantly reduced compared with T alone at 15, 30, 45, and 60 min after injection.

Type I males, FAD + 11kT treatment

Fictive call duration in type I males rapidly increased after 11kT treatment, even in the presence of FAD (data not shown) ($n = 3$; relative to baseline = 100% means were as follows: 5 min, 128.27; 15 min, 149.37; 30 min, 142.02; 45 min, 150.18; 60 min, 119.47; 90 min, 129.95; 120 min, 119.18). The effect of 11kT treatment was highly significant in the presence of FAD ($F = 24.42$; $df = 7,14$; $p < 0.005$), supporting the steroid specificity of the effects of FAD.

5- α -Dihydrotestosterone

There were no observed effects of DHT on fictive call duration in any adult morph (type Is, type IIs, or females). The overall effects of DHT were not significant for either the main treatment effect ($F = 0.35$; $df = 2,42$; $p = 0.72$) (data not shown) or for the treatment $\times$ morph interaction ($F = 0.50$; $df = 14,42$; $p = 0.92$) (data not shown). When examined within each adult morph, DHT produced no significant effects on fictive calling in type I males ($F = 0.87$; $df = 7,14$; $p = 0.55$) (data not shown), type II males ($F = 1.75$; $df = 7,14$; $p = 0.18$) (data not shown), and females ($F = 1.49$; $df = 7, 14$; $p = 0.25$) (data not shown). Although the rapid effects of 11kT alone in type I males and of T alone in type II males and females already show morph-dependent androgen specificity, the lack of an effect of DHT (which can act as androgen agonist; see Materials and Methods) further emphasizes the distinct nature of the rapid androgen mechanisms in each morph.

Mifepristone (RU486)

The rapid, suppressive effects of cortisol were reduced in the presence of the glucocorticoid receptor antagonist RU486 in both females and type II males.

Type II males, between-group effects

The glucocorticoid receptor antagonist RU486 significantly altered the response to cortisol in type II males (Fig. 5A). Both the main effect of treatment (RU486 + cortisol vs cortisol, $F = 33.29$; $df = 1,35$; $p = 0.002$) and the interaction of treatment over time ($F = 3.36$; $df = 7,35$; $p = 0.007$) were significantly different between RU486 + cortisol versus cortisol-alone treatments. *Post hoc* tests revealed that RU486 significantly ($p < 0.05$) reduced cortisol suppression of fictive calling at 5, 15, 30, and 45 min after injection. In addition, there was no significant difference between RU486 + cortisol versus RU486-alone treatments for the main effect ($F = 1.85$; $df = 1,12$; $p = 0.25$) or the interaction effect over time ($F = 2.60$; $df = 3,12$; $p = 0.09$).

Females, between-group effects

RU486 also significantly altered the response to cortisol in females (Fig. 5B). Both the main effect of treatment (RU486 + cortisol vs cortisol, $F = 11.97$; $df = 1,42$; $p = 0.01$) and the interaction of treatment over time ($F = 2.86$; $df = 7,42$; $p = 0.01$) were significantly different between RU486 + cortisol versus cortisol-alone treatments. *Post hoc* tests revealed that RU486 significantly ($p < 0.05$) reduced cortisol suppression of fictive calling at 15, 45, and 60 min after injection. In addition, there was no significant difference between RU486 + cortisol versus RU486-alone treatments for the main effect ($F = 3.65$; $df = 1,28$;

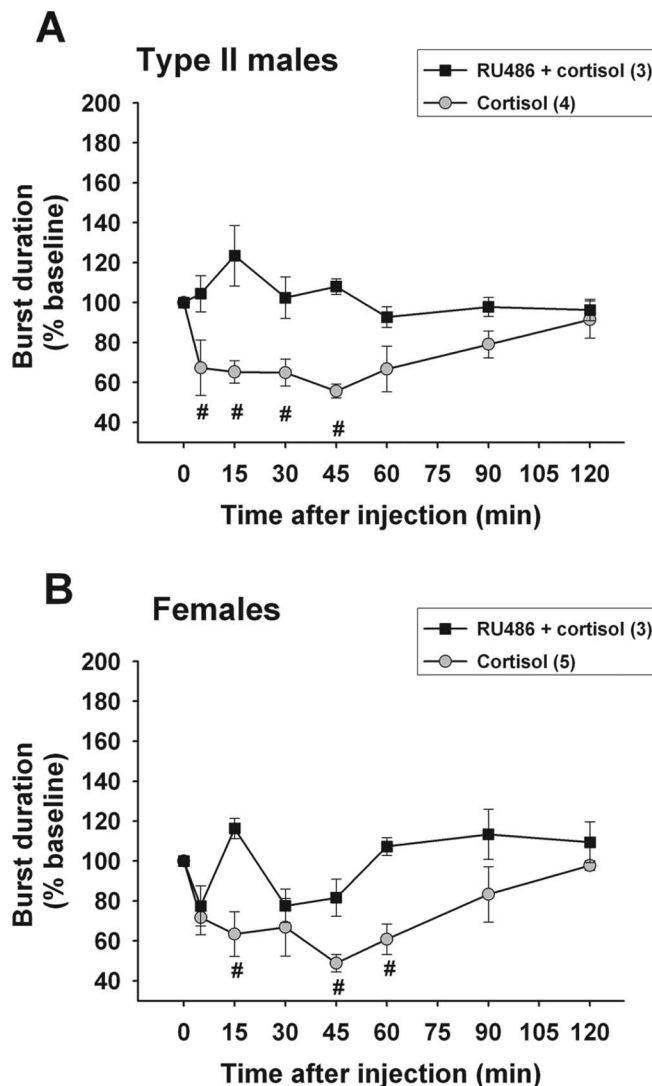


Figure 5. The glucocorticoid receptor antagonist mifepristone (RU486; 0.25 mg/kg) blocks the rapid actions of cortisol (same dose as in Fig. 3) in type II male (**A**) and female (**B**) midshipman fish. # $p < 0.05$ for significant between-group differences (cortisol vs RU486 + cortisol) in both morphs; sample sizes are indicated in parentheses; values are mean $\pm$ SEM.

$p = 0.12$) or the interaction effect over time ($F = 0.88$; $df = 7,28$; $p = 0.53$).

Discussion

In this study, divergent profiles of circulating steroid hormones have direct consequences for within- and between-sex differences in the neurophysiological patterning of a social behavior. Based on these findings, we propose that (1) the organization/activation theory of steroid hormone action can include the rapid actions of steroids on central pattern generators, and (2) comparable mechanisms play a major role in the widespread distribution of divergent neuroendocrine phenotypes among vertebrates.

Mechanisms of rapid steroid action

In addition to long-term (days to weeks) effects on brain and behavior (Crews 2005), steroid hormones can act in a rapid (seconds to minutes) manner to alter neuronal excitability (Teyler et al., 1980; Rose et al., 1995; Joels, 1997; Kelly and Levin, 2001). The rapid time course (within 5 min) for the steroid-specific effects observed here are consistent with a nontranscriptional mode of

action, via extranuclear (membrane) actions, and with the ligand specificity of extranuclear steroid binding sites in the brain (Towle and Sze, 1983; Grazzini et al., 1998) and periphery (Loomis and Thomas, 2000). The present results suggest that extranuclear actions on neurons can be inhibited by conventional nuclear steroid receptor antagonists, indicating a similarity of ligand-binding domains between nuclear and extranuclear sites of steroid action (for estrogens, see Razandi et al., 1999). Notably, the specificity of rapid androgen actions (11kT vs T vs DHT) implies that neuronal androgen binding sites are markedly “tuned” in midshipman, consistent with the specificity of both nuclear and membrane androgen receptors observed in other teleosts (Olsson et al., 2005; Thomas et al., 2006).

Intact and surgical isolation experiments in type I male midshipman localize the rapid actions of steroids to the VPG region (Remage-Healey and Bass, 2004), and neuroanatomical evidence demonstrates that estrogen and androgen receptors and the enzyme aromatase are expressed within the VPG (Fig. 1) (Forlano and Bass, 2005; Forlano et al., 2005a,b). Therefore, the VPG is a candidate locus for the rapid events observed here via nuclear and/or extranuclear steroid receptors (glucocorticoid receptor expression is under study).

Morph-specific patterns of rapid steroid sensitivity

The diversity of responses to androgens observed here reflects the predominant circulating androgen in each adult morph (11kT in type I males vs T in type II males and females). Fictive calling in type II males and females is rapidly modulated by T but not 11kT, whereas a previous study of type I males reported the inverse pattern (Remage-Healey and Bass, 2004). A comparable result occurs in rat hippocampal slices, in which sex differences in the rapid actions of steroids on neuronal excitability are related to the relative levels of circulating T and E2 (Smith et al., 2002). Together, these data indicate that one functional consequence of differences in adult steroid levels is the steroid-specific, rapid modulation of neural function and behavior (for a supporting microdialysis study, see Castner et al., 1993).

The rapid actions of E2 on fictive calling are similar in direction (increase), time course, and magnitude among all three adult morphs (Remage-Healey and Bass, 2004; this report), consistent with evidence for rich expression of aromatase, the enzyme that converts T to E2, in the VPG of all morphs (Schlinger et al., 1999; Forlano and Bass, 2005). Rapid estrogen regulation of neuronal activity is widespread in vertebrates (for review, see Joels, 1997; McEwen, 2002), consistent with evidence that the ancestral steroid hormone receptor was an estrogen-like receptor (Thornton et al., 2003).

In females alone, the rapid actions of T are completely eliminated by aromatase inhibition but not androgen receptor blockade, suggesting that T effects are entirely dependent on rapid T conversion to E2. This neuromodulatory pattern is reversed in type II males. Therefore, whereas some central aromatization of androgens and/or neurosteroid precursors into E2 may provide a local E2 source for rapid neural effects in type II males, aromatization does not account for the predominant effects of T (for similar results in hippocampal slices, see Teyler et al., 1980). Rapid changes in the androgen/estrogen ratio may yet modulate call patterning in midshipman, as suggested for reproductive behaviors in other teleosts (Black et al., 2005) and birds (Cornil et al., 2006).

The influence of cortisol on fictive calling is distinct: it rapidly suppresses fictive call duration in type II males and females (this report) but increases fictive call duration in type I males

Table 1. The rapid effects of steroid hormones on fictive call duration in plainfin midshipman are convergent between type II males and females but divergent from type I males

Sex/steroid	11kT	Testosterone	Estradiol	Cortisol
Type I male	↑	↔	↑	↑
Type II male	↔	↑	↑	↓
Female	↔	↑	↑	↓

Upward arrows indicate significant rapid elevation, downward arrows indicate significant, rapid suppression, and even arrows indicate no significant changes after steroid injection. Data for type I males are adapted from Remage-Healey and Bass (2004).

(Remage-Healey and Bass, 2004). This suggests that cortisol acts via a similar receptor mechanism (sensitive to RU486) in all three morphs but that downstream effectors such as second messengers (Abraham and Herbison, 2005) or coactivators (Balthazart et al., 2003) within vocal neurons are divergent. These results suggest that acute stress is associated with increased versus decreased vocal production in, respectively, type I males versus type II males and females. Consistent with this, type I males display agonistic grunting and/or biting when other type I or II males approach their nest, whereas type II males and females are usually silent at such times (Brantley and Bass, 1994). The prediction for type I males is consistent with elevated cortisol levels in calling, territorial males of the closely related toadfish *Opsanus beta*, which have been proposed to support the energetic demands of extensive calling through energy mobilization (Remage-Healey and Bass, 2005). The prediction for type II males and females is consistent with studies of (1) elevated glucocorticoids in type II-like male sunfish (Knapp, 2003) and in nondominant individuals in other teleosts (Johnsson et al., 2006), and (2) glucocorticoid suppression of reproductive vocal behaviors in tetrapods (Wingfield and Silverin, 1986; Marler and Ryan, 1996; Leary et al., 2006).

Field studies are just beginning to reveal the relationship between steroids and vocalizations in teleosts. Territorial male toadfishes show elevated plasma 11kT and cortisol when transitioning from non-calling to calling states (Knapp et al., 2001; Remage-Healey and Bass, 2005). 11-Ketotestosterone treatment elevates fictive call duration into the range of natural advertisement hums in type I male midshipman (Remage-Healey and Bass, 2004), whereas the current results show that E2 and T elevate fictive call duration into the range of natural grunts produced by type II males and females during the breeding season (>50 ms) (Brantley and Bass, 1994) (A.H.B., unpublished observations). We therefore predict that circulating T and/or E2 levels undergo rapid plasma and/or central fluctuations during periods of agonistic/reproductive grunting in type II males and females.

Together, these results are significant in two primary ways. First, type II males and females achieve similar neuromodulation by steroids via a mixture of separate (T) and convergent (E2, cortisol) mechanisms. Rapid T action is mainly achieved via T acting as an androgen in type II males and as an estrogen, via aromatization, in females. Thus, the patterns of steroid neuromodulation of the VPG are morph specific and reflect behavioral, and not gonadal, phenotype (Table 1). Second, however, both type I and II males exhibit neuromodulation by androgens (11kT and T, respectively), which is dependent on androgen receptors and not on aromatization (Table 2). Type II males, therefore, represent a blend of female-like vocal characters (neuronal morphology and behavior) and rapid androgen mechanisms that reflect a male gonadal phenotype.

Table 2. The mechanisms of rapid steroid action are dependent on gonadal phenotype

Sex/steroid action	Androgen action blocked by CA?	Androgen action blocked by FAD?	Cortisol action blocked by RU486?
Type I male	Yes	No	Yes
Type II male	Yes	No	Yes
Female	No	Yes	Yes

The androgen receptor antagonist CA eliminates the rapid effects of androgens in type I and type II males, but not females, whereas the aromatase inhibitor 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 for CA and RU486 are adapted from Remage-Healey and Bass (2004).

Evolution of alternative neuroendocrine phenotypes

Type II males and females: ancestral neuroendocrine phenotype

We propose that type II males and females express a suite of ancestral, “vertebrate-typical” neuroendocrine mechanisms. In type II males and females, reproduction-related motor output is augmented by both E2- and T-dependent mechanisms but suppressed by the glucocorticoid cortisol. Such roles for these steroids are present throughout vertebrates (Moore and Miller, 1984; Sapolsky, 1993; Meddle et al., 2002; White et al., 2002; Crews, 2005; Moore et al., 2005), suggesting an ancestral group of neuroendocrine mechanisms.

Type I territorial males: novel neuroendocrine phenotype

In contrast, we propose that type I males express a suite of novel neuroendocrine mechanisms. First, type I males are the only adult morph in which the teleost-specific androgen 11kT rapidly affects neural patterning. Second, unlike most vertebrates (see above), in type I males, cortisol augments, rather than suppresses, reproduction-related vocal output (Remage-Healey and Bass, 2004).

The novel phenotype adopted by type I males is closely linked to circulating 11kT, an innovation apparently specific to actinopterygian fishes (Borg, 1994), among which teleosts predominate (Nelson, 1994). Importantly, there is no sex difference in 11kT levels in either the basal teleost genus *Anguilla* (Lokman et al., 2002) or the chondrosteian *Acipenser baeri* (Cuisset et al., 1995), a more basal actinopterygian (Nelson, 1994). Thus, the generally higher plasma levels of 11kT in male teleosts represents a more derived character within this lineage and is also characteristic of the territorial/parental morph in species with ARTs (Brantley et al., 1993; Oliveira, 2004). 11-Ketotestosterone could therefore be a “basal” innovation that was permissive for the evolution of teleost ARTs. The principle role of 11-ketotestosterone in regulating the expression of type I male-like secondary sex traits (Brantley et al., 1993; Borg, 1994) and exclusive male parental care (Magee et al., 2006; Rodgers et al., 2006) suggests a key innovation in the widespread evolution of ARTs among teleosts (Mank and Avise, 2006). Recent discoveries of a teleost androgen receptor specifically activated by 11kT (Olsson et al., 2005) and an 11kT-responsive membrane androgen receptor (Thomas et al., 2006) may provide clues about the molecular origins and refinement of 11kT-dependent mechanisms.

Concluding comments

Each adult midshipman morph is characterized by a suite of traits (Bass, 1996), which can apparently evolve independently (Goodson and Bass, 2000; Lee and Bass, 2005). Accordingly, individual ART traits can be shaped by activational and/or organizational steroid mechanisms to provide a rich array of phenotypic variation. Consistent with this conceptual framework, the plasticity of rapid neuroendocrine mechanisms shown here appears to reflect

the influences of developmental organization (male vs female gonadal phenotype) on patterns of rapid neuromodulation during adulthood (morph-specific phenotype). Therefore, we propose that the fundamental organization/activation theory of steroid hormone action, [that steroids bind to nuclear receptors during development and adulthood to shape neural/behavioral phenotypes (Phoenix et al., 1959; Arnold and Breedlove, 1985; Emerson, 2000)], can be expanded to include the rapid actions of steroid hormones on the neurophysiological mechanisms of adult behavior. Comparable steroid mechanisms may explain the widespread distribution of divergent neural/behavioral patterns among teleosts with ARTs, as well as the many examples of intra-sexual divergence in behavioral phenotypes among tetrapods (Rhen and Crews, 2002; Roselli et al., 2004), including humans and other primates (Gladue et al., 1984; Maggioncalda et al., 2002; Morris et al., 2004).

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