Aromatase activity in the hindbrain vocal control region of a teleost fish: divergence among males with alternative reproductive tactics

Barney A. Schlinger¹, Christina Greco¹ and Andrew H. Bass^{2*}

¹Department of Physiological Science and the Laboratory of Neuroendocrinology of the Brain Research Institute, UCLA, Los Angeles, CA 90095-1527, USA

²Section of Neurobiology and Behavior, Cornell University, Ithaca, NY 14853, USA, and UC Bodega Marine Laboratory, Bodega Bay, CA 94923, USA

Type I male midshipman fish acoustically court females, whereas type II males do not but instead sneak or satellite spawn to compete with type I males for fertilizations. 'Singing' type I males diverge from type II males and females in the organization of an expansive hindbrain pacemaker–motoneuron circuit that establishes the physical attributes of vocalizations. Here, levels of aromatase activity were determined in homogenates of brain by measuring the conversion of ³H-androstenedione (AE) to ³H-oestrone (E₁) and ³H-oestradiol (E₂). Levels were highest in the telencephalon–preoptic area and similar for all morphs. Lower levels were in a region including the diencephalon, midbrain and cerebellum, although levels were significantly higher in females compared with type I males. In the vocal hindbrain region, aromatase levels were three- to five-fold higher in type II males and females than in type I males, and in castrated type II males than in castrated type I males. Conversion of testosterone to oestrogen in type II males and females may effectively prevent testosterone-induced maturation of the vocal system that characterizes type I males. Aromatase may thus be a key enzyme regulating the expression of individual-specific brain circuitry and behaviours among members of one sex.

Keywords: aromatase; androgen; hindbrain; oestrogen; sexual differentiation

1. INTRODUCTION

Alternative male mating tactics are known for a wide range of animals (Caro & Bateson 1986; Andersson 1994, pp. 379–395; Taborsky 1994; Gross 1996). Parallel divergence in brain organization has been most thoroughly investigated in the midshipman fish, *Porichthys notatus*, which has two male morphs, types I and II, distinguished by a suite of traits including morph-specific spawning and vocal behaviours (Bass 1992, 1996). Only type I males build a nest under a rocky shelter from where they generate mate calls ('hums') to attract females; type II males 'sneak' or 'satellite' spawn to compete with type I males for fertilizations (Brantley & Bass 1994). Although both male morphs and females generate brief 'grunts', only type I males produce trains of grunts during agonistic encounters.

A pacemaker–motoneuron circuit, extending from the caudal hindbrain into the rostral spinal cord, establishes a vocalization's fundamental frequency and duration (figure 1; Bass & Baker 1990; Bass *et al.* 1994). Sexual maturation in type II males is uncoupled from an expansive growth of the vocal circuit that accompanies maturation in the 'singing' type I males (Bass *et al.* 1996).

Androgens, but not oestrogens, can induce type I male-like vocal traits in juveniles (Brantley *et al.* 1993a; Bass 1995). One mechanism that could uncouple sexual maturation events from vocal motor growth is a decreased sensitivity to androgens in nascent type II males (Brantley *et al.* 1993a); this could be achieved by a variety of mechanisms including differential expression of sex steroid receptors or enzymes that convert androgens into active or inactive metabolites. Two of these enzymes, 5 α -reductase and aromatase, are found in the teleost brain (Callard *et al.* 1990a,b). 5 α -reductase converts testosterone into 5 α -dihydrotestosterone, whereas aromatase converts testosterone into oestradiol.

Here, we demonstrate that aromatase activity in the CNS vocal centre is several-fold greater in type II males than in type I males, either intact or castrated; our results suggest that these differences are not dependent on gonadal activity. Hence, aromatase, which has been implicated in mechanisms of sexual differentiation and maturation, and the activation of male-specific behaviours (see, for example, Adkins-Regan 1985; Schlinger & Callard 1989; Elbrecht & Smith 1992; Piferrer *et al.* 1994), may also be a key enzyme regulating the expression of divergent male phenotypes.

A preliminary report of these results has appeared in abstract form (Bass *et al.* 1997).

*Author for correspondence (ahb3@cornell.edu).

2. MATERIALS AND METHODS

Aromatase activity was determined by measuring the conversion of 1,2,6,7- ^3H -androstenedione (AE_1) to ^3H -oestrone (E_1) and ^3H -oestradiol (E_2) in homogenates of fish tissues by using procedures described previously (Pasmanik & Callard 1985; Pasmanik *et al.* 1988; Schlinger & Arnold 1991). Type I males (14.3–18.0 cm standard length), type II males (8.5–11.0 cm) and females (11.5–16.0 cm) were collected in northern California during the summer breeding seasons of 1994–1996. After deep anaesthetization with MS222 (Sigma Chemical, St. Louis, MO), whole brains, microdissected brains and gonads were removed, immediately frozen in liquid nitrogen and stored on dry ice or at -80°C until analysed (within two months of collection). For castrations, specimens were lightly anaesthetized in tricaine methane sulfonate (MS222, Sigma, St. Louis, MO) and after a ventral midline incision, the blood supply to the testes was sutured shut and the testes then removed. The incision was sutured shut, sealed (Vetbond; 3M, St. Paul MN) and the animal returned to its home aquarium. Inspection after killing at the end of the experiment verified complete removal of testes. All experimental procedures follow guidelines approved by the Cornell University Institutional Animal Care and Use Committee.

For enzyme analysis, tissues were homogenized with a Teflon-glass homogenizer in sucrose (250 mM)–phosphate (50 mM) buffer (pH 7.0). Homogenates were incubated with 133 nM or 266 nM ^3H -AE (87.5 Ci mmol $^{-1}$) in the presence of an NADH–NADPH-generating system at 22°C in a shaking water bath. Control tubes contained ^3H -substrate and cofactors but no tissue. Procedural losses of formed androgens and oestrogens were determined by measuring the recovery of 100 000 cpm ^3H -AE (on average 60%) and ^3H - E_1 (on average 20%). Reactions were terminated by snap-freezing in a methanol–dry ice bath and stored frozen until analysed.

Homogenates were extracted with ethyl ether (three times (2 ml)). Ether residues were later separated by phenolic partitioning (twice, in equal parts ice-cold 1M NaOH and CCl_4). Oestrogens remaining in the NaOH fraction were extracted with ethyl acetate. The ethyl acetate residues (to which radio-inert E_1 and E_2 were added as carriers) were then chromatographed twice on thin-layer silica gel (TLC) plates in ether–hexane (3:1). E_1 and E_2 carriers were visualized by exposure to iodine vapours. Silica gel was scraped from each product region and was eluted in 15% aqueous methanol. Aliquots of product were then added to scintillation vials containing 5 ml of Scintisafe (Fisher Scientific, Fair Lawn, NJ) for the estimation of radioactivity. Proteins were measured by Bradford's method (1976) and aromatase activity in homogenates is reported as picomoles (pmol) or femtomoles (fmol) of product (^3H - E_1 + ^3H - E_2) per minute per milligram protein.

All measures are presented as mean $\pm$ standard error of the mean. Data were analysed with a two-way ANOVA followed by post hoc Tukey–Kramer pairwise comparisons, or two-tailed *t*-tests with Systat (Systat, Inc., Evanston, IL).

3. RESULTS

To first validate methods appropriate for quantifying activity, homogenates of whole brain (one each type I and II males, female), testis (one each type I and II males), and ovary (one female) were incubated for 10, 30, or 90 mins with 133 nM or 266 nM ^3H -AE. As in other teleosts (Callard *et al.* 1990a,b), activity in brain

tissue was extremely high under these conditions (1.73–3.36 pmol min $^{-1}$ mg $^{-1}$ protein; maximal at 10 min). Oestrogens were formed in only very small amounts, of the order of 1000-fold less, in either testes (type I or type II male) or ovaries even after 90 min (0.018–0.894 fmol min $^{-1}$ mg $^{-1}$ protein). Oestrogens formed under these conditions were confirmed by recrystallization ($\times 3$): brain E_1 (994 cpm in crystals, 902 cpm in mother liquor; 2.8% error, 100% recovered), brain E_2 (343 cpm in crystals, 353 cpm in mother liquor; 1.4% error, 84.1% recovered) and ovary E_2 (52.4 cpm in crystals, 54.9 cpm in mother liquor; 2.3% error, 85.3% recovered).

To validate the aromatase reactions detected in the brain, tissue (type I male hindbrain) was incubated for 5 min with 100 nM [1,2,6,7]- ^3H -testosterone (T; specific activity 85.0 Ci mmol $^{-1}$) with or without 5 min pre-incubation of homogenates with 100 nM of the aromatase inhibitor fadrozole HCl (Ciba Geigy, Suffern, NY). As expected, in the presence of ^3H -T, ^3H - E_2 was the dominant oestrogen produced, whereas ^3H - E_1 was the dominant oestrogen formed when ^3H -AE was used as the substrate in previous experiments. Fadrozole reduced the formation of ^3H - E_2 by 82% from 0.054 to 0.010 pmol min $^{-1}$ mg $^{-1}$ protein.

A second experiment established more appropriate reaction times because of the extremely high reaction rate in the brain and low rate in gonads. Homogenates of whole brain (one each type I and II males) were incubated for 1, 5 and 10 min with 266 nM ^3H -AE; homogenates of testes (one each type I and II males) and ovaries (one female) were incubated for 30, 90 and 180 min, also with 266 nM ^3H -AE. Under these conditions, activity in testes was undetected and was very low in ovaries (0.080 fmol min $^{-1}$ mg $^{-1}$ protein). In the brain, maximal activity (9.96 pmol min $^{-1}$ mg $^{-1}$ protein) was detected at 1 min but continued to increase to 10 min. Because it was difficult to ensure uniformity in processing with the brief (1 min) incubation time, and because product formation continued to increase up to 10 min, an intermediate time (5–6 min) was used in subsequent experiments with brains. Based on these data, we concluded that the gonads express little aromatase, and no further experiments were performed on gonads. Some oestrogens could have been formed but were undetected in our assay. It remains possible that the gonads contain conjugating enzymes capable of converting free oestrogens to glucuronated and sulphated forms, which might be recovered by methods other than those used here (see, for example, Scott & Sorensen 1994).

In these preliminary time-course experiments, 5α -reduced metabolites of AE (5α -androstenedione and 5α -dihydrotestosterone) were undetected or very low in the brain. Low activity may have been the result of the relatively low substrate concentrations used (≤ 266 nM). 5α -reductase has a lower affinity for androgen (higher K_m) than for aromatase, so the abundant expression of aromatase may have obscured our ability to detect 5α -reductase under these conditions.

In the third experiment, whole frozen brains of females, type I males and type II males ($n=5$ per group) were grossly dissected into anterior (mainly telencephalon and preoptic area), medial (mainly diencephalon and

midbrain) and posterior (cerebellum and hindbrain) regions. Tissues were incubated with 133 nM ^3H -AE for 5 min and all tubes were processed together. In general, activity was highest in anterior and lowest in posterior brain regions. Values, expressed as mean $\text{pmol min}^{-1} \text{mg}^{-1}$ protein, were (type I males, type II males, females) as follows. Anterior, 16.07 ± 5.07 , 38.8 ± 12.3 , 26.6 ± 5.55 ; medial, 5.72 ± 1.73 , 8.1 ± 0.94 , 6.07 ± 0.74 ; posterior, 1.5 ± 0.4 , 6.47 ± 0.86 , 4.25 ± 0.9 . A two-way ANOVA identified a significant effect of region ($F=19.43$; d.f.=2; $p \leq 0.0001$). There was no statistically significant trend for differences across groups ($F=3.09$; d.f.=2; $p=0.058$). No interaction between group and region was observed ($F=1.26$; d.f.=4; $p=0.31$).

On average, 80% of the aromatizable substrate (^3H -AE or ^3H -T) remained after 5 min incubations of the various brain regions. In addition, only a small amount of radioactivity co-migrated on TLC plates with 5α -androstenedione; this observation suggests that there are low levels of 5α -reductase (amounting to less than 9% of the formed tritiated oestrogens). These results suggest that, under these conditions, the reaction rate for aromatization is not limited by the concentration of substrate or by competition with other androgen-metabolizing enzymes. Moreover, we counted the radioactivity remaining in the aqueous fraction after ether extraction of incubates of the different brain regions. Although we did not determine the molecular nature of this radioactivity, we saw no differences between type I and type II males; this observation suggests that differential conjugation was not occurring in brain regions of the different male morphs and hence was not interfering with our aromatase measures.

In experiment four, aromatase activity was measured in more discretely dissected brain regions of adult females, type I males and type II males ($n=6$ per group). Unlike experiment three, the brain was dissected into three regions before freezing: (i) the telencephalon and most of the preoptic area (A–B, figure 1b); (ii) a large region including the caudal aspect of the preoptic area, diencephalon, midbrain, cerebellum and an anterior segment of the hindbrain (DMC; B–C, figure 1b); and (iii) a region extending mainly from the vagal lobes to just caudal to the second pair of occipital nerve roots, which is inclusive of the entire hindbrain vocal centre (C–D, figure 1b). Homogenates of tissues from each brain region were incubated with 133 nM ^3H -AE for 6 min and processed and analysed separately. Aromatase activity in the telencephalon was high in animals from each group (figure 2a), but groups did not differ significantly (ANOVA; $F=1.048$; d.f.=2; $p=0.377$). Activity in DMC was much lower than that seen in the telencephalon (figure 2b). In this case, there was a significant group effect (ANOVA, $F=3.916$; d.f.=2; $p=0.043$) with activity in females significantly greater than in type I males ($p<0.05$). In the vocal hindbrain region (figure 2c), activity in type II males and females was similar but both were greater than the activity found in type I males (ANOVA, $F=29.379$; d.f.=2; $p<0.001$; post hoc, $p<0.0001$).

Experiment five evaluated whether differences in brain aromatase between the two male morphs was due to differences in circulating gonadal steroids (figure 3). Activity was measured in microdissected brain regions as in experiment four, but in tissue from type I ($n=5$) and

Figure 1. (a) Midshipman fish generate sounds by the contraction of paired sonic muscles attached to the lateral walls of the swimbladder. (b) Dorsal view of brain showing the position of the vocal pacemaker-motoneuron (VM-PN-SMN) circuit (see Bass *et al.* 1994). Sonic motor axons exit the brain via occipital nerves (OC) that form a single nerve, which innervates the ipsilateral sonic muscle. Also indicated are the levels where the brain was microdissected into three anterior-posterior regions (A–B, B–C, C–D). C, cerebellum; M, midbrain; OB, olfactory bulb; OL, olfactory nerve; PLLN, posterior lateral line nerve; PN, pacemaker neurons; SMN, sonic motoneurons; SP, spinal cord; T, telencephalon; V, trigeminal nerve; VM, ventral medullary neurons; VIII, eighth nerve; IX, glossopharyngeal nerve; X, vagus nerve.

type II males ($n=4$) castrated for 38 days. Homogenates of tissues from each brain region were incubated with 133 nM ^3H -AE for 5 min and processed and analysed separately for aromatase activity. As in other experiments, there was no significant difference in aromatase activity in the telencephalons of castrated type I and II males ($t=1.06$, d.f.=6, $p=0.33$). However, in both DMC and hindbrain, activity was significantly greater in type II males than in type I males (in DMC, $t=8.60$, d.f.=7, $p<0.0001$; in hindbrain, $t=16.41$, d.f.=7, $p<0.0001$).

We were concerned that morph-typical differences in body size (see § 2), and hence in brain size, could account for the significant differences in aromatase activity per milligram of protein in the hindbrain. Thus, if male morphs had equivalent total aromatase activity, the differences in hindbrain mass (with the larger type I males having the larger mass) might indicate differences in activity when expressed per milligram of protein. However, in experiment five, where the standard length of type I males was on average 43% greater than that of type II males, total hindbrain aromatase activity was still significantly greater in type II males than in type I males (0.676 ± 0.042 compared with 0.313 ± 0.009 pmol; $t=9.58$, d.f.=7; $p<0.0001$).

4. DISCUSSION

Characterization of the vocal motor system of the midshipman fish distinguishes a developmental trajectory

Figure 2. Aromatase activity ($\text{pmol E}_1 + \text{E}_2 \text{ min}^{-1} \text{ mg}^{-1}$ protein) from brain regions of type I male, type II male and female midshipman fish (legend at top). (a) Activity in the telencephalon was high in animals from each group ($4.83\text{--}6.61 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein), but groups did not differ significantly. (b) Activity in the diencephalon–midbrain–cerebellum (DMC) was much lower than in the telencephalon ($0.14\text{--}0.28 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein). Female activity was significantly greater than that of type I males ($*p < 0.05$). (c) Activity across groups in the ‘vocal’ hindbrain region was more variable ($0.096\text{--}0.544 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein). Type II male and female values were similar but significantly greater than type I males ($**p < 0.0001$).

and adult phenotype for type I males distinct from that of type II males and females (Bass 1996). Both type II males and females undergo a gonadal and behavioural maturation, which is uncoupled from the extensive growth of the vocal system that accompanies sexual maturation among type I males (Brantley *et al.* 1993b; Bass *et al.* 1996). The present demonstration of a dramatic difference between type I males and type II males + females in aromatase activity in the hindbrain region that includes the expansive vocal motor circuitry adds an important new biochemical character to the suite of traits that defines each reproductive morph (Bass 1992, 1996). The results also suggest that aromatase may be a key enzyme in a biochemical ‘switch’ mechanism that regulates the adoption and/or maintenance of a type I or a type II male- or female-like brain phenotype.

Figure 3. Aromatase activity ($\text{pmol E}_1 + \text{E}_2 \text{ min}^{-1} \text{ mg}^{-1}$ protein) from brain regions of castrated type I and type II male midshipman fish (legend at top). Telencephalon (TEL): there was no significant difference in aromatase activity between type I and II males. Diencephalon–midbrain–cerebellum (DMC): activity was significantly greater ($**p < 0.0001$) in type II males ($0.730 \pm 0.034 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein) than in type I males ($0.396 \pm 0.022 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein). ‘Vocal’ hindbrain (HB): activity was significantly greater ($**p < 0.0001$) in type II males ($0.492 \pm 0.004 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein) than in type I males ($0.160 \pm 0.002 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein).

Aside from differential brain aromatase activity, expression of a type I male vocal brain phenotype could be regulated by morph-specific androgens and/or a greater sensitivity of juvenile males to the inductive influences of androgens. Type II males and females have higher testosterone levels than type I males, whereas 11-ketotestosterone (KT), a non-aromatizable androgen in teleosts (Callard *et al.* 1981), is mainly detectable only among type I males (Brantley *et al.* 1993c). Both testosterone and KT stimulate sonic muscle growth with greatest sensitivity among juvenile type I males (Brantley *et al.* 1993a). Testosterone also induces type I male-like vocal neuron traits (both neuron size and pacemaker firing frequency (Bass 1995)). Comparable neuron data are unavailable for KT, although given KT's very low binding affinity to brain tissue (Pasmanik & Callard 1988b) testosterone's androgenic action in the vocal hindbrain may be largely due to its direct influence or conversion to another androgenic steroid.

Given the relatively high levels of aromatase and low levels of 5 α -reductase in most brain regions, most of testosterone's central action may be mediated via its aromatization to oestrogens. Oestrogens formed locally in the hindbrain of type II males and females might act directly to inhibit the development of type I male vocal circuitry. Although there is no direct evidence supporting the latter, oestrogens themselves do not induce type I male-like traits in juveniles at either peripheral or central levels (Brantley *et al.* 1993a; Bass 1995). Alternatively, aromatase might function to inactivate androgens, by converting them into oestrogens. Hence, rather than directly stimulating female characteristics, aromatase might act to ‘protect’ type II males and females against the development of type I male characteristics. This effect would be restricted to the androgenic effects of aromatizable androgens, such as testosterone, which have comparable levels in type II males and females, and

would not include the non-aromatizable androgen KT (which is essentially undetectable in these morphs (Brantley *et al.* 1993c)).

A previous study in a closely related toadfish showed higher aromatase levels in the anterior spinal cord of females than in that of males, although this was not evaluated statistically (Pasmanik & Callard 1985). The vocal motor system in toadfish resembles that of midshipman fish (Bass & Baker 1991, 1997) and the spinal cord region in this earlier study probably compares to our vocal hindbrain region. Anatomical studies in toadfish support the hypothesis that the central vocal circuit is an androgen-target tissue; testosterone-binding neurons are abundant in a reticular formation region (Fine *et al.* 1990, 1996) corresponding to the vocal circuit's ventral medullary nucleus (Bass *et al.* 1994).

The pattern of results shown here are similar to those from studies of other teleosts, which reported very high aromatase levels in forebrain and higher activity in some regions in females (Callard *et al.* 1981; Pasmanik & Callard 1985; Borg *et al.* 1987a,b; Timmers *et al.* 1987; Andersson *et al.* 1988; Mayer *et al.* 1991). High aromatase activity in teleost brain is correlated with the presence of abundant androgen-receptor expression (Pasmanik & Callard 1988b; Gelinas & Callard 1997), but is inversely correlated with expression of 5 α -reductase (Pasmanik & Callard 1988a). Because aromatase is expressed at high levels when 5 α -reductase is low, it is likely that less androgen will be available for binding to androgen receptors and more oestrogen will be formed. The present results also confirm others showing low aromatase activity in gonadal tissue, including ovarian tissue of reproductively active adult teleosts (Callard *et al.* 1981).

Aromatase expression in midshipman brain could be controlled by gonadal factors, although this seems unlikely given the maintained difference in aromatase activity in castrated type I and II males. Alternatively, it could be under direct genetic control and established early in development by the action of organizational signals (see Bass *et al.* 1996). Dimorphic male development in midshipman fish may be one of many examples where neural aromatase expression has evolved to play a role in regulating divergence of male phenotypes for any one species. Alternative male reproductive tactics are widespread among teleost fishes (Taborsky 1994); regulation of brain aromatase expression may represent one mechanism explaining the development and evolution of divergent male morphs (see, for example, Grober 1998). Given the known role of brain aromatase in the development of masculine neural structure and function in other vertebrates (Lephart 1996), the intra- and intersexual divergence in aromatase expression demonstrated here for midshipman fish may also represent a more general mechanism among vertebrates permitting the development and/or maintenance of individual-specific brain circuitry and behaviours among members of one sex.

Thanks to Margaret Marchaterre for experimental assistance. Supported by US National Science Foundation (IBN9602139 to B.A.S. and IBN9421319 to A.H.B.).

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