Steroid Regulation of Brain Aromatase Expression in Glia: Female Preoptic and Vocal Motor Nuclei

Paul M. Forlano,* Andrew H. Bass

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

Received 7 March 2005; accepted 14 March 2005

ABSTRACT: Expression of the enzyme aromatase, which converts androgens to estrogens, is known to be regulated by gonadal steroids in brain areas linked to reproduction and related behaviors in several groups of vertebrates. Previously, we demonstrated in a vocal fish, the plainfin midshipman, that both males and females undergo seasonal changes in brain aromatase mRNA expression in the preoptic area (POA) and the dimorphic sonic/vocal motor nucleus (SMN) that parallel seasonal variation in circulating steroid levels and reproductive behavior. We tested the hypothesis that steroids are directly responsible for seasonal modulation of aromatase in females because they show the most dramatic fluctuations of testosterone (T) and 17β-estradiol (E2) throughout the year. Adult female midshipmen were ovariectomized and administered T, E2, or blank (control) implants. We then quantified aromatase mRNA expression within the POA and SMN by in situ hybridization. Both T- and E2-treated females had elevated mRNA expression levels in both brain areas compared to controls. T affected aromatase expression in a level-dependent manner, whereas E2 showed a decreased effect at higher circulating levels. This study demonstrates that seasonal differences in brain aromatase expression in female midshipman fish may be explained, in part, by changes in levels of circulating steroids.

INTRODUCTION

Estrogen is synthesized in the brain by the enzyme aromatase and its regulation by gonadal steroid hormones is well documented in several vertebrate groups (review: Balthazart and Ball, 1998). Teleost fish have the highest levels of brain aromatase activity compared to other vertebrates (Callard et al., 1990). Several studies in adult teleosts have focused on the regulation of brain aromatase activity by steroid hormones (Borg et al., 1987, 1989; Pasmanik et al., 1988; Melo and Ramsdell, 2001). By contrast, few studies have investigated the regulation of brain aromatase mRNA by steroids, which was measured either by Northern blot in large brain homogenates of pooled sexes or by competitive RT-PCR (Gelinas et al., 1998; Halm et al., 2002). Although several studies have also begun to examine the effects of either synthetic or environmental steroids on brain aromatase mRNA (e.g., see Contractor et al., 2004; Kazeto et al., 2004), these are outside the context of this study, which focuses on naturally occurring steroids.

The companion study showed that both male and female midshipman fish undergo seasonal changes in brain aromatase mRNA expression (Forlano and Bass, 2005) that parallel sex-specific changes in circulating steroid levels and reproductive behavior (Sisneros et al., 2004b). In that study, mRNA expression was quantified in two brain areas: the inter- and intrasexu-
ally dimorphic sonic motor nucleus (SMN) and the gigantocellular division of the magnocellular preoptic area (PMg). The current study experimentally investigated the direct effect of testosterone (T) and 17β-estradiol (E2) on aromatase mRNA expression in the SMN and PMg to test the hypothesis that brain aromatase mRNA levels could be dependent upon variation in circulating steroid levels. Because the focus here is on the general question of steroid regulation of mRNA expression, we used adult females for all of the experimental manipulations. Although T and E2 are found in all three adult reproductive phenotypes (females and type I males: Brentley et al., 1993, Knapp et al., 1999 and Sisneros et al., 2004b; type II males: Sisneros et al., unpublished observations), wild-caught females alone have the most dramatic fluctuations of these steroids throughout the year and so we hypothesized would also be the most conducive to experimental stimulation. There may yet be sex and/or male morph differences in steroid regulation of aromatase mRNA expression, but this question must await future study.

MATERIALS AND METHODS

Animals

A total of 17 female midshipman fish were used in this study. Females (10.5–16 cm standard length) were collected when plasma levels of steroids were already low, which is during either the non-reproductive period in sites offshore from Monterey Bay, California, or the nesting period in the intertidal zone of Tomales Bay, California (Sisneros et al., 2004b). Two of six E2-implanted and three of five T-implanted females were collected from the nesting period and all others, including controls, were from the non-reproductive period. There were no obvious differences in the results between animals collected at these different times. The variances in treated animals appeared relatively low (see Fig. 2). All experimental protocols were approved by the Cornell University Institutional Animal Care and Use Committee.

Hormone Treatments and Steroid Assays

Methods follow Sisneros et al. (2004a). Fish were anesthetized in 0.025% benzocaine (Sigma Chemical, St. Louis, MO) in seawater, ovaries were surgically removed, and T (n = 5), E2 (n = 6), or control (blank) (n = 6) implants were placed in the body cavity, which was resealed with suture and Vetbond tissue adhesive (3M, St. Paul, MN), and allowed to survive 20–30 days in artificial seawater tanks. The goal of the implants was to either boost circulating steroids close to physiological levels found during the pre-nesting period or obtain basal levels found during the non-reproductive/nesting period using controls. This indeed was accomplished (see Results). Two types of implants were used because we experimented early on with different methods to obtain physiologically relevant levels of steroids. The surgical procedure was identical for both types of implants and the sizes were very similar. The controls included four animals with a silastic tube implant (1.57 mm ID × 3.18 mm OD × 5 mm length; Dow Corning model 508-008) and two with a silicone elastomer implant (RTV 6382; Factor II Inc., Lakeside, AZ) that lacked any steroid. T implants were constructed from a silastic tube to which powdered T crystals were added. Each tube end was sealed with silastic adhesive. For the E2 implants, elastomer was mixed with a measured amount of powdered E2 crystals (2 mg E2/4 mL elastomer), cured, and formed into 2 mm diameter × 5 mm thick disks. This protocol was the same used to effectively induce natural seasonal changes in auditory encoding by T and E2 in female midshipman fish (Sisneros et al., 2004a).

Females were anesthetized as above, and blood was taken by cardiac puncture via 25 g heparinized syringe and immediately perfused (see below). Blood samples were kept at 4°C for 6–12 h, centrifuged at 10,000 g for 10 min, and plasma removed and stored at −20 or −80°C until assays were performed. T assays were measured by radioimmunoassay and conducted at the Cornell Diagnostics Laboratory (New York State College of Veterinary Medicine, Ithaca, NY) and E2 assays were conducted by using an EIA kit (Cayman Chemical, Ann Arbor, MI). Circulating hormone levels were measured in all animals used in this study. All control animals were measured for T and two of these same animals were also measured for E2.

In Situ Hybridization and Quantification of mRNA Levels

The procedure for tissue preparation and in situ hybridization detection of aromatase mRNA in midshipman brain was previously published (Forlano et al., 2001). The methods for quantification also follow those from a companion study (Forlano and Bass, 2005).

Statistics

Results for mRNA quantification are expressed as mean ± standard error of the mean. Differences across treatments in the same brain area were determined by a one-way ANOVA and posthoc comparisons were performed using Fisher’s Protected Least Significant Difference tests (PLSD). Regression analysis was performed for the relationship between plasma steroid level and grain density measurements and α set at 0.05. Two-tailed t tests (α = 0.05) were used to compare expression levels of implanted females to wild-caught females (see Discussion).

RESULTS

Four brain areas, previously shown to express aromatase mRNA during the nesting or reproductive period
(Forlano et al., 2001), were the focus here, as in the companion study on seasonal and reproductive morph differences (Forlano and Bass, 2005; see this for Nissl-stained sections at similar levels as those studied here). These areas were chosen for study because they are either part of a vocal motor network [anterior parvocellular preoptic nucleus (PPa), midbrain periaqueductal gray (PAG), and sonic motor nucleus (SMN)], or involved in the neuroendocrine regulation of reproduction [gigantocellular division of the magnocellular preoptic nucleus (PMg)] (Bass et al., 1994; Goodson and Bass, 2002; Goodson et al., 2003). Levels of mRNA were quantified in only the SMN and PMg because their nuclear boundaries are the best defined, they are representative hindbrain-spinal and forebrain regions that express aromatase mRNA and protein, and their functional significance (vocal and neuroendocrine) is well established (see above).

Figure 1 shows in situ hybridization results from representative sections in all four of the brain areas studied here; both T and E2 had a similar effect on aromatase mRNA expression across all brain regions. The level of expression throughout different treatments was consistent so that relative changes seen across treatments in one area were representative of others. Thus, when SMN and PMg were high, so were PAG and PPa. Because expression in the SMN was highest along the dorsal periphery (also see Forlano et al., 2001; Forlano and Bass, 2005), measurements were separated into peripheral SMN (SMN-p) and central SMN (SMN-c) regions. Levels in SMN-p and PMg were highest and within a similar range. SMN-c levels were much lower, likely due to aromatase mRNA expression mainly in glial processes rather than their somata as in the SMN-p (Forlano et al., 2001; Forlano and Bass, 2005).

Figure 2 shows a quantitative comparison of aromatase mRNA expression (silver grain densities) in the SMN and PMg across treatments groups. There was a significant effect of treatment on the SMN-p \( F(2, 14) = 5.127; p = 0.021 \). Estradiol-treated females had significantly higher mRNA levels than blank-implanted females (ovariectomized without steroid replacement) \( (p = 0.020, \text{ Fisher’s PLSD}) \). T-treated females showed the same pattern \( (p = 0.012, \text{ Fisher’s PLSD}) \), and were no different from E2-treated females \( (p = 0.717) \). By contrast, there was no significant effect of treatment on the SMN-c \( F(2, 14) = 1.248; p = 0.317 \). There was a highly significant effect of treatment on the PMg \( F(2, 14) = 14.060; p = 0.0004 \). Similar to the SMN-p, E2-treated females had significantly higher expression levels in PMg than blank-implanted females \( (p = 0.0002, \text{ Fisher’s PLSD}) \) and T-treated females showed the same pattern \( (p = 0.0016, \text{ Fisher’s PLSD}) \). Also like the SMN-p, mRNA levels in this brain area were similar for T and E2 females \( (p = 0.393) \).

Figure 3(A) shows a regression analysis between plasma T and aromatase mRNA (grain density) in T-treated and control females. Females given T implants had elevated levels of T \( (\bar{X} = 16.72 \pm 8.24 \text{ ng/mL}) \), while T levels of blank-implanted females were low \( (\bar{X} = 1.13 \pm 0.73 \text{ ng/mL}) \). Within individual T-treated females, the PMg or the SMN-p had the highest overall expression, whereas expression in SMN-c was comparatively much lower. The samples with the lowest T levels in Figure 3(A) \( (0.28–2.28 \text{ ng/mL}) \) were from control females. Increasing T levels were highly correlated with increasing aromatase mRNA expression in the SMN-p \( (t = 9.904; p < 0.0001) \) and the PMg \( (t = 6.244; p = 0.0002) \). Expression in SMN-c was not correlated with plasma T levels.

Figure 3(B) shows a regression analysis between plasma E2 and aromatase mRNA (grain density) in E2-treated and control females. Females given E2 implants had several fold higher levels of E2 \( (\bar{X} = 7.65 \pm 2.76 \text{ ng/mL}) \) than blank-implanted females \( (\bar{X} = 0.18 \pm 0.11 \text{ ng/mL}) \). The lowest values in Figure 3(B) \( (0.1–0.26 \text{ ng/mL}) \) were from control females (all six control animals were measured for T and two of the same animals were also measured for E2). Aromatase mRNA expression was not positively correlated with plasma E2 levels in either SMN region or in the PMg. The relationship between aromatase mRNA and E2 levels resembled an inverted u-shaped function such that a mid-range of 4–8 ng/mL of plasma E2 had the greatest effect on expression levels.

**DISCUSSION**

Both T and E2 induced an up-regulation of brain aromatase mRNA expression in female midshipman fish, whereas expression levels in females with empty implants remained basal. Thus, manipulation of circulating steroids resulted in a subsequent change in brain aromatase expression, which parallels the change in circulating steroids and mRNA expression between non-reproductive and pre-nesting females in the wild, when steroid levels are at their natural low and high, respectively (Fig. 2; Forlano and Bass, 2005). These results are consistent with studies in adult goldfish in which T and E2 up-regulated aromatase mRNA in forebrain and mid/hindbrain homoge-
nates compared to gonadectomized controls, and controls showed a reduction in transcript levels compared to intact (reproductive) fish (Gelinas et al., 1998).

For ease of comparison with the results from the companion study (Forlano and Bass, 2005) examining seasonal and morph differences in aromatase mRNA levels, we have added the results from that study for both pre-nesting (P) and non-reproductive (N) females to Figure 2. Mean absolute levels of quantified aromatase mRNA expression in the SMN-p were remarkably similar in both steroid-treated (T and E2 implanted) and control females compared to wild-caught pre-nesting and non-reproductive females, respectively. Levels in the SMN-c in control females were also similar to those in wild-caught non-reproductive females. While expression levels in E2-
treated females were similar to wild-caught pre-nesting females, T-treated females had significantly less expression than wild-caught pre-nesting females in the SMN-c ($p = 0.04$, two tailed $t$ test). Lastly, although mean expression levels in the PMg were higher in wild-caught pre-nesting females compared to steroid-treated females, there was no significant difference between the two groups ($p > 0.22$, two tailed $t$ test), while the levels of wild-caught nonreproductive females were significantly greater (approximately 3.75 times) than control females ($p = 0.023$, two tailed $t$ test). The differences in expression levels in the PMg between wild-caught females and the control females may reflect the intact gonadal state of wild-caught animals that have a mixture of circulating T and E2 at all times of the year (Sisneros et al., 2004b). Thus, a synergistic effect of both T and E2 on brain aromatase, as seen in mammals and birds (review: Balthazart and Ball, 1998), may account for the higher aromatase expression levels in either non-reproductive, intact animals compared to controls (e.g., the PMg), or in pre-nesting females compared to steroid-treated ones (e.g., the SMN-c of T-treated females).

Our results show that experimental manipulation of circulating steroids can entirely mimic natural variation in aromatase mRNA expression in the SMN; however, this does not seem to be the case in the PMg. One explanation for differences in regulation between these two nuclei is that the SMN is linked to a seasonally dependent behavior (vocalization), whereas the PMg, part of the preoptic area, as in all vertebrates, is linked to a myriad of functions, which include but are not exclusive to reproduction.

Although both T and E2 clearly up regulate aromatase mRNA, each steroid showed a different relationship across a range of plasma steroid levels. T had a clear positive linear relationship while E2 did not [compare Fig. 3(A) and (B)]. T levels in T-implanted and blank-implanted females overlapped, respectively, the natural range for the spring pre-nesting period when T levels peak and the winter non-reproductive period when T levels are basal (Sisneros et al., 2004b). Although T levels beyond 10 ng/mL are outside the known physiological range, the relationship to aromatase mRNA remained positive. Interestingly, one T-implanted female with very high plasma T levels (52 ng/mL) that was not included in
the data set exhibited a grain density level in the SMN-p (3873) that was no different than that found in females with levels of 23 and 27 ng/mL, while grain density in SMN-c (129) was lower than other T-treated females, suggesting that the relationship does begin to asymptote at such levels. For E2-implanted females, E2 levels were well within the range of wild-caught females undergoing gonadal recrudescence during the pre-nesting period (Sisneros et al., 2004b). Although linear regression analysis showed aromatase mRNA expression was not positively correlated with plasma E2 levels in either SMN region or in the PMg, there clearly was a dramatic effect of elevated plasma E2 on aromatase mRNA in most individuals (Figs. 1 and 2). These data suggest that a midrange (4–8 ng/mL) level of plasma E2 has the greatest effect on expression.

One possible explanation for the difference in the relationship between aromatase mRNA levels and either T or E2 levels is that local aromatization (con-

---

**Figure 3**  (A) Regression analysis between circulating testosterone (T) and aromatase mRNA expression in the peripheral (SMN-p) and central (SMN-c) regions of the sonic motor nucleus and the gigantocellular division of the magnocellular preoptic area (PMg). For each T concentration there are three brain measurements. The lowest six T levels are blank-implanted fish. (B) Regression analysis between circulating estradiol (E2) and aromatase mRNA expression in the peripheral (SMN-p) and central (SMN-c) regions of the sonic motor nucleus and the gigantocellular division of the magnocellular preoptic area (PMg). For each E2 concentration there are three brain measurements. The lowest two E2 levels are blank-implanted fish.
version of T to E$_2$) and subsequent formation of E$_2$ in the brain may exert additional positive feedback on expression and activity levels (Pasmanik et al., 1988; Callard et al., 2001; Melo and Ramsdell, 2001; Trant et al., 2001). Estrogen, either from the circulation or made in brain from circulating T, is thought to primarily regulate brain aromatase expression in fish, because the promoter region of both the goldfish and zebrafish brain aromatase specific gene (CYP19b or CYP19A2) contains an estrogen response element (ERE) (Callard et al., 2001; Kazeto et al., 2001; Tchoudakova et al., 2001). Consistent with this, estrogen receptor alpha is highly expressed in many of the same areas as aromatase in the brain of midshipman fish (Forlano et al., 2005). In all studies to date in teleost fishes, estradiol treatment alone up-regulates brain aromatase activity and/or mRNA expression in adults (Pasmanik et al., 1988; Gelinas et al., 1998; Melo and Ramsdell, 2001; Halm et al., 2002) and embryos (Kishida and Callard, 2001; for studies using synthetic or environmental estrogens, e.g., see Trant et al., 2001; Kishida et al., 2001; Con-tractor et al., 2004; Kazeto et al., 2004). However, some studies in adults have also demonstrated that aromatase activity and mRNA can either be down-regulated or fail to induce expression by exogenous estradiol treatment at higher concentrations (Melo and Ramsdell, 2001; Halm et al., 2002). These findings may help explain the nonlinear relationship of circulating estradiol with brain aromatase in female midshipman.

Unlike T- and E$_2$-treated fish, the nonaromatizable androgen 5$\alpha$-dihydrotestosterone (DHT) had no effect on aromatase activity in goldfish (Pasmanik et al., 1988). Similarly, DHT did not affect aromatase transcripts in the forebrain but decreased aromatase mRNA in the mid/hindbrain of goldfish (Gelinas et al., 1998). In contrast, aromatase activity in theickleback can be restored after castration with either androstenedione or the nonaromatizable androgen, 11-ketoandrostenedione (Borg et al., 1989). Thus, in teleosts, there appears to be species differences in regulation of aromatase by androgens. To our knowledge, however, DHT may not naturally occur in goldfish (or most teleosts in general), thus, what may appear to be species differences may be somewhat artificial. Although 11-ketotestosterone, the principal circulating androgen in the courting type I male midshipman and many other teleosts (see Introduction), and DHT and are both nonaromatizable androgens, they have very different binding properties to the classical androgen receptor (Pasmanik and Callard, 1988). Thus, the effects of either nonaromatizable androgens alone or both estrogen and nonaromatizable androgens deserve further study in midshipman and other teleosts. Future studies will also require knowledge of the neuroanatomical distribution of androgen receptor(s) in relation to aromatase, which at this time is unknown in any teleost species.

In mammals, androgens (both T and DHT) appear to be key regulators of brain aromatase, and work through the androgen receptor, which overlaps in distribution with aromatase in the medial preoptic area and anterior hypothalamus. However, androgen regulation of aromatase is specific to brain areas (e.g., medial-basal hypothalamus and preoptic area) involved in sexual behavior and regulation of gonadotrophin release, so that aromatase in brain areas outside of “reproductive centers” may be mainly regulated by estrogens (Roselli et al., 1997, 1998; Resko et al., 2000). In birds, estradiol alone up-regulates aromatase; however, a synergistic effect between estradiol and DHT is also found in the medial preoptic area (Balthazart et al., 1996; Panzica et al., 1996).

As discussed previously (Forlano et al., 2001), aromatase in the preoptic area (including PMG) is likely involved in feedback of gonadotrophin release, while aromatase in the dimorphic sonic motor nucleus likely serves morph-specific functions. Up regulation of aromatase within this area is caused by elevated T levels in females (this study) as well as in type II males (Bass and Forlano, 2005). This positive feedback of testosterone on brain aromatase may function as a buffering system to regulate the amount of circulating steroid reaching specific brain nuclei, such as the SMN, which is androgen sensitive (Bass, 1995). The close coupling between circulating T levels and aromatase mRNA levels for the SMN may reflect the local, rapid, and perhaps inter-individual effects of E$_2$ on the duration of the sonic motor volley (which is predictive of the duration of natural vocalizations) generated by the SMN in all reproductive morphs including females (Remage-Healey and Bass, unpublished observations). Thus, regional differences in steroid production appear to contribute to sex, morph, and seasonal differences in brain aromatase, which in turn may contribute to inter- and intrasexual divergence of neurophysiological events and behavior in vertebrate species in general.

Thanks to Joseph Sisneros for performing implant surgeries and assistance with hormone assays, Christina de Roos for quantification of in situ hybridization, Luke Remage-Healey and Nick Santangelo for statistical advice, and Elizabeth Adkins-Regan for helpful comments on the manuscript. We also thank two anonymous reviewers for comments that greatly improved the manuscript.
REFERENCES


