Published in final edited form as:
Horm Behav. 2007 January ; 51(1): 164–170.

Social Dominance Regulates Androgen and Estrogen Receptor Gene Expression

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Abstract

In *Astatotilapia burtoni*, dominant males have higher levels of sex steroid hormones than subordinate males. Because of the complex regulatory interactions between steroid hormones and receptors, we asked whether dominance is also associated with variation in sex steroid receptor gene expression. Using quantitative PCR, we compared the expression of specific subtypes of androgen (AR) and estrogen (ER) receptor genes between dominant and subordinated males in 3 divisions of the brain, the pituitary, and the testes. We measured mRNA levels of AR-α, AR-β, ER-α, ER-βa, and ER-βb, gonadotropin releasing hormone 1 (GnRH1), and GnRH receptor 1 (GnRH-R1) relative to 18S rRNA. In the anterior part of the brain, we found that dominant males had higher mRNA expression of AR-α, AR-β, ER-βa, and ER-βb, but not ER-α, compared to subordinate males. This effect of dominance was reflected in a positive correlation between testes size and AR-α, AR-β, ER-βa, and ER-βb in the anterior brain. In addition, mRNA levels of all ARs and ERs in the anterior brain were positively correlated with mRNA level of GnRH1. In the middle and posterior portions of the brain, as well as the testes, steroid receptor mRNA levels were similar among dominants and subordinates. In the pituitary, ER-α mRNA level was positively correlated with testes size and AR-α mRNA was positively correlated with GnRH-R1 mRNA level. These data suggest that dominant male brains could be more sensitive to sex steroids, which may contribute to the increased complexity of the behavioral repertoires of dominant males.

Keywords

steroid hormone receptor; testosterone; estradiol; social dominance; evolution; cichlid; teleost; fish

In the cichlid fish *Astatotilapia (Haplochromis) burtoni*, social status regulates male fertility by controlling the brain-pituitary-gonad (BPG) axis. Consequently, dominant males have 40% larger testes relative to body size compared with subordinate males. Ultimately, dominance affects testes size through regulation of gonadotropin-releasing hormone 1 (GnRH1) production by neurons in the anterior parvocellular preoptic nucleus (aPPn) of the hypothalamus (Davis and Fernald, 1990; Francis et al., 1993; White et al., 2002). The enlarged testes of dominant males contain a higher proportion of mature sperm (Fraley and Fernald, 1982), and produce higher levels of testicular hormones, including 11-ketotestosterone and testosterone (Parikh et al., 2006). The higher levels of circulating androgens in dominant males have behavioral (Francis et al., 1992) and physiological (Soma et al., 1996) consequences. To

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more fully understand the potential influence of differences in sex steroid hormone levels, it is important to understand whether and how sex steroid receptors might also be regulated by social status. For example, if the higher androgen levels of dominant males are accompanied by lower expression of steroid receptors in the brain due to negative feedback regulation, higher androgens might not have behavioral consequences for the dominant males. If, on the other hand, increased levels of androgens are accompanied by similar or higher levels of steroid receptor expression, the tissue might be more sensitive to steroid levels. Because effects of androgens are sometimes mediated by estrogen receptors following aromatization, we were interested in both androgen and estrogen receptor expression. Thus, we asked whether dominant and subordinate males differ in the expression of androgen (AR) or estrogen (ER) receptor genes at multiple points of the BPG axis.

Among teleosts, two androgen receptors (AR-α, AR-β) and three estrogen receptors (ER-α, ER-βa, ER-βb) of the regulatory transcription factor type have been identified. This elaboration of the number of steroid receptor gene paralogs, like in other gene families, was the result of an early genome duplication in the lineage leading to teleosts (Hoegg et al., 2004). The three ERs have distinct pharmacological characteristics (Hawkins and Thomas, 2004), and they have different tissue distributions (Choi and Habibi, 2003; Halm et al., 2004; Pinto et al., 2006). All ERs are expressed in the brain, pituitary, and gonad (Choi and Habibi, 2003) and, in the female brain, all three ERs are expressed in the hypothalamus, in overlapping but distinct patterns (Hawkins et al., 2000; Menuet et al., 2002), suggesting that each receptor type has a role in some aspects of neuroendocrine function. Direct comparison of neural expression levels of the three ERs outside of the hypothalamus has not been reported. Less is known about the functional attributes or tissue distribution of the two androgen receptors of teleosts. Like the ERs, the two ARs show distinct binding profiles (Sperry and Thomas, 2000) and their expression varies with seasonal changes in gonadal growth (Sperry and Thomas, 1999). Less is known about the tissue distribution of the AR subtypes. More generally, little is known about how these receptors are regulated by reproductive capacity in males.

To address this question, we compared the relative levels of gene expression for these receptor subtypes in the brain, pituitary, and testes of dominant and subordinate males using quantitative polymerase chain reaction (PCR). In addition, we determined whether variation in steroid receptor gene expression corresponded to different measures of reproductive capacity in the brain (GnRH1 expression), the pituitary (GnRH receptor 1 expression), and the testes (testes size relative to body size). Although three forms of GnRH are expressed in *A. burtoni*, we did not investigate GnRH2 or GnRH3 because their expression does not vary with reproductive capacity in *A. burtoni* (White et al., 2002). Likewise, although there are two receptors for GnRH, we focused on GnRH-R1 because it is expressed in gonadotropes (Chen and Fernald, 2006), and its expression is regulated by reproductive capacity (Au et al., 2006).

Methods

All work was performed in accord with accepted standards of humane animal care and was approved by the Stanford University Administrative Panel on Laboratory Animal Care committee.

Identification of ER-βa and ER-βb cDNA sequence

Although *A. burtoni* specific sequences for AR-α, AR-β, and ER-α cDNA were available through Genbank (see Table 1), at the start of the study sequence for *A. burtoni* ER-βa and ER-βb were unknown. To identify the cDNA sequence for these transcripts, we used a combination of PCR and library screening as follows.

To identify ER-βa cDNA sequence, we used PCR followed by a phage library screen. To prepare the cDNA for PCR, we extracted total RNA from estradiol-primed female liver using Trizol (Invitrogen, Carlsbad CA) and synthesized cDNA using an anchored poly-T primer and Superscript II reverse transcriptase (Invitrogen). To amplify ER-βa cDNA, we designed the following primers based on the sequence of Nile tilapia ER Type II: forward 5'-GCC CAT CTG TAT CCG CTC AC-3' and reverse 5'-ATC ATG ACG CTT GCT TCG GTC-3'. We amplified an 800 base pair fragment on a thermal cycler (PCR Express, Thermo Electron, Waltham, MA) using the following program: 1 min at 94° C followed by 5 cycles of 30-sec at 94° C, 30-sec at 60° C, 90-sec at 72° C, followed by 10 cycles of 30-sec at 94° C, 30-sec at 60-51° C (decreasing 1 degree per cycle), 90-sec at 72°C, followed by 20 cycles of 30-sec at 94°C, 30-sec at 50°C, 90-sec at 72°C, and a final step of 5 min at 72°C. We gel extracted the amplicon and reamplified it before cloning and sequencing. We identified the fragment as ER-βa based on sequence similarity to known ER-βa sequences using BLAST.

To obtain a full-length ERβa clone, we screened an *A. burtoni* brain cDNA phage library (Stratagene, La Jolla CA) using a 174 bp probe corresponding to nucleotides 906..1079 of the full length clone. Duplicate membranes were incubated with the ^{32}P-deoxy CTP labeled probe produced by random priming (DECAprime II, Ambion, Austin, TX) overnight at 65°C in hybridization buffer (30% formamide, 0.6M NaCl, 0.04 M sodium phosphate, 2.5 mM EDTA, 1% SDS). For the primary screen, we washed the membranes twice at room temperature and twice at 55°C with 2× SSC containing 0.1% SDS, and exposed the membranes to film to identify potential clones. These potential clones were excised and screened a second time as above, yielding three positive full-length cDNA clones (Genbank Accession No. DQ862128).

To isolate a subclone of ERβb, we designed the following degenerate primers to regions that differentiate ERβa and ERβb in other teleosts: forward 5'-GGG GAT GCA GAC GGT C-3', reverse 5'-GAC CTG AAG AAT CCN TA-3'. RNA extraction, cDNA synthesis, and PCR reaction assembly was carried out as described above for the cloning of ERβa. To amplify the 1410 bp fragment, we used the following PCR cycling program: 1 min at 94° C, followed by 5 cycles of 30-sec 94° C, 30-sec 60° C, 90-sec 72° C, followed by 10 cycles of 30-sec at 94° C, 30-sec at 60-50°C (decreasing 1 degree per cycle), 90-sec 72°C, followed by 20 cycles of 30-sec at 94°C, 30-sec 50°C, 90-sec 72°C, and final elongation for 5 min at 72°C. The PCR product was sequenced and determined to have high similarity to ERβb sequences. We then used rapid amplification of cDNA ends (SmartRACE, Clontech, Palo Alto, CA) with the *A burtoni* gene-specific primer 5'-GTA AGG GTT GGA CTG GGT TCT CGG G-3' to isolate the 3' end of the cDNA (Genbank Accession No. DQ862129).

Animals and tissue collection

Adult male and female *A. burtoni* were housed together in aquaria maintained under conditions mimicking those of their natural habitat (12:12 L:D, full spectrum lights, pH = 8.0, 26 C; Fernald and Hirata, 1977). We used focal observations to determine the dominance status of individual males over a greater than 3-week period as follows. We observed each male in a tank every 1-3 days for a 3-min period to determine whether a male was dominant or subordinate. We categorized a male as dominant if he defended a territory and displayed dominant coloration (eyebar and body coloration). We categorized a male as subordinate if he did not defend a territory and did not display dominant coloration. Only males whose status was consistent and unambiguous for at least 3 weeks were included in the study. This period of time is sufficient to identify males whose status-related physiological characteristics (e.g., GnRH1 neuron size) are stable (White et al., 2002).

Using the above criteria, we chose three subordinate and three dominant males from three different community tanks (total n = 18), and we accounted for potential variation due to community tank in our statistical analysis (see Statistics below). We weighed subjects, killed

them by rapid decapitation, and then collected their brains, pituitaries, and testes. Testis weight was used to determine gonadosomatic index (GSI; ratio of testes mass to body mass multiplied by 100). Using a scalpel, we divided the brain into anterior, middle, and posterior parts by transversely sectioning the tissue at the anterior commissure and between the optic tectum and cerebellum. As a result, the anterior portion contained the entire telencephalon and a portion of the aPPn. The middle portion contained the diencephalon, including the remainder of the aPPn, the pretectum, and the midbrain; the posterior portion contained the hindbrain. *Post facto* we determined that the anterior portion of the brain included part, but not all, of the aPPn because both the anterior and middle portions show GnRH1 expression (see Results) and GnRH1-expressing neurons are located in the most anterior part of the aPPn (Davis and Fernald, 1990; Fernald and Shelton, 1985).

Following dissection, tissue was immersed in Trizol, frozen in an ethanol-dry ice bath, and stored at -80° C until RNA extraction. To extract RNA, we homogenized the tissue and followed the Trizol protocol. Because of the large number of samples (total = 90), we extracted RNA in multiple groups organized by tissue and tank (e.g., we extracted RNA from all the testes of males from tank 1 simultaneously). We accounted for possible variation introduced by RNA extraction group in our statistical analysis (see Statistics below). RNA integrity and concentration was estimated from spectrophotometric absorbance at 260 nm and 280 nm. We treated the RNA with DNase (Turbo DNA-free, Ambion, Austin TX) to remove contaminating genomic DNA before synthesizing cDNA using random hexamer primers and Transcriptor reverse transcription (Roche Applied Science, Indianapolis IN).

Quantitative PCR

We used quantitative PCR to measure mRNA expression of two ARs, three ERs, and the reference transcript 18S in all 5 tissues. In addition, we measured mRNA expression of GnRH1 in the anterior and middle portions of the brain as well as GnRH-R1 mRNA from the pituitary. We designed primers (Table 1) using a strategy described in Greenwood et al. (2003) that maximizes reaction efficiency. For the real-time PCR reaction, we used iQ Sybr Green Supermix (Bio-Rad Laboratories, Hercules CA) and our primers at 0.5 μM concentration. The concentration of cDNA included in the reaction varied, and this variation was accounted for by our measurement of 18S for each sample (see below). The PCR parameters were 3 minutes at 95°C followed by 40 cycles of 95°C, 60°C, and 72°C for 30 seconds each. We used PCR Miner (Zhao and Fernald, 2005) to calculate the reaction efficiencies and cycle thresholds from the fluorescence readings of individual wells during the reaction. The reaction efficiencies were 1.9-2.1. For each sample, we calculated a mean cycle threshold of three PCR reactions. We calculated the expression of each target gene of interest relative to 18S using the equation: relative target gene expression $=100 * (E_{18S}\text{^}CT_{18S})/(E_{target}\text{^}CT_{target})$, where E was reaction efficiency and CT was cycle threshold (Pfaffl, 2001). Thus, expression of the target genes are expressed as a percent of 18S rRNA expression.

Statistics

In our comparison of dominant to subordinate males, the factor of interest in the experiment was social status. In addition to this factor, we accounted for error attributable to the confounded variables of community tank and RNA extraction group by including the factor “tank/group” in the analyses. To test for effects of status on mRNA expression of ARs, ERs, GnRH1, GnRH-R1, and on GSI, we used multiple two-way analysis of variance (ANOVA) tests with status and tank/group as between subject factors although we did not include the interaction between status and tank/group in the model. We do not report or discuss effects of tank/group, and in all ANOVA models we used Type III sums of squares. When comparing dominants to subordinates in our graphical representations, we used the estimated marginal means and

standard errors, which represent the effect tested by the ANOVA model (i.e., status with effect of group/tank removed); we present graphs of data for which $p < 0.1$.

In addition to the effects of social status, we were interested in whether the ARs and ERs covaried with our different measures of reproductive capacity, namely, GnRH1 expression level in the brain, GnRH-R1 expression level in the pituitary, and GSI. Specifically, we examined the following relationships: within the anterior and middle parts of the brain, covariation of each SR mRNA expression with GSI; within the anterior and middle parts of the brain, covariation of each SR mRNA expression with GnRH1 expression; within the pituitary, covariation of each SR mRNA expression with GnRH-R1 expression; and, within the testes, covariation of each SR mRNA expression with GSI. For our analysis of covariation with GnRH1, we used the sum of the expression level in the anterior and middle brain parts to represent GnRH1 expression by the entire population of GnRH1 neurons. Because we wanted to examine all of these relationships independently of the influence of tank/group, we used multiple regression analysis with tank/group coded by dummy variables. Specifically, the model included three independent variables (two dummy variables representing tank/group and either GSI, GnRH1, or GnRH-R1) and one dependent variable (the AR or ER). Using this model, we ignored the overall explanatory power of the combination of independent variables, and focused on the independent contribution of the variable representing the reproductive axis (GSI, GnRH1, or GnRH-R1) on AR or ER expression. We used the significance test of the beta weights (also called the standardized coefficients) to evaluate whether the reproductive axis variable influenced a specific receptor's expression, and the partial correlation to assess the direction of the relationship. Finally, to represent the relationships, we graphed the partial plots, which were the unstandardized residuals of each variable after the effect of tank/group is removed; we present graphs of data for which $p < 0.1$.

Sample sizes were 18 for each tissue and gene except for the testes. During the PCR reaction for the testes, air bubbles were inadvertently introduced into a small number of wells which resulted in abnormal fluorescence curves and necessitated the exclusion of these samples from the analyses; the resulting sample sizes were 16 (ER-α), 16 (ER-βa), 15 (ER-βb), 15 (AR-α), and 14 (AR-α).

Results

Replicating previous results (e.g., White et al., 2002), the dominant males in our study had larger testes than subordinate males, as measured by GSI ($F_{1,14} = 5.5$, $p = 0.034$), and higher expression levels of GnRH1 mRNA in the brain ($F_{1,14} = 7.3$, $p = 0.017$). However, unlike a previous study which found higher GnRH-R1 expression in dominant males (Au et al., 2006), we found similar GnRH-R1 in the pituitaries of dominants and subordinates (estimated marginal means ± SE: dominant males, 23.5 ± 7.0; subordinate males, 21.2 ± 7.0; $F_{1,14} = 0.6$, $p = 0.82$). Further examination suggested that data from one subordinate male was an outlier with respect to GnRH-R1 expression; this male had GnRH-R1 expression levels that were 95% of 18S compared to a range from 3.0 to 29.0% for all other males in the study. This male did not appear to be an outlier on any other measure, including GSI and GnRH1 expression. When this subject was removed from the analysis of pituitary GnRH-R1 mRNA levels, dominant males had slightly higher mean levels than subordinate males, although the difference was not statistically significant (estimated marginal means ± SE: dominant males, 21.2 ± 2.6; subordinate males, 14.4 ± 2.8; $F_{1,13} = 3.1$, $p = 0.1$). All subsequent analyses that involved GnRH-R1 expression exclude this subordinate male.

Effects of social status on expression of AR and ER mRNA levels are reported in Table 2. In the anterior portion of the brain, dominant males had higher expression of AR-α, AR-β, ER-βa, and ER-βb (Fig. 1A-D), whereas ER-α was similar in dominants and subordinates. In the

middle portion of the brain, AR-α was higher in dominants, but this difference was not statistically significant (Fig. 1E); mRNA expression of the other SRs was similar in dominants and subordinates. In the posterior part of the brain, dominant and subordinate males had similar levels of receptor expression (all $p > 0.1$). In the pituitary, dominant males had higher ER-α expression than subordinates, but this difference was not statistically significant (Fig. 1F); all other SRs had similar expression levels. Status had no detectable effect on any steroid receptor mRNA expression in the testes (all $p > 0.2$).

Given that dominance is associated with enhanced activity of the reproductive axis, we asked whether variation in receptor expression level was correlated with variation in GSI, GnRH1 expression in the brain, or GnRH-R1 expression in the pituitary. For GSI, the pattern of correlations with receptor expression level generally matched the pattern for the effect of status (Fig. 2). Specifically, we found that, in the anterior part of the brain, GSI was positively correlated with AR-α (Fig. 2A; $r = 0.73$, $p = 0.001$), AR-β (Fig. 2B; $r = 0.57$, $p = 0.02$), ER-βa (Fig. 2C; $r = 0.66$, $p = 0.005$), and ER-βb (Fig. 2D; $r = 0.55$, $p = 0.028$). In the middle part of the brain, GSI was weakly positively associated with AR-α (Fig. 2E; $r = 0.44$, $p = 0.085$). In the pituitary, we found a strong positive correlation between GSI and ER-α (Fig. 2F; $r = 0.74$, $p = 0.001$). In the testes, we did not detect any relationship between any steroid receptor mRNA expression and GSI (all $p > 0.2$). We also examined the correlation between GnRH1 mRNA and SR mRNA expression levels. We found significant positive correlations for all SRs in the anterior portion of the brain with GnRH1 (Fig. 3; AR-α: $r = 0.75$, $p = 0.001$; AR-β: $r = 0.74$, $p = 0.001$; ER-α: $r = 0.71$, $p = 0.002$; ER-βa: $r = 0.72$, $p = 0.002$; ER-βb: $r = 0.71$, $p = 0.002$), including ER-α, which did not show an effect of status or GSI. In the middle portion of the brain, we did not detect any significant correlations, including for AR-α, for which we detected weak relationships with status (Fig. 1E) and GSI (Fig 2E).

Since regulation of GnRH receptors in the pituitary may be an important part of social regulation of the BPG axis and SRs may contribute to mediating this relationship, we examined their correlation within the pituitary. We detected a strong relationship between GnRH-R1 and AR-α ($r = 0.73$, $p = 0.002$) and a weaker relationship with AR-β ($r = 0.48$, $p = 0.07$) that appears to be weakened statistically primarily by a single outlying point (Fig. 4). In addition, ER-α was correlated with GnRH-R1 (Fig. 4; $r = 0.54$, $p = 0.038$) although the data are quite scattered, thus reducing our confidence in this conclusion.

Discussion

In *A. burtoni*, social dominance determines reproductive capacity of males through regulation of the brain-pituitary-gonad (BPG) axis. The increased activity of the BPG axis results in increased production of sex steroid hormones (Parikh et al., 2006) which in males are primarily androgens. Using quantitative PCR, we found that dominance and reproductive capacity were associated with increased mRNA expression of two androgen receptors and two estrogen receptors in the brain. In the pituitary, only ER-α was regulated by reproductive capacity, although expression of pituitary AR-α was correlated with GnRH-R1. We did not find a difference in GnRH-R1 mRNA expression in the pituitary between dominant and subordinate males unlike in a previous study (Au et al., 2006). The reasons for the discrepancy between the studies is not clear, although it suggests that GnRH-R1 mRNA expression is highly labile and may be regulated by factors in addition to social status. In the testes, we found no evidence that reproductive capacity was associated with changes in expression of ARs or ERs, in spite of the fact that enlarged testes are associated with increased production of mature sperm (Fraley and Fernald, 1982) and increased androgen production (Parikh et al., 2006).

One of the striking aspect of our results was the fact that, in the anterior brain, expression of all ARs and ERs were regulated by reproductive capacity: all but ER-α were expressed at higher

levels in dominant males and were positively correlated with GSI, and expression of all ARs and ERs were positively correlated with GnRH1 expression. ER-α was the only receptor, among those measured, that did not show a robust relationship between reproductive capacity and expression levels, suggesting that ER-α may have a different role in the brain than the other ERs or ARs. In addition, this increase in expression of the SRs, if reflected in increased protein levels (You and Yin, 2000), implies that the anterior part of the brain could have increased sensitivity to steroid hormones. Such increased sensitivity may enhance the well described negative feedback responses of GnRH1 neurons to circulating steroid levels (e.g., Soma et al., 1996), or increase steroid sensitivity of brain regions involved in behavior. Given that dominant males have higher, not lower, androgen levels, it seems unlikely that GnRH1 neurons, or the brain regions regulating them, have enhanced negative feedback, a conclusion supported by castration studies (Soma et al., 1996). Recent data describing the neuroanatomical distribution of AR-α and AR-β in dominant males shows that AR-β has a wider distribution than AR-α, with AR-β expressed in the pallium and subpallium in addition to hypothalamic regions (L.K. Harbott, S.S. Burmeister, R. White, M. Vagell, and R.D. Fernald, unpublished). This distribution pattern suggest that, at least for AR-β, increased expression in dominant males has the potential to influence brain regions involved in sensory processing, behavioral motivation, as well as regulation of the BPG axis. Information about receptor distribution (particularly the ERs) and the relative binding capacity of the receptors will further inform the implications of the current study. Without additional studies that incorporate tissue localization, our conclusions must remain cautious given our relatively imprecise anatomical resolution.

We were surprised to find robust effects of differences in reproductive capacity on AR and ER mRNA expression in the anterior, but not middle, portions of the brain since all but part of one hypothalamic nucleus (the aPPn) were included in the middle portion whereas the anterior portion consisted primarily of the telencephalon. The hypothalamus is generally known to express high levels of steroid hormone receptors and is likely to be a central player in generating behavioral and physiological responses to changes in steroid hormone levels. Studies from *A. burtoni*, as well as other teleost, suggest that all AR and ER subtypes are expressed in regions of the preoptic area and hypothalamus (Harbott et al. unpublished; Hawkins et al., 2000; Menuet et al., 2002). Our inability to detect an effect for the middle portion of the brain should be interpreted with caution, however, since our quantitative PCR approach lacked a high degree of neuroanatomical resolution. Future studies using better neuroanatomical resolution (e.g., using quantitative *in situ* hybridization or PCR on micropunches) may shed light on the present results. Nonetheless, it is intriguing that the steroid hormone receptor mRNA expression was increased in the telencephalon which is associated more generally with sensory and cognitive processes rather than with reproduction.

Social status has now been shown to regulate reproduction at multiple levels in *A. burtoni*- from GnRH1 neurons in the brain to steroid hormone production by the testes. Here we showed that the receptors that respond to steroids were also regulated by social status. This raises the question of whether social status *per se* directly regulates these receptors in the brain or, alternatively, whether social status regulates steroid hormones which, in turn, regulate expression of their own receptors? Because steroids are known to regulate expression of their own receptors, it is a reasonable prediction that social status regulates steroid receptors indirectly through changes in steroid hormone levels. Here we have only observed two components of this relationship at one point in time (i.e., social status and receptor expression). In order to disentangle the two, future studies that independently control social status and hormones are required.

Understanding the function of the steroid receptor gene paralogs can help elucidate the evolutionary consequences of gene duplication. The fate of gene paralogs is believed to follow one of three trajectories: one paralog either becomes nonfunctional or evolves a novel function,

or the two paralogs evolve divergently to subserve functions of the ancestral gene (Ono, 1972). In *A. burtoni*, the regulation of both AR subtypes and two ER subtypes by social dominance and the correlation between GnRH1 expression with each of the AR and ER subtypes strongly suggest that all the receptors regulate some aspect of reproduction. Nonetheless, data from *A. burtoni* and other teleosts showing distinct neuroanatomical distribution (Harbott et al. unpublished; Hawkins et al., 2000; Menuet et al., 2002) and distinct steroid binding sensitivities (Hawkins and Thomas, 2004; Olsson et al., 2006) of the SR subtypes suggest they serve different functions. Thus, our data contribute to the growing evidence that the steroid receptor paralogs have diverged to serve related functions. Although the distinction between the evolution of novel functions versus subfunctionalization cannot be determined without additional evidence from a representative of the common ancestor of teleosts, the patterns, on the whole, suggest subfunctionalization. Such an elaboration in function is consistent with recent theories on the important role of steroid hormone receptors in the evolution of vertebrates (Baker, 2003).

In conclusion, we have found that in the anterior part of the brain, the two ARs and two ER-βs are regulated by social status and reproductive capacity. Although it has long been established that teleosts have elaborated the number of AR and ER genes through genome duplication, the functional role of these steroid hormones in reproductive behavior is unclear. Here we show that the expression levels of the genes for these receptors are regulated by dominance and reproductive capacity in males. Although much more work remains before we fully understand the role of these receptors in reproductive behavior, these data provide the first direct evidence that these steroid receptors are regulated by social context in males.

Acknowledgements

We thank Dr. Anna K Greenwood for advice during the course of the study and for comments on an earlier version of this manuscript. We also thank Ms. Amanda Knier for assistance with RNA extraction and Dr. Sheng Zhao for assistance with data analysis. This work was funded by NIH NRSA NS42984 to SSB, an undergraduate HHMI research grant to VK, and NIH J. Javits Award NS34950 to RDF.

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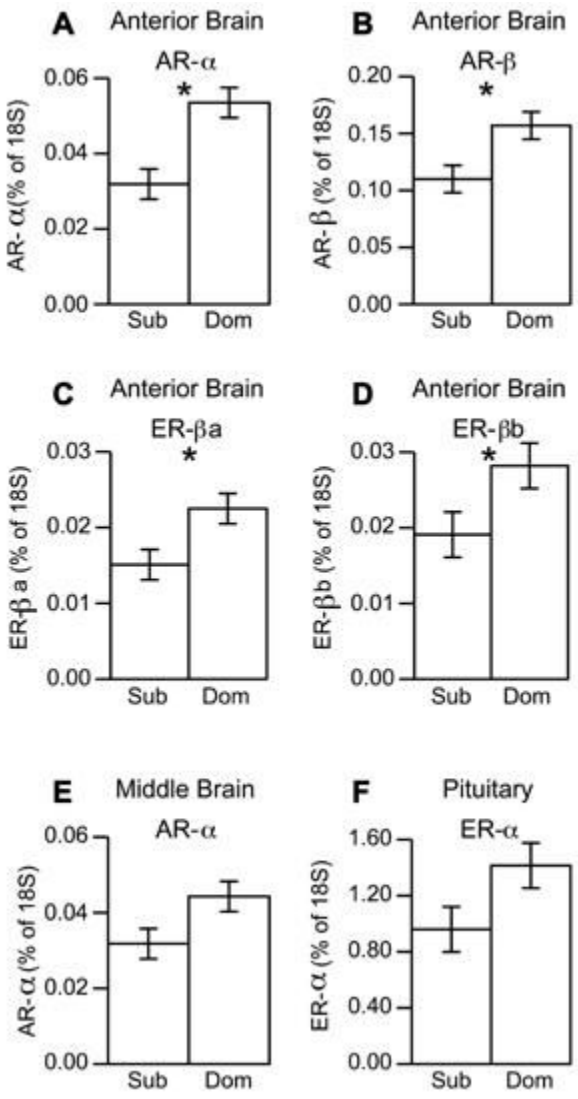

Fig. 1.

Effects of reproductive status on expression of steroid receptor genes in subordinate (sub) and dominant (dom) males. Asterisks indicate $p < 0.05$.

A-D Estimated marginal means (± SE) of AR and ER gene expression in subordinate and dominant males in the anterior portion of the brain.

E Estimated marginal means (± SE) of AR-α gene expression in subordinate and dominant males in the middle portion of the brain.

F Estimated marginal means (± SE) of ER-α gene expression in subordinate and dominant males in the pituitary.

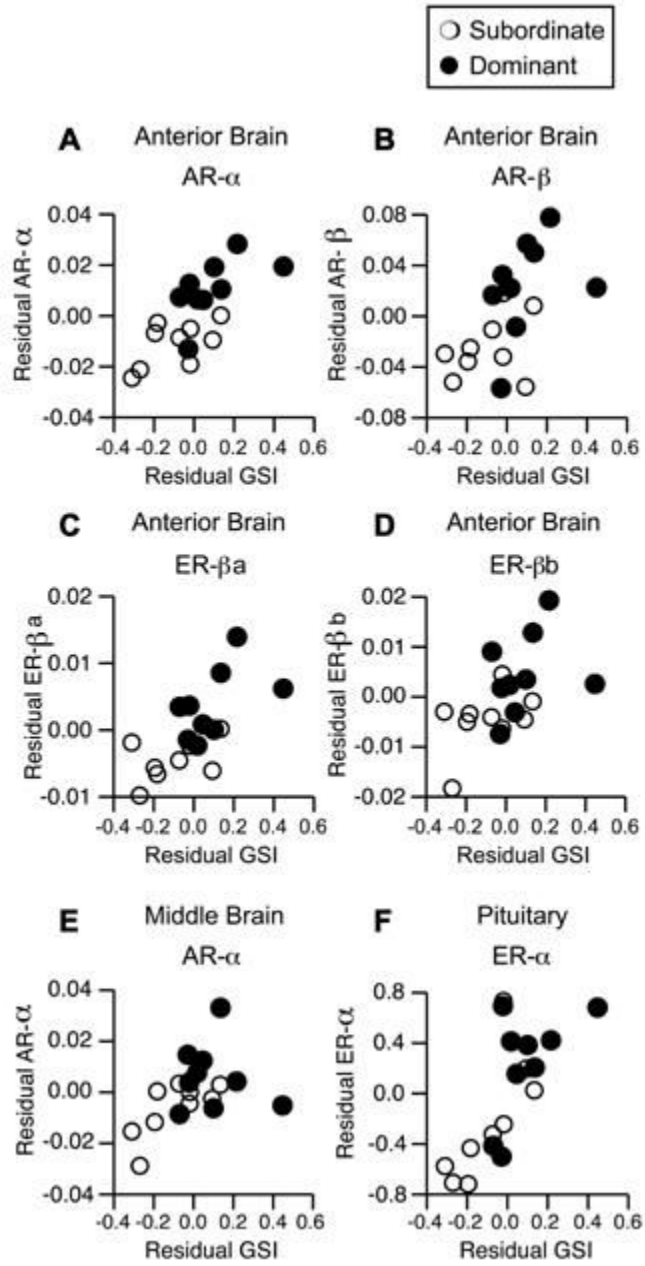

Fig. 2.

Covariation between steroid receptor gene expression and gonadosomatic index (GSI).

A-D Partial plots showing covariation between AR or ER gene expression in the anterior portion of the brain and GSI.

E Partial plots showing covariation between AR-α gene expression in the middle portion of the brain and GSI.

F Partial plots showing covariation between ER-α gene expression in the pituitary and GSI.

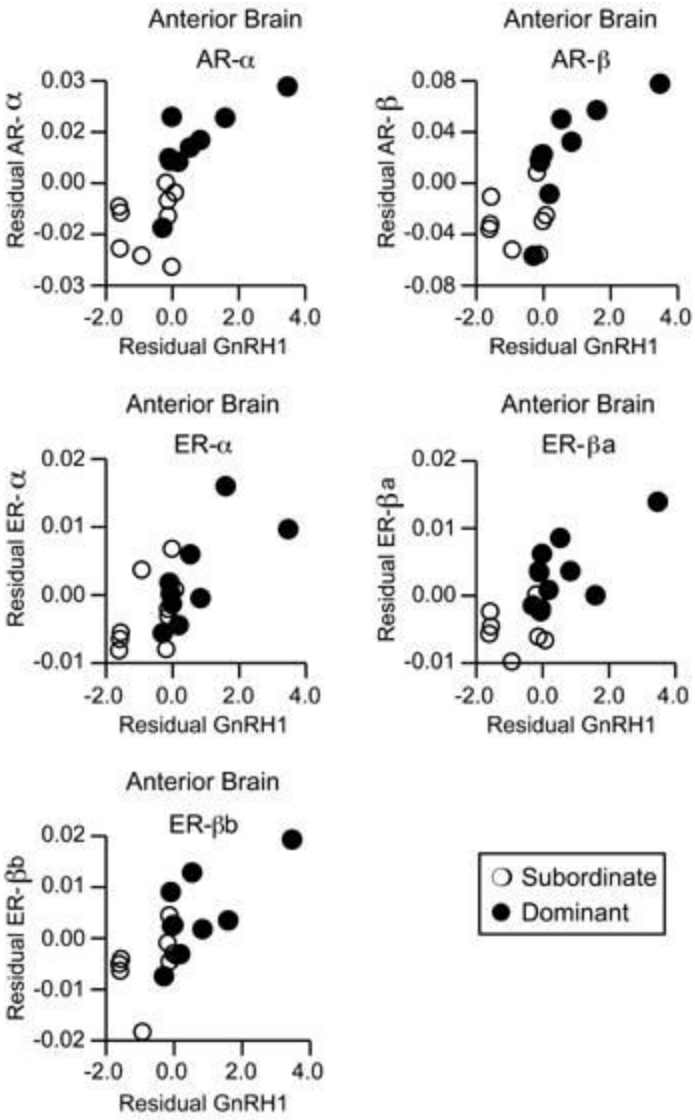

Fig. 3.

Covariation between GnRH1 gene expression and steroid receptor gene expression in the anterior portion of the brain graphed as partial plots.

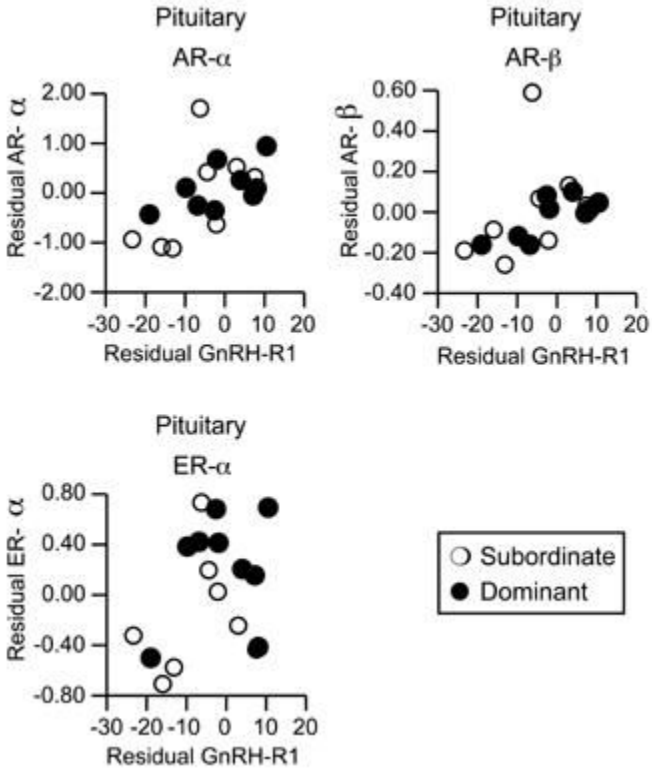

Fig. 4.

Covariation between GnRH-R1 gene expression and steroid receptor gene expression in the pituitary graphed as partial plots.

Table 1

Primers used to amplify the cDNAs of interest.

Gene	Genbank #	Forward primer	Reverse primer	Amplicon Length
AR-α	AF121257	CGC TGT ATC TGG TAC GGT AG	TGA GGA ATC GCA CTT GG	104
AR-β	AY082342	TTC GGC GAC AAG TAC AAC TC	ACT GTT CAC GGC GCA TTA	124
ER-α	AY422089	CCG GTT CCC AGA GAC GAC CAG	CTC GCC CAA GCC GTA T	57
ER-βa	DQ862128	ACA AGA AGG TTT GCC GTGT C	GCC CGT CTC CTA GTA TTC A	49
ER-βb	DQ862129	AAT CTG AAG GAG CGG AAG G	CTA GCG CAG ATG AGC ACG AT	51
GnRH1	U31865	CAG ACA CAC TGG GCA ATA TG	GGC CAC ACT CGC AAG A	128
GnRH-R1	AF356598	TCA GTA CAG CGG CGAAAG	GCA TCT ACG GGC ATC ACG AT	187
18S	U67333	CCC TTC AAA CCC TCT TAC CC	CCA CCG CTA AGA GTC GTA TT	82

Table 2

Effects of social status on mRNA expression level of andogen and estrogen receptor subtypes. Degrees of freedom were 1,14 for all F statistics with the exception of the testes which were 1,12 (ER-α, ER-βa), 1,11 (ER-βb, AR-α), and 1,10 (AR-β) .

	AR-α		AR-β		ER-α		ER-βa		ER-βb	
	F	p	F	p	F	p	F	p	F	p
Anterior Brain	17.53	0.001	8.19	0.013	2.43	0.142	9.67	0.008	6.39	0.024
Middle Brain	4.34	0.056	2.38	0.145	2.63	0.127	0.66	0.430	1.02	0.331
Posterior Brain	0.08	0.787	0.10	0.755	2.02	0.177	0.01	0.941	0.63	0.441
Pituitary	0.35	0.565	0.19	0.670	3.96	0.066	0.01	0.934	0.20	0.663
Testes	<0.01	0.976	1.62	0.231	0.02	0.882	0.62	0.446	0.23	0.641