SOCIAL REGULATION OF CORTISOL RECEPTOR GENE EXPRESSION

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Abstract:

In many social species, individuals influence the reproductive capacity of conspecifics. In a well-studied African cichlid fish species, *Astatotilapia burtoni*, males are either dominant (D) and reproductively competent or non-dominant (ND) and reproductively suppressed as evidenced by reduced gonadotropin releasing hormone (GnRH1) release, regressed gonads, lower levels of androgens and elevated levels of cortisol. Here, we asked whether androgen and cortisol levels might regulate this reproductive suppression. *A. burtoni* has four glucocorticoid receptors (GR1a, GR1b, GR2 and MR) encoded by three genes, and two androgen receptor (ARα and ARβ) encoded by two genes. We previously showed that ARα and ARβ are expressed in GnRH1 neurons in the preoptic area (POA) that regulates reproduction and that the mRNA levels of these receptors are regulated by social status. Here we show that GR1, GR2 and MR mRNAs are also expressed in GnRH1 neurons in the POA, revealing potential mechanisms for both androgens and cortisol to influence reproductive capacity. We measured AR, MR and GR mRNA expression levels in the microdissected region of POA containing GnRH1 neurons, comparing D and ND males. Using quantitative PCR (qPCR), we found D males had higher mRNA levels of ARα, MR, total GR1a and GR2 in the POA compared to ND males. In contrast, ND males had significantly higher levels of GR1b mRNA, a receptor subtype with reduced transcriptional response to cortisol. Through this novel regulation of receptor type, neurons in the POA of an ND male will be less affected by the higher levels of cortisol typical of low status suggesting GR receptor type change as a potential adaptive mechanism to mediate high cortisol levels during social suppression.
Introduction

Social status hierarchies are a ubiquitous organizing principle of social systems in many animal species from ants (Wilson, 2000) to primates (Cheney and Seyfarth, 1990) including humans (Chiao et al., 2009). Such hierarchies typically regulate access to resources including territories, food, and/or mates, granting individuals of high status access to these resources and requiring those of low status to find alternative solutions to survive. In many species, the reproductive capacity of low status individuals is reduced and mating is not possible due to behavioral and physiological changes (e.g., Willsch et al., 2012). Typically, aggressive encounters establish and maintain social rank, which, in turn, produce significant differences in reproductive capacity. In such social systems, levels of stress and reproductive hormones directly reflect the social rank of individuals and may play a causal role in behavioral and physiological changes.

The relationship between social dominance and reproductive physiology has been well studied in an African cichlid fish, *Astatotilapia burtoni*, in which status regulates both reproductive access and competence. In *A. burtoni*, there are two types of adult males: those with and those without territories (Fernald, 1977). Dominant (D) males are brightly colored, whereas non-dominant (ND) males are cryptically colored, making them difficult to distinguish from the substrate and from females that are similarly camouflaged. In their natural habitat, the shallow shore pools and river estuaries of Lake Tanganyika (Fernald and Hirata, 1977a; Coulter, 1991), *A. burtoni* live in a lek-like social system in which D males vigorously defend contiguous territories (Fernald and Hirata, 1977 a, b). This social system exerts potent control over the reproductive capacity of ND animals (Davis and Fernald, 1990; Francis et al., 1993). As is typical of many social hierarchies, D males have high levels of testosterone and low levels of the
The stress response to social subordination inhibits the reproductive axis in many species and results in chronic elevation of stress hormones, which, if sustained, is generally considered detrimental (Webster et al., 2008; Kaplan and Manuck, 2004; Kaplan, 2008). Thus it is a puzzle how socially suppressed individuals survive chronic elevation of glucocorticoid levels until an opportunity for social ascent occurs. One possible mechanism for modulating the responsiveness of non-dominant individuals to stress would be to change the amount and/or sensitivity of the cortisol receptors that mediate cortisol’s potentially damaging effects on the body (Avitsur et al., 2001) and brain (Sapolsky, 1996).

Localizing androgen and cortisol receptors and quantifying their expression patterns is essential for discovering what role these hormones might play in social regulation of reproduction. In *A. burtoni* males circulating testosterone is significantly more abundant than 11-ketotestosterone, a teleost-specific androgen, but D males have significantly higher levels of both hormones than ND males (Parikh et al 2006). *A. burtoni* has two AR receptors, ARα and ARβ (Harbott et al., 2007). *A. burtoni* ARα is part of the ARa/AR1 group in teleosts, which has been shown to have a higher affinity for testosterone than 11-ketotestosterone (Pasmanik and Callard, 1988; Pottinger, 1987; Slater et al., 1995). In contrast, ARβ has been shown to have a higher affinity for 11-ketotestosterone than testosterone in other fish (Olsson et al., 2005).

*A. burtoni*, like many other teleost species, has three receptor genes for cortisol: two glucocorticoid receptors (GR) and a mineralocorticoid receptor (MR) (Greenwood et al., 2003; note that the nomenclature of the two GR genes has been changed since the original report by...
Greenwood et al., 2003 to be consistent with GR genes subsequently cloned from other species; i.e., GR1 is now GR2 and vice-versa). In mammals, MR binds cortisol and aldosterone equally (Funder et al., 1988), however, teleost fish do not produce aldosterone and MR is highly responsive to cortisol and may act as a cortisol or 11-deoxycorticosterone receptor \textit{in vivo} (Sturm et al., 2005).

One of the teleost GR genes (GR1) has two splice variants, GR1a and GR1b. These forms differ by a nine-amino acid insertion in the DNA-binding domain in GR1b (Greenwood et al., 2003; Stolte et al., 2006). This splice insertion has a strong influence on the transcriptional response to receptor binding. GR1b has been shown to act as a dominant negative inhibitor of transcription in zebrafish (Schaaf, et al., 2008) and it drives significantly less reporter gene activity than GR1a in \textit{A. burtoni} (Greenwood et al., 2003).

Here, we asked whether these steroid receptors might influence reproductive capacity in \textit{A. burtoni}. Reproduction is controlled in \textit{A. burtoni}, as in all vertebrates, by the hypothalamic-preoptic area (POA) of the brain by gonadotropin releasing hormone (GnRH1)-containing neurons, named because their main product is the GnRH1 decapeptide. This peptide is delivered to the pituitary gland where it causes release of hormones that regulate the gonads. Since these neurons are the critical control point through which the brain signals the gonads, we wanted to understand what hormonal signals might influence their activity. We previously used \textit{in situ} hybridization to show that androgen receptors are expressed in GnRH1 neurons (Harbott et al., 2007), and here, we used this technique to localize GR and MR. To quantify effects, we used qPCR on microdissected regions of the POA containing GnRH1 neurons to determine expression levels in the steroid receptor genes were changed in ND males.

**Results**
**Behavior**

To quantify behavioral effects, the dominance index (DI) was calculated as the sum of the number of aggressive acts minus the number of submissive acts that occurred during a given observation period (White et al., 2002). DI values were averaged over the number of days an animal spent in one social setting, as were the number of reproductive displays. These daily mean values were used for comparison. As anticipated from previous studies (White et al., 2002), D males’ dominance index was significantly higher than that of ND males ($N=17; t = 6.476, P < 0.0001$; data not shown).

**Steroid Hormone Levels**

The levels of circulating hormones in males were compared as a function of social status. As expected, circulating cortisol levels were significantly elevated in ND males compared to D males ($N=17; \text{Fig. 1, } t = -3.181, P < 0.006$). Circulating testosterone and 11-ketotestosterone levels were significantly higher for D males as compared to ND males ($N=18; \text{Fig. 1, } t = 6.317, P < 0.0001$ and $N=15; \text{t} = 5.717, P < 0.0001$, respectively).

**GnRH1 and GR/MR double in situ hybridization**

We next performed double in situ hybridization to ask whether GR1, GR2, and/or MR were expressed within GnRH1-expressing neurons. Both GR1 and MR were highly expressed within the POA and also specifically within GnRH1 neurons (Fig. 2). GR2 was also expressed in the POA, but was missing or expressed only at very low levels within GnRH1 neurons (Fig. 2).

**GnRH1 mRNA levels**

GnRH1 mRNA levels in the microdissected POA were significantly higher in D males as compared with ND males ($N = 13, \text{Fig. 3, } t = -5.11, P < 0.0001$, consistent with previous data.
from whole brain analyses (Au et al., 2006) and from dissection of larger brain regions (Burmeister et al., 2007).

**Androgen Receptors**

ARα mRNA levels in the POA were significantly elevated in D males compared with ND males (N=11, t = 2.705, P < 0.024) while ARβ mRNA levels in the POA were similar for both D and ND males (Fig. 3).

**Glucocorticoid and Mineralocorticoid Receptors**

Dominant males had significantly higher levels of MR mRNA levels in the POA compared to ND males (Fig. 4, N=11, t = 5.885, P < 0.0001). GR2 mRNA was also significantly higher in D males (N=11, t = 2.183, P < 0.045). Dominant males had significantly higher levels of GR1(a+b) mRNA expression in the POA compared to ND males compared by qPCR (Fig. 5, N=10, t = 3.546, P < 0.002). We used a primer situated within the splice insertion to selectively amplify GR1b, which revealed that GR1b is more highly expressed in ND males than D males compared to all GR1 mRNA (Fig. 5). Because of individual variability of GR1b levels in males (especially ND males) the ratio of GR1b to all of GR1 was calculated to assess within each individual the potential contribution of splice variant GR1b (GR1b/(GR1b+GR1a+b) Fig. 5, N = 12, t = 2.288, P < 0.035).

**Discussion**

**Androgen, Glucocorticoid and Mineralocorticoid Receptors**

Reproductive and stress hormones signaling through their receptors are important regulators of many behaviors and more generally for homeostatic balance. Mathematical models incorporating feedback and feedforward regulation of gene expression by high and low affinity nuclear cortisol receptors supports the idea that these effects improve network robustness in the
face of moderate-frequency stressors (Kolodkin et al., 2013). In A. burtoni, previous work has established that the mRNA levels of several receptors, including the main steroid receptors, are regulated by social status in distinct brain regions as well as in the pituitary and testes (reviewed in Maruska and Fernald, 2014). In the POA, ARα, ARβ, ERβα, and ERββ have all been shown to be upregulated in dominant males relative to the levels found in non-dominant males. This regulatory action extends to GnRH1 and its receptors suggesting it is an integral part of the mechanisms through which animals respond to changes in social status. The data presented here are an extension of the general principle of receptor regulation since we show that the relative abundance of a particular GR receptor subtype can also be altered as a function of social status.

Glucocorticoid and Mineralocorticoid Receptor expression in the POA

We found GR1, GR2, and MR mRNA are expressed in the POA both using in situ hybridization and by qPCR. In addition, GR1 and MR are expressed in GnRH1 neurons, as revealed by double in situ hybridization, and are thus poised to play a direct role in the regulation of GnRH1 neurons by cortisol as a function of social status.

Using microdissected POA regions, we found that MR levels are higher in D males compared to ND males. A clearly defined role for MR in fish remains elusive. In vertebrates cortisol binds and activates both GR and MR, but MR has 10 times higher sensitivity to cortisol than GR and can also be activated by aldosterone (Bury and Sturm, 2007; Krozowski and Funder, 1983). Plasma aldosterone levels are usually lower than those of cortisol in tetrapods such as humans (Baker et al., 2007). On the other hand, in all teleosts tested to date, aldosterone levels are at or close to non-detectable levels (Prunet et al., 2006). It has been proposed that MRs are occupied by cortisol under basal conditions for both tetrapods and fish (Bury and Sturm, 2007; Krozowski and Funder, 1983). Sturm et al. (2005) have shown an alternative
glucocorticoid, 11-deoxycorticosterone (DOC) exhibits a higher affinity for MR compared to cortisol. DOC levels circulating in teleosts are comparable to resting cortisol levels and a potential role of DOC signaling through MRs has been proposed to counter increased cortisol levels following stressful events (Sturm et al., 2005). Mineralocorticoid receptors are thought to be occupied by cortisol (or another glucocorticoid like DOC) under basal conditions and in theory could decrease the signaling of cortisol through GRs. In the case of D males, an increase of MR levels in the POA with low levels of cortisol available could increase neuronal glucocorticoid signaling compared to ND males, possibly during transient increases caused by fighting.

Messenger RNA levels of MR in the brain of juvenile rainbow trout are higher than any other tissues measured using RT-PCR (Sturm et al., 2005). Recent work by Johansen et al. (2011) showed that MR mRNA levels are elevated in the hypothalamus of rainbow trout that have been selectively bred for low cortisol response to stress compared to trout that were bred for high cortisol release. These low response fish tend to become dominant when paired in agonistic interactions with high response fish (Johansen et al., 2011). Thus, MR may play a conserved role in regulating dominance or cortisol levels in *A. burtoni* and rainbow trout. 

Fish have two GR1 isoforms that differ in the spacing between two DNA zinc finger binding domains due to an insertion as first described by Greenwood et al., 2003. These isoforms show differential activation via a reporter construct *in vitro* (Greenwood et al., 2003) and similar insertions have been found in GR receptors of other teleost species (Lethmonier et al., 2002; Takeo et al., 1996). We found that the ratio of GR1b to total GR1 levels was significantly higher in ND males, suggesting a differential role for GR1b vs. GR1a in the regulation of social status.
It is possible that the GR1a and GR1b isoforms recognize different promoter sequences, and thereby have different target genes. It is interesting to speculate that GR1b might be upregulated in ND males because a different suite of target genes is expressed in ND vs. D males. An alternative scenario is that changes in cortisol receptor mRNA in the POA might directly or indirectly effectively protect the POA region of ND males from the chronically elevated levels of cortisol they endure. It is interesting to note that GR2 and MR are both downregulated in ND males. However, GR2 expression levels are very low to undetectable in GnRH1 neurons suggesting that GR2 expression in neighboring neurons could modify GnRH1 release but not directly within GnRH1 neurons. In addition, the GR1 isoform that is upregulated in ND males is one that shows reduced DNA transcription (Greenwood et al., 2003). If the effect (direct or indirect) is protection of the POA and other regions of the telencephalon, it could be adaptive for a species with flexible reproductive strategy.

A recent study in another teleost compared GR mRNA levels of males by status. Similar to A. burtoni, plainfin midshipman (Porichthys notatus) males occur in one of two phenotypes: a dominant male and a non-dominant male (sneaker male). Non-dominant plainfin midshipman have two-fold more circulating cortisol than D males (Arterbery et al., 2009) and also exhibit elevated GR1b mRNA levels in all brain regions tested. Moreover, when GR mRNA levels were measured in the vocal muscles, Genova et al. (2012) found increased GR mRNA in non-singing males. Modulation of teleost GR1b expression may provide an explanation for the survival of ND males and modulation of their neurons, especially in brain nuclei that are critical for the behavioral and physiological plasticity exhibited in male A. burtoni.

Androgen Receptors ARα and ARβ expression in the POA
Similar to other teleosts, cichlids have two androgen receptor subtypes, ARα and ARβ (Harbott et al., 2007). Previous measurements on *A. burtoni* using qPCR has shown that both ARα and ARβ are present in the brain (Burmeister et al., 2007) and *in situ* hybridization revealed that they are co-localized with GnRH1 neurons in the POA of *A. burtoni* (Harbott et al., 2007). *In situ* data shows high levels of expression of both ARα and ARβ in the POA of male *A. burtoni* (Harbott et al., 2007). Moreover, when whole brains from D males were macrodissected into 3 different regions, Burmeister et al. (2007) found that the anterior portion, which contains the POA, had elevated ARα and ARβ mRNA levels compared to ND males. In our study, microdissection of the POA revealed that D males had significantly higher mRNA levels of ARα compared to ND males (Fig. 3). However, unlike in the larger brain regions, ARβ levels were not different in the POA of D and ND males. This may be due to elevated ARβ expression non-POA regions in the anterior brain. Similarly to D male *A. burtoni*, D male plainfin midshipman also have elevated circulating levels of 11-ketotestosterone and express higher levels of ARα mRNA in vocal muscles compared to ND males (Genova et al., 2012). Our data combined with previous work showing that the higher levels of testosterone combined with the increased affinity for testosterone by ARα and increased ARα expression in the POA suggest it may be the key androgen receptor associated with maintenance of GnRH1 neuron size and elevated androgen levels in D males (Fig. 6).

**GnRH1**

The role of the hypothalamic-pituitary-gonadal axis in regulating reproductive competence is well documented in vertebrates (e.g., Sower et al., 2009). Gonadotropin releasing hormone 1 producing cells, located in the pre-optic area of the hypothalamus (POA), project into the portal vasculature in mammals and directly into the pituitary in fish (Fridberg and Ekengren, 1977).
where they cause release of gonadotropin hormones (follicle stimulating hormone and luteinizing hormone). In *A. burtoni*, social status regulates reproductive competence directly (Davis and Fernald, 1990; Fernald, 2012) and our data measuring local GnRH1 levels in the hypothalamus showed a twofold increase in GnRH1 mRNA expression in the POA of D as compared to ND males (Fig 3). These levels are consistent with previous measurements that sampled larger brain regions (Au et al., 2006; Burmeister et al., 2007).

**Hormones and Behavior**

Our data (not shown) confirmed previous laboratory studies reporting significant differences in agonistic and reproductive behavior as well as differences in the levels of stress and reproductive hormones between D and ND male *A. burtoni*. D males had higher levels of aggressive and reproductive behaviors than ND males. Similarly, we also confirmed previously reported glucocorticoid (cortisol) and androgen (testosterone and 11-ketotestosterone) level difference between D and ND males (Fig 1). Taken together behavioral endocrine data verify the key characteristics of male *A. burtoni* when they are housed in pairs.

**Conclusions**

The social regulation of reproductive capacity is an important evolutionary adaptation in *A. burtoni*. We have shown behavioral, hormonal, and mRNA expression level differences between the two distinct social states of males, consistent with previous data using larger tissue samples from the brain. Our data suggest that in D males increased expression of ARα, MR and GR2 occurs during normal feedback signaling for reproductive stimulation in the POA, a region involved in both neuroendocrine control of the gonads and regulation of male reproductive behaviors. The low levels of cortisol may require increased production of a highly active receptor for proper feedback signaling during normal daily cycling of cortisol. In ND males,
decreases of MR and GR2 and an increase of GR1b expression may be important for protection
of neurons, including those containing GnRH1, from high cortisol levels and thus allow the
neuronal circuit important for future social opportunity to remain intact (Figure 6). We
hypothesize that possibly other brain areas are similarly protected though we do not have data to
test that hypothesis.

As noted, we measured mRNA levels to assess the changes in expression of the receptors
of interest. How are these changes in GR mRNA related to GR protein expression? Though this
is not known in A. burtoni directly, it is known that cortisol levels and their receptors are tightly
controlled in the homeostatic regulation of the HPA axis. For example, in response to an acute
cortisol antagonist, rainbow trout GR protein levels decrease and in response, GR mRNA levels
increase (Alderman, et al., 2012), consistent with the tight regulation of the HPA axis.
Correspondingly, chronic treatment with GR agonists generally results in a coincident decrease
in both GR mRNA and GR protein levels, suggesting that using mRNA as a proxy for protein
levels is appropriate (e.g. Svec & Rudis, 1981; Okret et al., 1986). These data suggest that
mRNA levels are reasonable predictors of protein levels for the GR receptors.

Our results suggest a novel potential mechanism for the regulation of the HPA axis. In
their dynamic social interactions, A. burtoni are subjected to rapid and dramatic changes in their
social interactions from social dominance to social suppression with concomitant cortisol level
changes. Modulation of cortisol receptor subtype expression that could mitigate the
consequences of socially induced increases in cortisol levels in non-dominant males.

**Methods:**

**Animals**
A. burtoni bred from wild caught individuals were housed in aquaria under conditions mimicking their natural habitat: 29°C; pH 8; 12:12 light:dark cycle with full spectrum illumination (Fernald and Hirata, 1977). A layer of gravel (~3 cm) covered the bottom of the aquaria and terracotta pots in each tank facilitated the establishment and maintenance of territories by males. Animals were fed *ad libitum* every morning with cichlid pellets and flakes (AquaDine, Healdsburg, CA). Animals were marked using randomized combinations of colored beads attached just beneath the dorsal fin. All animals were treated in accordance with the Stanford University Institutional Animal Care and Use Committee (IACUC) guidelines.

**Pairing of males**

Reproductively active adult A. burtoni were kept in aquaria (85 l x 53.7 w x 30 d cm). Two pairs of size-matched D males (N = 24) from different aquaria were introduced with females to a new experimental aquarium, identical to their former aquarium except that a perforated divider separated the tank into two equal sections with a pair of males and three females in each section. Within minutes of being transferred to the aquaria, one male asserted his prior D status and the other became ND. They were kept for 30 days in this new condition (Fox et al., 1997) and then sacrificed. Males were used only once in each. Tissue collection as well as pairing of animals and all other behavioral procedures followed procedures described in detail previously (Korzan et al., 2008).

**Behavior**

Behavior of the paired males and their neighboring pair was recorded with a digital video camera (MiniDV, JVC) for 30 minutes six hours after lights turned on each day for the first three days (days 1-3) as well as the final three consecutive days (days 28-30) of the experiment. Aggressive behavior for each male was coded by observers blind to the experiment and ranked using a
dominance index, calculated from the sum of aggressive behavioral acts (e.g. bites and chases) minus subordinate behavioral acts (e.g. flee) performed per ten minute interval (White et al., 2002). Immediately after the final observation, both pairs of D and ND males in a tank were killed and brains and plasma were collected. Plasma was collected using a hypodermic needle (25 gauge) coated with heparin, inserted on the midline ~5 mm behind the anal fin into the caudal vein (Fox et al., 1997). Approximately 200 µl of blood was collected and centrifuged for 3 min (13.5 x 10³ g) to separate plasma from blood cells. The plasma was then transferred into a clean collection tube (1.5 ml). Plasma and brains were immediately frozen on dry ice and stored at -80°C until assayed. Behavioral data for some pairs were excluded due to significant recording time (more than 5 min) outside field of view of the recording equipment.

Steroid hormone measurements

Testosterone, 11-ketotestosterone and cortisol levels in A. burtoni plasma were measured using commercially available reagents (testosterone and cortisol; Assay Design, Ann Arbor, MI; 11-ketotestosterone Cayman Chemical Ann Arbor, MI) following protocols established by Parikh et al. (2006a,b). Plasma cortisol concentrations were measured in triplicate from blood plasma using a standard competitive immunoassay. Cortisol levels were measured using standard procedures and calculating the concentration from the standard curve. Similar protocols were used for 11-ketotestosterone and testosterone quantification with the addition of an extraction step utilizing diethyl ether and ethyl acetate/hexane respectively to remove the hormone from the whole plasma (Korzan et al., 2008). Because of the small size of A. burtoni collecting blood samples is a challenge and very small volumes (<100 ul) are normally obtained. Therefore, the volume of plasma needed to run multiple tests was not always possible.
In situ hybridization co-localization of glucocorticoid receptor mRNA and GnRH1 mRNA in POA

In situ hybridization followed standard procedures as used in the Fernald laboratory (Burmeister et al., 2007; Grens et al., 2005). Male brains were frozen in Tissue-Tek OCT compound (Ted Pella, Redding, CA) inside Peel-A-Way plastic molds (Polysciences, Inc., Warrington, PA) on dry ice and stored at -80°C. Brain tissue was sectioned coronally in three series at 14 μm using a Microm HM 550 cryostat (Thermo Scientific, Waltham, MA) and mounted on glass charged slides that were then stored at -80°C until use. Slides were brought to room temperature, fixed for 10 minutes in 4% paraformaldehyde in phosphate buffered saline (PBS), rinsed twice for 3 minutes each in PBS, immersed in 0.1 M triethanolamide (TEA) buffer for 3 minutes, acetylated in 0.25% acetic anhydride in 0.1 M TEA for 10 minutes, rinsed twice for 3 minutes each in 2× sodium citrate sodium chloride (SSC) buffer, dehydrated in an ethanol series, and air dried. Radioactive riboprobes labeled with S-35 UTP were diluted to $5 \times 10^6$ cpm/ml, and DIG probes were diluted to 1 ng/ml in hybridization solution (Sigma-Aldrich, St. Louis, MO) supplemented with 1 g/ml dithiothreitol (DTT). Preheated probe mix was added to each slide and then slides were coverslipped and immersed overnight in a 60°C mineral oil bath. After removing slides from the mineral oil, residual oil was removed by immersion in chloroform. Probe and coverslips were removed in two rinses of 4X SSC, and then washed with 2X SSC with DTT. To detect the GnRH1 probe, slides were incubated in anti-DIG-peroxidase primary antibody (Roche, Indianapolis, IN), then amplified using biotinyl tyraminde (Tyramide Signal Amplification kit, NEN Life Sciences, Boston, MA), incubated in avidin-HRP and stained using 0.055 diaminobenzidine (DAB). Finally, slides were dehydrated in ethanol, air dried, and dipped in nuclear emulsion diluted 1:1 in water (NBT-2; Eastman Kodak, Rochester, NY), air dried, and
stored in a light-tight box at 4°C for 3-4 weeks. Following development, slides were stained with cresyl violet, dehydrated in an ethanol/xylene series, and coverslipped. We used sense versions of each radioactive probe to test the specificity of our in situ hybridization results, and in no instance was any signal above background evident.

Micro-dissection of GnRH1 brain regions and mRNA isolation/quantification

Brains were cut coronally at 300 µm and freeze-thawed onto uncoated specimen slides (Korzan et al., 2000). The part of the POA containing GnRH1 neurons, the anterior parvocellular preoptic nucleus (aPPn), was identified using reference images from prior in situ and immunohistochemistry experiments in this species (Harbott et al., 2007; Burmeister and Fernald, 2005) and an anatomical map of GnRH1 expression (White, et al., 1995). The POA, including aPPn was microdissected from coronal slices using a 300-µm diameter punch (Korzan, et al., 2000).

Microdissected punches were expelled into lysis buffer (RNeasy Plus Micro, Qiagen) and RNA was isolated using the protocol provided (Purification of total RNA from microdissected cryosections; Qiagen kit # 74034), a standard procedure which removes DNA from the samples. We also treated the samples with a DNase (Turbo DNA-free, Ambion; Austin, TX) before synthesizing cDNA using random hexamer primers and reverse transcription (Roche Applied Science, Indianapolis IN).

Quantification of mRNA levels was performed using standard quantitative PCR procedures (e.g., Harbott et al., 2007). PCR primer pairs for GnRH1 and ARα and ARβ (Burmeister et al., 2007), MR, GR1a+b, GR1b and GR2 (Greenwood et al., 2003) were synthesized and used to amplify the samples using SYBR Green Supermix and the MyIQ system (BioRad). Results were normalized to the geometric mean of the internal control genes actin
(Zhao and Fernald, 2005), 18s (Burmeister et al., 2007) and G3PDH (Greenwood et al., 2003) for quantification and comparison. mRNA levels were calculated from the raw data using the Real Time PCR Miner program (Zhao and Fernald, 2005). GR1 ratio was calculated based on mRNA levels from qPCR reaction for primers that select for GR1a +b and primers for GR1 b. Then calculating the ratio of GR1b/(GR1b+GR1a+b). For all qPCR reactions the extremely small amount of tissue and mRNA extracted from punches did not allow for reanalysis of some reactions that failed.

**Data analysis**

Dominance index, hormone levels, and qPCR data of ND and D male pairs were compared using two-tailed paired t-tests. The level of significance was p<0.05 (SPSS 13.0, SPSS Inc., Chicago, Ill.). All error bars for figures are standard errors of the mean (SE).

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**Author Contributions**

W.J.K. and R.D.F designed the study. W.J.K. and B.P.G. performed the experiments and collected and analyzed the results. W.J.K. and R.D.F wrote the manuscript.

**Competing Interests**

No competing interest declared.

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References


Figure captions

Figure 1: Cortisol and androgen levels depend on social status. ND males have higher cortisol levels compared to D males (p<0.006). In contrast, androgens [testosterone (p<0.001) & 11-ketotestosterone (p<0.001)] are significantly higher in D males as compared to ND males.

Figure 2: Cortisol receptor expression within GnRH1 neurons. Photomicrographs of the preoptic area containing DAB-labeled GnRH1 neurons (brown). The receptors responsive to cortisol are labeled with in situ hybridization using $^3$H thymidine where silver grains show double label with MR (A1, A2) and GR1 (C1, C2) but not GR2 (B1, B2). Panels D1 and D2 show in situ labeling with a sense probe for GR1 as a control. Scale bar 20 μm.

Figure 3. GnRH1, ARα and ARβ mRNA levels in microdissected POA regions. D males have significantly higher levels of GnRH1 (p<0.0001) and ARα compared to ND males (p<0.024), but ARβ levels do not differ.

Figure 4: MR and GR2 mRNA levels in microdissected POA regions. Expression levels of MR (p<0.0001) and GR2 (p<0.045) are elevated in D males compared to ND males.

Figure 5: mRNA levels for GR1b, GR1a+b and GR1b/GR1b+a). The mRNA quantity normalized to housekeeping genes of GR receptor 1b and the combined GR receptor 1a+1b are plotted. The ratio of glucocorticoid receptor 1b mRNA to 1a mRNA in the POA is significantly higher (p<0.05) in non-dominant males compared to dominant males.

Figure 6: Dominant males (left column) have higher amounts of both testosterone and 11-ketotestosterone and larger GnRH1 neurons and higher ARα mRNA levels compared to ND males (Right column). Dominant males also have higher mRNA levels for MR, and GR1a. Non-dominant males have a reduced level of GR1a mRNA for high transcriptional response receptors in GnRH1 neurons and high levels of GR1b, low transcriptional response receptors.
Figure 1

The bar graph shows the comparison of cortisol, testosterone, and 11-ketotestosterone levels between territorial and non-territorial groups. The y-axis represents hormone levels in ng/ml, starting from 0 up to 45. The x-axis indicates the type of group (Territorial, Non-territorial) for each hormone. The graph illustrates significantly higher levels of cortisol in the non-territorial group, while testosterone and 11-ketotestosterone levels are nearly negligible and not significantly different between groups.
Figure 3

The graph shows mRNA levels relative to housekeeping genes for different conditions:

- **GnRH**:
  - Territorial: Approximately 3.0
  - Non-territorial: Approximately 1.0

- **AR alpha**:
  - Territorial: Approximately 1.5
  - Non-territorial: Approximately 1.0

- **AR beta**:
  - Territorial: Approximately 1.0
  - Non-territorial: Approximately 1.0

All values are shown with error bars, indicating variability. The asterisks (*) indicate statistical significance.
Figure 4

The graph illustrates mRNA levels relative to housekeeping genes for Mineralcorticoid and Glucocorticoid Receptor 2 in territorial and non-territorial conditions.
Figure 5

The figure shows mRNA levels relative to housekeeping genes for different conditions:
- **Glucocorticoid Receptor 1b**
- **Glucocorticoid Receptor 1a+b**
- **GR1 Ratio (GR1b/GR1b+a)**

The data is presented for both territorial and non-territorial groups, with error bars indicating variation. The results indicate significantly higher mRNA levels for Glucocorticoid Receptor 1b under territorial conditions compared to non-territorial conditions. Similar trends are observed for the GR1 Ratio, with a noticeable increase in territorial conditions.
Figure 6

GnRH1 Neurons in POA

Androgen signaling

Glucocorticoid signaling

Dominant

Non-dominant

T, 11-KT

Cort