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Somatostatin Regulates Aggressive Behavior in an African Cichlid Fish

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Animals respond to environmental and social change with plasticity in the neural substrates underlying particular behavioral states. In the African cichlid fish *Astatotilapia burtoni*, social dominance status in males is accompanied by reduced somatic growth rate as well as increased somatostatin neuron size in the preoptic area. Although somatostatin is commonly studied within the context of growth, we show here for the first time that this ancient neuropeptide also plays a role in controlling social behavior. Somatostatin antagonists increased aggressive behavior in a dose-dependent fashion and the potent somatostatin agonist octreotide decreased aggression. We cloned and sequenced the genes encoding two somatostatin receptor subtypes in this species to study transcription in the gonads. When we examined somatostatin receptor gene expression in testes, expression of the somatostatin type 3 receptor was negatively correlated with an aggressive display and androgen levels. However, octreotide treatment did not reduce plasma testosterone or 11-ketotestosterone levels, suggesting that the behavioral effects of somatostatin are not mediated by androgens. These results show that somatostatin has important effects on social behavior. In dominant male *A. burtoni*, somatostatin may function to contain energetically costly processes such as somatic growth and aggressive behavior. (*Endocrinology* 147: 5119–5125, 2006)

INVESTIGATIONS INTO THE physiological bases of aggressive behavior have historically focused on steroid hormones (1) and more recently serotonergic action in the brain (2) as well as certain neuropeptides, such as arginine vasotocin and arginine vasopressin (3). Often studied in isolation, these neuroendocrine systems are highly integrated, and there is growing evidence that other hormones may be involved in the control of aggressive and dominance behaviors. Somatostatin, which was initially identified for its effects on GH secretion (4), is an important regulatory peptide in a variety of physiological contexts. In addition to inhibiting GH secretion by the pituitary, somatostatin acts as a neuromodulator in the brain. Somatostatin also regulates motor activity (5, 6), probably by affecting dopaminergic systems (7), and recent evidence suggests that somatostatin has important neuroprotective effects in the brain (8). Somatostatin also influences the reproductive axis by inhibiting secretion of LH from the pituitary (9).

These diverse functions of somatostatin are reflected by the complexity of the somatostatin receptor (sstR) family. In mammals, five different subtypes of the sstRs have been identified. The transcripts of four of these subtypes have also been discovered in fish (10). There appears to be at least some functional homology between teleost and mammalian sstRs. The somatostatin agonist octreotide suppresses GH secretion in trout (*Oncorhynchus mykiss*) (11) as it does in mammals (12), and the pharmacological properties of sstR5 in goldfish appear to be similar to those of mammals (13). Pharmacological characterizations of the other subtypes have not yet been completed in any teleost fish. In general, distributions of the different somatostatin subtypes in the brain are unique but overlapping. This suggests that sstRs may act in parallel to a certain extent (14). In rodents, sstR3 mRNA expression is highest in the cerebellum and is observed in the hypothalamus, hippocampus, and midbrain (15, 16). A similar pattern of sstR3 mRNA distribution is found in goldfish (*Carassius auratus*) (17). SstR2 and sstR5 are the predominant transcripts in the pituitary of both rodents (18) and teleosts (17, 19). Selective somatostatin ligands provide evidence for subtype-specific function, because sstR2 and sstR5 agonists inhibit pituitary GH secretion in rats, whereas selective sstR1 and sstR3 agonists do not (20). The diversity of sstR subtypes is likely at the root of some of somatostatin's distinct biological functions (21), because differences in the expression of receptor subtypes may affect how somatostatin acts on various physiological processes in diverse tissues. Although developmental plasticity in the transcriptional expression of the five sstR subtypes has been explored (22, 23), plasticity in sstR gene expression in adults has not been examined in detail (but see Ref. 24).

Previous studies suggest that somatostatin is related to behavioral and neural plasticity in the cichlid fish *Astatotilapia* (formerly *Haplochromis*) *burtoni*. In this species, dominant males aggressively maintain a territory and are reproductively active, whereas subordinate males school with females and are reproductively suppressed (25). Subordinate males grow faster than dominant males (most teleosts grow throughout life), apparently representing a tradeoff between growth and reproduction (26). Frequent fluctuations of the physical environment are common in the natural habitat (27), and laboratory studies indicate that such fluctuations result in constant change in social dominance relationships (28).

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Abbreviations: RACE, Rapid amplification of cDNA ends; sstR, somatostatin receptor.

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Such transitions in social status are characterized by asymmetrical changes in growth and reproduction (28, 29), with up-regulation of the reproductive axis in socially ascending males occurring much more quickly (~7 d) than reductions in growth (>2 wk). In descending animals, reproductive physiology is down-regulated slowly (~2–3 wk), although sexual and aggressive behaviors cease as soon as a fish loses a territory, and growth remains inhibited. Somatostatin immunoreactive neurons in the preoptic area are about four times larger in dominant males compared with subordinate males, and preoptic area somatostatin neuron size is negatively correlated with growth rate (30). This finding suggested that the increased neuron size may be due to increased production of somatostatin along with increased release (with a subsequent reduction in growth).

We manipulated somatostatin function by treating dominant males with somatostatin antagonists or agonists and observed the effects on behavior. We also tested whether any behavioral effects occurred via changes in GH secretion, because previous studies on fish have found that GH increases aggression (31, 32). Finally, because castration reduces aggressive behavior in dominant *A. burtoni* (33), we examined sstR mRNA expression in the testes and considered whether the effects of somatostatin function influence androgen production. Together these experiments indicate that aggressive behavior should be added to the growing list of behavior systems modulated by somatostatin.

Materials and Methods

Fish

Fish were descendents of a wild-caught stock population and were group housed (five to seven males and six females per tank) in aquaria as previously described (27, 30). Each aquarium contained five overturned terracotta flowerpots in a standard layout (one in each corner plus one central location). The flowerpots mimic the natural substrate and are necessary for males to establish vigorously defended territories, to which they attract females for spawning (27). In each tank, there were two to three dominant males and three to four subordinate males. All males were tagged with colored beads attached to a plastic tag (Avery-Dennison, Pasadena, CA). The tag was inserted with a stainless steel tagging tool (Avery-Dennison) through the skin just below the dorsal fin at least 1 wk before behavioral observations were conducted. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Harvard University Institutional Animal Care and Use Committee (protocol no. 22-22).

Hormone manipulations

In experiment 1, pretreatment focal observations were conducted for 10 min on territorial males. In addition to behavioral observations, dominant males were identified by characteristic yellow or blue coloration, red humeral patch, and black vertical stripe on either side of the face. After behavioral observations, males were randomly assigned to treatment groups (see below). Treatments began the following day and consisted of one ip injection per day for 5 d. Territorial males were randomly assigned to be treated with saline (n = 7) or 1.2 mg/kg (n = 5), 12 mg/kg (n = 6), or 120 mg/kg (n = 6) of the general somatostatin antagonist cyclosomatostatin (Sigma Chemical Co., St. Louis, MO) (34). In experiment 2, different territorial males from established tanks were observed and then randomly assigned to be treated with saline (n = 19), 4 mg/kg octreotide (a somatostatin agonist; Bachem, Torrance, CA) (n = 8), 80 mg/kg of the GHRH antagonist JV-1-38 (35) (Bachem) (n = 14), or 10 mg/kg ovine GH (National Hormone and Peptide Program, Torrance, CA) (n = 8). Octreotide selectively binds to sstR2/sstR3/sstR5 but not sstR1/sstR4 (36), and the dose we used is known to reduce GH in fish (11). For both experiments 1 and 2, 10-min posttreatment behavioral observations were conducted 1 d after the last injection treatment. We tested whether somatostatin agonists and antagonists affected behavior using the differences between pretreatment and posttreatment scores.

Testicular sstR gene expression and androgens

We hypothesized that somatostatin may affect aggression by altering androgen function. In experiment 3, we collected blood samples from the caudal peduncle from a separate set of dominant (n = 9) and subordinate (n = 9) males to confirm phenotypic differences in plasma androgen levels. In experiment 4, we tested whether there were phenotypic differences in somatostatin sensitivity in testes; we measured sstR2 and sstR3 expression in testes of dominant and subordinate males. We conducted behavioral observations on a set of unmanipulated dominant (n = 10) and subordinate (n = 9) males in undisturbed aquaria. Males were killed by decapitation and the testes dissected and stored in RNA later. Finally, in experiment 5, we tested whether somatostatin regulated androgen production by randomly assigning dominant males to be treated with saline (n = 5) or 4 mg/kg octreotide (n = 5). After 5 d of treatment, a blood sample was drawn from the caudal peduncle for androgen measurements.

Cloning of A. burtoni sstR cDNAs

We sequenced the *A. burtoni* sstR2 and sstR3 homologues because these receptors are known to bind octreotide. For cloning of sstR2, we used a nested set of degenerate primers based on goldfish (*C. auratus*) and pufferfish (*Takifugu rubripes*) sequences using genomic DNA as a template. In the first reaction, we used the external primers, forward primer 5′-TACGTCATCCTGCGCTSCG-3′ and reverse primer 5′-GAAGGCGTAGSGGATGGGGTT-3′. Thirty cycles of PCR amplification were performed with denaturation for 45 sec at 95 C, annealing for 1 min at 58 C, extension for 1 min at 73 C, and final extension for 5 min at 73 C after the last cycle. Then we performed a second PCR using the product of the first reaction as template. We used the same reaction conditions, this time using a set of internal primers, forward primer 5′-GTSATGAGCWTCGAYCGMTA-3′ and reverse primer 5′-GGGTTGGCGAGCTGTTGGCGTA-3′. This reaction produced a 500-bp product that was purified, subcloned using a TOPO cloning kit (Invitrogen), and sequenced. Analysis of the nucleotide sequence indicated that it was highly similar to goldfish and pufferfish sstR2 sequences. Based on this sequence, we designed primers for 5′ (5′-ACCATGAGGGGCAGGAAGAAGCCTAGA-3′) and 3′ (5′-CCCCTCATGGTCATCTGCCTTTGCTAC-3′) rapid amplification of cDNA ends (RACE) using a SMART RACE kit (Clontech, Mountain View, CA). A cDNA pool derived from brain tissue was used for the RACE reaction. The products of these reactions were gel purified, subcloned, and sequenced.

Degenerate primers for sstR3, forward primer 5′-GTCATGAGCATCGAYCGSTA-3′ and reverse primer 5′-GGGTTGGCGCASSTGTTRGCRTA-3′, were based on goldfish sstR3 sequences. This PCR on genomic DNA produced a 500-bp product that was purified, subcloned, and sequenced as described above. Based on this sequence, we designed primers for 5′ (5′-GAGGCAGATGATGAGAAGAGGGCAGA-3′) and 3′ (5′-GACACGCATGGTTGTGATTGTTGTTGC-3′) RACE using the SMART RACE kit. A cDNA pool derived from brain tissue was used for the RACE reaction.

Quantitative real-time PCR experiments

RNA was extracted from testicular tissue in 0.5 ml Trizol, and RNA quality was checked using the Nanochip on a Bioanalyzer (Agilent, Palo Alto, CA). For each RNA sample, 2 μg RNA was treated with DNase (amplification grade; Invitrogen, Carlsbad, CA), and RNA concentration was precisely determined in duplicate using the RiboGreen assay (Invitrogen). This assay allows for precise determinations of RNA concentrations, which alleviates the need for using so-called housekeeping genes when conducting quantitative real-time PCR (37). Although housekeeping genes are often used as standards in quantitative real-time PCR experiments, it has been shown repeatedly that their expression levels cannot be assumed to be constant across experimental conditions (37–39). Normalization of RNA using the RiboGreen method is not affected by differences in standard housekeeping gene expression such as G3PDH or 18S rRNA in subsequent real-time PCR (37, 38). Based on the RiboGreen measurements of RNA, 1 μg RNA from each sample was reverse transcribed using Superscript (Invitrogen) for use in quantitative real-time PCR.

Quantitative real-time PCRs were conducted on an MJ DNA Engine Opticon 2 thermocycler. Primers for sstR2 and sstR3 were designed using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). For each primer set, we determined the efficiency of the PCR using standard curves using the plasmids that were created when the cDNAs were cloned. The efficiency was calculated using the formula $E = 10^{[-1/\mathrm{slope}]} - 1$ and was over 90% for the somatostatin pre-propeptide, for sstR2, and for sstR3. Cycling conditions were 5 min at 95 C and then 40 cycles of 30 sec at 95 C, 30 sec at 52 C, and 30 sec at 72 C, followed by a 5-min extension period and a melt curve analysis. All reactions were run in duplicate.

Hormone assays

We measured testosterone with a direct RIA (DSL Diagnostics, Webster, TX) on caudal peduncle plasma samples that were diluted 1:12.5. We measured 11-ketotestosterone using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) on samples that were diluted 1:33.3. For each hormone assay, when assay concentrations for serial dilutions of an *A. burtoni* plasma pool were compared with standards, computed regression lines did not differ in slope ($P > 0.05$). Quality control pools were assayed in duplicate in each assay. The intraassay coefficient of variation was 3.31% for testosterone and 2.51% for 11-ketotestosterone.

Statistical analysis

We used Q-Q plots to assess normality and Hartley's Fmax test to assess homogeneity of variance for all variables used in statistical analyses. Behavioral data met the requirements for parametric statistics and were thus analyzed with ANOVA and planned comparisons (*vs.* saline controls). Gene expression data were not normally distributed so we used nonparametric U tests and rank correlations to analyze these data. Hormone levels were log transformed for all analyses.

Results

Isolation and sequencing of somatostatin genes in A. burtoni

The *A. burtoni* sstR2 and sstR3 cDNAs contain open reading frames of 1110 and 1449 bp encoding for 370- and 483-amino-acid receptor proteins, respectively (Fig. 1). Both sstR2 and sstR3 contained the YANSCANP motif in the putative seventh transmembrane domain, which is characteristic for mammalian sstRs. The amino acid sequences of the *A. burtoni* sstR2 have 60 and 68% identity to mouse and human sstR2s, respectively, whereas the *A. burtoni* sstR3 sequences have 45 and 46% identity to mouse and human sstR3s. Both sequences have been deposited in GenBank (accession numbers AY585718 and AY585719).

Hormone manipulations

In experiment 1, treatment with the somatostatin antagonist cyclosomatostatin increased aggressive chasing in territorial males in a dose-dependent fashion (Fig. 2). Territorial males treated with the medium and high doses exhibited significant increases in chases ($P < 0.05$). Also, males treated with the low, medium, and high doses exhibited significant increases in border threats compared with saline-treated males ($P < 0.05$). Importantly, cyclosomatostatin treatment did not affect courtship behavior in territorial males ($F_{3,20} = 0.80$; $P = 0.51$), indicating that its actions are fairly specific.

In experiment 2, treatment with the somatostatin agonist octreotide significantly reduced chasing behavior (Fig. 3, $P < 0.05$) but not border threats or courtship behavior, again indicating a specific role for somatostatin in the control of behavior. Treatment with the GHRH antagonist reduced chasing (Fig. 3; $P < 0.05$) but not border threats or courtship behavior. We observed no significant effects of GH on chasing (Fig. 3), border threats, or courtship behavior (all P values > 0.3).

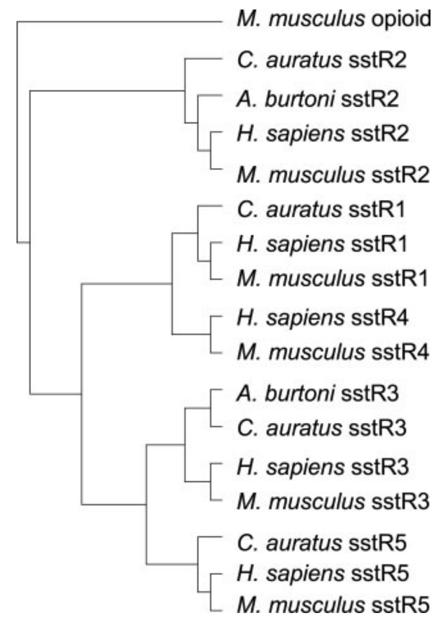

FIG. 1. Alignment of *A. burtoni* sstR sequences with receptor sequences from *C. auratus* (goldfish), *M. musculus* (mouse), and *Homo sapiens* (human).

SstR gene expression in testes and androgen levels

In experiment 3, dominant males had significantly higher testosterone ($t_{18} = 2.19$; $P = 0.04$) and 11-ketotestosterone ($t_{18} = 2.46$; $P = 0.02$) levels than subordinate males. In experiment 4, we observed no significant differences in sstR2 (Mann-Whitney, $U = 40$; $P = 0.68$) or sstR3 ($U = 38$; $P = 0.57$) expression in the testes of dominant and subordinate males. However, in dominant males, both sstR2 (Spearman $\rho = -0.73$; $P = 0.01$) and sstR3 (Fig. 4A; Spearman $\rho = -0.68$; $P = 0.03$) expression were negatively correlated with border threats. SstR3 expression in testes was negatively correlated with testosterone (Fig. 4B; Spearman $\rho = -0.63$; $P = 0.048$) and 11-ketotestosterone (Spearman $\rho = -0.72$; $P = 0.045$) levels in dominant males. In experiment 5, we tested whether somatostatin suppresses androgen secretion in *A. burtoni* by treating dominant males with octreotide (somatostatin agonist). Surprisingly, plasma testosterone was significantly higher ($t_8 = 2.61$; $P = 0.03$) in males treated with octreotide (mean ± SE, 156.6 ± 21.4 ng/ml) compared with males treated with saline (81.0 ± 19.5 ng/ml).

In subordinate males, sstR3 was not significantly correlated with testosterone (Spearman $r = 0.08$; $P = 0.83$) or 11-ketotestosterone (Spearman $\rho = 0.18$; $P = 0.7$). Androgen levels were not significantly correlated with aggressive behavior or courtship behavior.

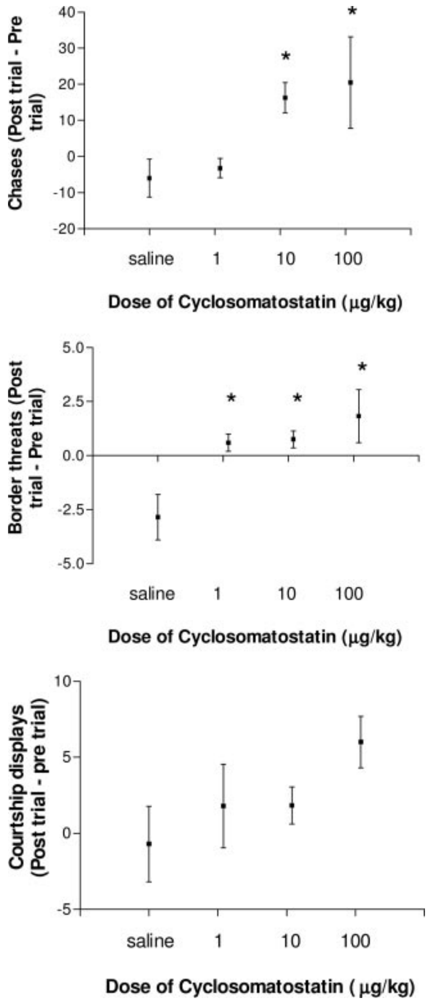

FIG. 2. Dose-dependent effects of the somatostatin antagonist cyclosomatostatin (μg/kg) on behavior in dominant males. All panels show the change in behavior between pretreatment observations and posttreatment observations during 10-min tests for chasing, border threats, and courtship displays. *, $P < 0.05$ compared with saline.

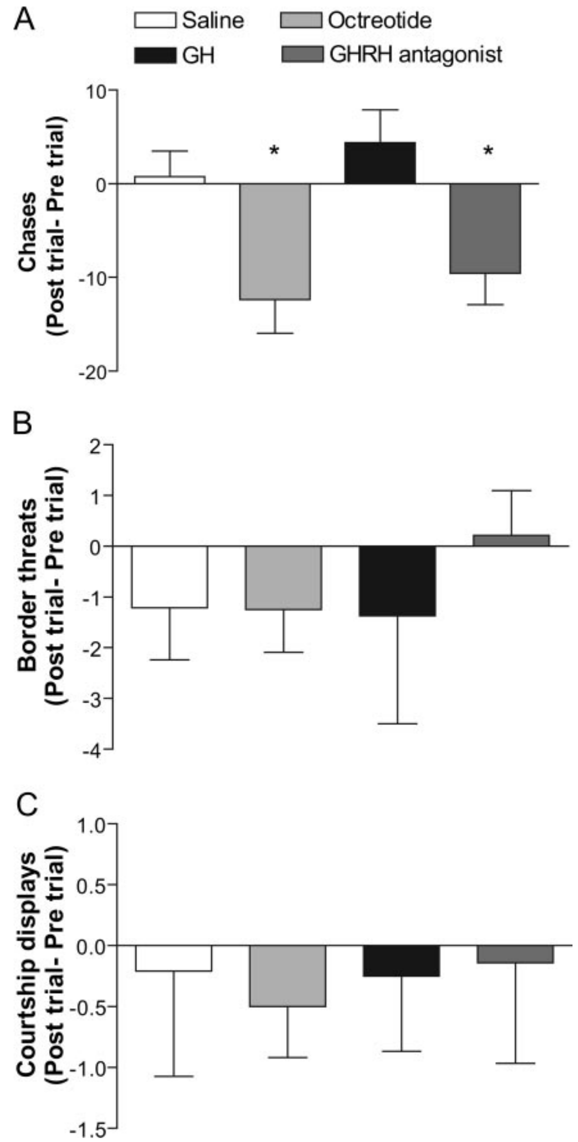

FIG. 3. Behavioral effects of pharmacological manipulations in dominant males. Dominant males were injected over a 5-d period with saline, the somatostatin agonist (octreotide), GH, or a GHRH antagonist (JV-1-38). All panels show the change in behavior between pretreatment observations and posttreatment observations for chasing (A), border threats (B), and courtship displays (C). *, $P < 0.05$ compared with saline.

Discussion

We have demonstrated that somatostatin has significant effects on aggressive behavior and that sstR expression in the testes is correlated with aggressive behavior. Treatment with a somatostatin agonist decreased aggressive behavior in dominant males, and treatment with a somatostatin antagonist increased aggression in a dose-dependent manner. These results were unexpected because more aggressive dominant males have slower growth rates (suggestive of increased somatostatin function) than less aggressive subordinate males. These observations suggest that the effects of somatostatin are complex and that variability in tissue-specific and/or receptor-specific hormone action may be involved in differential regulation of growth and behavior.

Our experiments are the first to demonstrate a role for somatostatin signaling in the regulation of aggressive behavior. Currently, the precise mechanisms for the behavioral actions of somatostatin in *A. burtoni* are unclear. An obvious mechanism for the effects of somatostatin on behavior is via regulation of GH secretion by the pituitary. GH treatment increases aggression in juvenile rainbow trout (*O. mykiss*) (31) and Atlantic salmon (*Salmo salar*) (32), an effect that has also been reported in wild house mice (*Mus musculus*) (40). Increased GH is also associated with increased aggression toward human handling in male mithuns (*Bos frontalis*), an Asian ruminant (41). Although both somatostatin analogs and a GHRH antagonist affected aggressive behavior in *A. burtoni*, we observed no effect of GH treatments on behavior (with one hormone-treated animal per tank under stable social conditions).

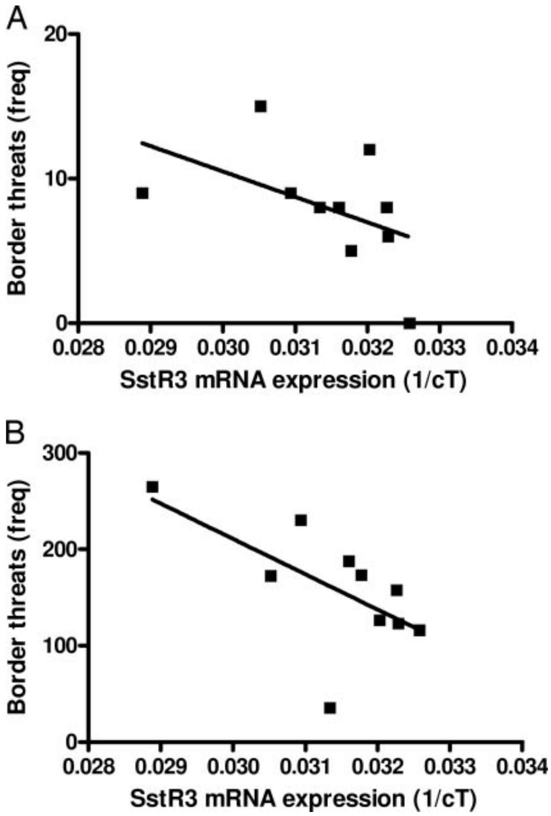

FIG. 4. Correlations between sstR3 mRNA levels in testes with testosterone and aggressive behaviors in dominant males. A, Correlation between sstR3 and testosterone; B, correlation between sstR3 and border threats.

However, when all males in a tank were treated with either GH or saline, the resulting social instability favored social ascent of GH-treated animals (42). It is also possible that effects of GH in *A. burtoni* may be dependent upon the fluctuating nature of GH release (43), which was not replicated with daily GH injection treatments. Thus, manipulations of systems that modulate GH (such as somatostatin or GHRH) could be more effective in changing behavior because they allow for rhythmic GH release. A final possibility is that the somatostatin compounds we used affected behavior via sstR5 subtypes, which do not have strong effects on GH regulation (20). It is not yet known whether sstR5 is expressed in *A. burtoni* brain.

We also tested whether somatostatin influences behavior via regulation of androgens, because somatostatin acting at the testes modulates androgen secretion in mammals (44–46) and castration reduces aggression in dominant *A. burtoni* (33). Interestingly, sstR3 expression was negatively correlated with border threats and testosterone. This led us to hypothesize that somatostatin acts on sstR3 in the testes to reduce aggression by decreasing androgen release. When we tested this hypothesis by measuring testosterone after octreotide injections, we observed that octreotide caused a significant increase in plasma testosterone. This result suggests that the behavioral effects of octreotide treatment are not a result of decreased testosterone. It is possible that octreotide treatment could interfere with transient increases in androgen levels (47–49), which can have important effects on dynamic changes in aggression (50). An alternative hypothesis is that somatostatin action in the testes alters inhibin A secretion. Inhibin A has been observed to increase steroidogenesis in testes (51) and inhibit GH secretion (52). If somatostatin increases inhibin A in *A. burtoni* testes, this could explain 1) increased testosterone resulting from octreotide treatment and 2) decreased aggression resulting from octreotide treatment (via regulation of GH).

Recent evidence suggests that somatostatin may affect general arousal systems and locomotor activity (53). The behavioral effects of somatostatin in *A. burtoni* could therefore be a result of modulation of more general behavioral systems, particularly because dominant fish that are more aggressive display more locomotor activity than less aggressive dominant fish. Although we did not take measurements on locomotor activity directly, it is unlikely that the effects of somatostatin on aggressive behavior result solely from general effects on locomotor behavior because somatostatin manipulations did not affect courtship behavior (which involves intricate motor displays such as fast approach, tail quivering, and leading to the nest). It is also possible that the pharmacological properties of sstRs in teleost fish differ from what has been described in mammals and that these differences may contribute to the observed effects of somatostatin analogs on *A. burtoni* behavior. We assert that there is at least some homology in the function of sstR2 and/or sstR3 between teleosts and mammals because octreotide decreases GH in both teleosts and mammals. Given the complexity in sstR subtypes that has been described in goldfish (17), additional pharmacological characterization is necessary to understand how diversity in receptor expression relates to physiological function.

Previous observations that dominant territorial males have larger preoptic somatostatin neurons than subordinate nonterritorial males (30) suggested that somatostatin might promote aggression. However, the hormone manipulations clearly reject this hypothesis. If somatostatin acts in the preoptic area to affect aggression in *A. burtoni*, this would suggest that preoptic somatostatin release is inhibited in dominant males. Future experiments will characterize somatostatin and sstR gene expression within the brain to determine possible sites of action. The effect of somatostatin on physiology and behavior may depend on whether release occurs centrally or peripherally. The effect of somatostatin release to the periphery via the pituitary is well characterized. However, there is also a growing literature describing the neuromodulatory effects of central somatostatin (54), with several studies suggesting that somatostatin function is affected in neurodegenerative disorders (54). Our examination of sstR mRNA expression in testes and the effects of octreotide on androgens suggest that somatostatin could have important action in the testes. Future experiments will examine patterns of sstR protein expression within the brain and testes. The finding that the somatostatin system plays an important role in the control of social dominance behavior adds a new dimension to our understanding of the complex actions of somatostatin.

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