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Short communication

Somatostatin and somatostatin receptor gene expression in dominant and subordinate males of an African cichlid fish

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Abstract

Somatostatin is a neuropeptide best known for its inhibitory effects on growth hormone secretion and has recently been implicated in the control of social behavior. Several somatostatin receptor subtypes have been identified in vertebrates, but the functional basis for this diversity is still unclear. Here we investigate the expression levels of the somatostatin prepropeptide and two of its receptors, sstR2, and sstR3, in the brains of socially dominant and subordinate *Astatotilapia burtoni* males using real-time PCR. Dominant males had higher somatostatin prepropeptide and sstR3 expression in hypothalamus compared to subordinate males. Hypothalamic sstR2 expression did not differ. There were no differences in gene expression in the telencephalon. We also observed an interesting difference between dominants and subordinates in the relationship between hypothalamic sstR2 expression and body size. As would be predicted based on the inhibitory effects of somatostatin on somatic growth, sstR2 expression was negatively correlated with body size in dominant males. In contrast sstR2 expression was positively correlated with body size in subordinate males. These results suggest that in *A. burtoni* social status affects the relationships between somatostatin prepropeptide and receptor gene expression in the hypothalamus and the control of somatic growth.

Keywords: Aggression; Growth; Social behavior; Pre-optic area; *Astatotilapia burtoni*

1. Introduction

Studies of the peptide hormone somatostatin have revealed that this hormone has diverse physiological functions. Originally discovered for its negative effects on growth hormone secretion [1], somatostatin is now known to act in a variety of tissues to regulate energy balance and metabolism [2,3]. There is also growing evidence that somatostatin can act as a neuromodulator [4,5], modulating both motor [6] and even social behaviors [7]. This diversity in function is perhaps reflected in the diversity of somatostatin receptor subtypes.

Five different somatostatin receptor (sstR) subtypes have been identified in vertebrates [2]. Based on structural and pharmacological characteristics the five types can be classified into two subgroups: the sstR2/sstR3/sstR5 subgroup and the sstR1/sstR4 subgroup. The former subgroup binds octapeptide and hexapeptide somatostatin analogs whereas the latter subgroup does not [8]. All five receptor types are expressed in the mouse brain [9] whereas all subtypes but sstR4 have been identified in teleost brains [10,11]. An autoradiography study on goldfish (*Carassius auratus*) brain found that radiolabeled somatostatin binding sites occurred primarily within three systems [11]: the preoptic/hypothalamic area (involved in regulation of pituitary hormones), the facial and vagal lobes (involved in the regulation of ingestive behavior), and the optic tectum (involved in the integration of visual stimuli). Despite identification and localization of multiple somatostatin receptor subtypes, there has been relatively little resolution of the functional bases of differential receptor expression. Sst2 and sst5 are the predominant subtypes in the pituitary of both rodents [16] and teleosts [17,18]. Selective somatostatin ligands provide

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evidence for subtype specific function, as sst2 and sst5 agonists inhibit pituitary growth hormone secretion in rats, whereas selective sst1 and sst3 agonists do not [19]. The diversity of sst subtypes is likely a contributing factor to some of somatostatin's distinct biological functions [20], as differences in the expression of receptor subtypes may affect how somatostatin acts on various physiological processes in diverse tissues. While developmental plasticity in the expression of the five sst subtypes has been explored [21,22], plasticity in sst expression in adults has not been examined in detail (but see [23]).

In addition to the diversity in receptors, different forms of the somatostatin peptide itself have been identified in teleost fish [12]. Expression of the somatostatin I gene in the brain primarily leads to production of a 14 amino acid protein that is closely associated with neuroendocrine regulation (especially growth hormone). The somatostatin II gene has apparently arisen during a gene duplication event and its expression generally results in the production of a 28 amino acid peptide [3]. Its role in the brain and behavior is less clear than other forms of somatostatin. A growing number of studies suggest that somatostatin III (and its mammalian homolog cortistatin) has important effects on circadian rhythms [13]. In goldfish the three forms have distinct but overlapping distributions within the brain [14], all three transcripts being present in the hypothalamus. We focused on somatostatin I because of our interest in growth and our previous work implicating the neuroendocrine functions of somatostatin in regulating behavior [7].

Previous studies suggest that somatostatin is important in behavioral and neural plasticity in the cichlid fish *Astatotilapia* (formerly *Haplochromis*) *burtoni* [7]. In this species, dominant males aggressively maintain a territory and are reproductively active, whereas subordinate males school with females and are reproductively suppressed [15,16]. Subordinate males grow faster than dominant males (many teleosts grow throughout life), apparently representing a trade-off between growth and reproduction [17]. Frequent fluctuations of the physical environment are common in the natural habitat [18] and laboratory studies indicate that such fluctuations result in constant change in social dominance relationships [19]. Such transitions in social status are characterized by asymmetrical changes in growth and reproduction [19,20], with up-regulation of the reproductive axis in socially ascending males being more rapid than reductions. Somatostatin immunoreactive neurons in the preoptic area (POA) are about four times larger in dominant males compared to subordinate males, and POA somatostatin neuron size is negatively correlated with growth rate [21]. These results 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 characterized phenotypic differences in somatostatin-I prepropeptide and somatostatin receptor gene expression in the hypothalamus, telencephalon, and pituitary of dominant and subordinate male *A. burtoni*. Using real-time PCR we measured gene expression in the hypothalamus and pituitary because previous work has identified these regions as critical in the regulation of somatic growth. We also measured gene expression in the telencephalon because our previous observations demonstrated that somatostatin has inhibitory effects on aggressive behavior, and serotonergic activity in the telencephalon is known to modulate aggression [22]. In addition, somatostatin plays a neuromodulatory role in the telencephalon, particularly in the hippocampus [23]. If larger hypothalamic somatostatin neuron size in dominant males is related primarily to reduced growth of dominant males, then dominant males should have more somatostatin prepropeptide, sstR2, and sstR3 expression than subordinate males. In contrast, if changes in somatostatin receptor gene expression in the brain affect aggression, we expected to observe reduced hypothalamic sstR3 and sstR2 expression in dominant males. We also expected to observe a relationship between receptor gene expression and aggressive behavior either between dominant and subordinate males or within dominant males.

2. Methods

2.1. Subjects

Fish were descendents of a wild-caught stock population [18] and were group-housed (5–7 males, 6 females per tank) in 100 L aquaria as previously described [18,21]. Each aquarium contained 5 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 [18]. In each tank there were 2–3 dominant males and 3–4 subordinate males. All males were tagged with colored beads attached to a plastic tag (Avery-Dennison, Pasadena, CA), which was inserted with a stainless steel tagging tool (Avery-Dennison) through the skin just below the dorsal fin at least one week 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 # 22-22).

2.2. Behavioral observations for gene expression studies

To examine differences in somatostatin gene expression in the brain between dominant ($n = 12$) and subordinate ($n = 10$) males we conducted two 10 min behavioral observations over a period of two weeks. We identified dominant and subordinate males by quantifying three behaviors based on previous descriptions of *A. burtoni* [18]. Chasing was defined as the number of times a territorial male chased non-territorial males or females. Border display was defined as the number of times a territorial male displayed to an adjacent territorial male by flaring the gills. Courtship displays were scored when males adopted a quivering display directed towards a female. For gene expression analyses, we chose unambiguously subordinate males that did not engage in any courtship or aggressive behaviors. Only one dominant and one subordinate male per tank was chosen. Males were measured for standard length (body size), and rapidly decapitated. We collected whole telencephalon and hypothalamus (Fig. 1) from each brain and stored these tissues in RNAlater (Ambion, Austin, TX). Previous studies in *A. burtoni* and other fish demonstrate that the hypothalamic portion includes almost the entire preoptic area with the exception of the rostral portion of the anterior parvocellular preoptic nucleus [24–26]. This rostral portion of the anterior parvocellurlar preoptic nucleus contains only a small fraction of the somatostatin I expressing neurons present in the POA [14].

2.3. Cloning of somatostatin prepropeptide and real-time PCR

The full length cDNAs for sstR2 and sstR3 have been previously described. To clone the somatostatin I prepropeptide, primers were designed based on the highly conserved somatostatin-14 peptide, forward

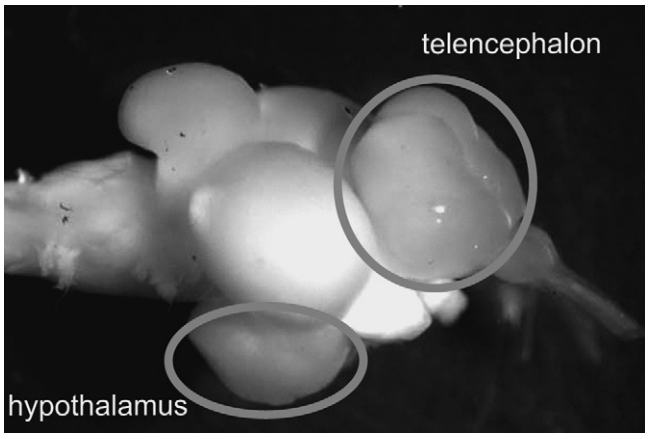

Fig. 1. Brain of male *Astatotilapia burtoni* with hypothalamus (including the preoptic area) and telencephalon indicated with circles.

primer [5′-TGCTCTTGTCGGACCTCCTGCAGG-3′], reverse primer [5′-TTCCAGAAGAAGTTCTTGCA-3′]. PCR was conducted and a 150 bp band was cloned and sequenced. Using the sequence of this fragment, the full length cDNA was obtained using 5′ [5′-CGCTCCAGGTCGACGCGGATGTCTTC-3′] and 3′ [5′-GGAGAACTTCCCTCTGGCCGACGGTGA-3′] RACE (Clontech, Mountain View, CA). The products of these reactions were gel purified, subcloned, and sequenced. We also attempted to sequence sstR1 and sstR5 transcripts but were unsuccessful. Future studies will probe an *A. burtoni* cDNA library for these transcripts.

RNA was extracted from whole hypothalamus and telencephalon samples in 0.5 mL of Trizol and RNA quality was checked using the Nanochip on a Bioanalyzer (Agilent, Palo Alto, CA.). For each RNA sample, 2 μg of 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 [28]. 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 [28–30]. Normalization of RNA using the Ribogreen method is not affected by differences in standard housekeeping gene expression such as G3PDH or 18 s ribosomal RNA in subsequent real-time PCR [28,29]. Based on the Ribogreen measurements of RNA, 1 μg of RNA from each sample was reverse transcribed using Superscript (Invitrogen) for use in quantitative real-time PCR reactions.

Quantitative Real-time PCR reactions were conducted on a MJ DNA Engine Opticon 2 thermocycler. Primers for all transcripts were designed using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). To design primers for sstR2 (Genbank accession no.: AY585718) and sstR3 (AY585719) we used previously published sequences for *A. burtoni* We determined the efficiency of the PCR reaction using standard curves using the plasmids that were created when the cDNAs were cloned. The efficiency was calculated using the formula $E = 10^{[-1/\text{slope}]} - 1$, and was over 90% for all primer sets used. Cycling conditions were: 5 min at 95 °C, then 40 cycles of 30 s at 95 °C, 30 s at 52 °C, and 30 s at 72 °C, followed by a five minute extension period and a melt curve analysis. All reactions were run in duplicate. Gene expression data were not normally distributed so we used non-parametric *U*-tests and rank correlations to analyze the data.

3. Results

The *A. burtoni* somatostatin I peptide gene contains an open reading frame of 366 bp encoding a 119 amino acid prepropeptide and has 45% identity to both mouse and human sequences. The last 14 amino acids, which form the bioactive somatostatin peptide, are identical in *A. burtoni*, mouse, and human. This sequence has been deposited in Genbank (Genbank Accession no.: AY585720).

3.1. Somatostatin and somatostatin receptor gene expression in brain

In the hypothalamus, dominant males had significantly increased expression of somatostatin prepropeptide (Mann–Whitney, $U = 21$, $p = 0.01$) and sstR3 ($U = 13$, $P < 0.01$) compared to subordinates (Fig. 2A). There was no difference in sstR2 expression between dominants and subordinates ($U = 39$, $p = 0.28$). In the telencephalon, there were no significant differences between dominants and subordinates in

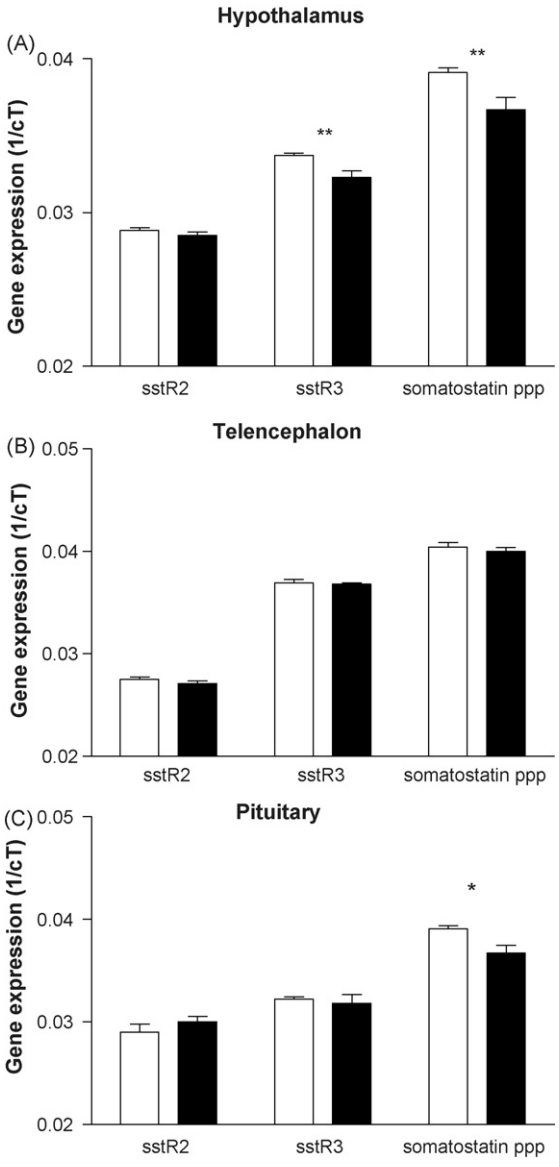

Fig. 2. Somatostatin and somatostatin receptor expression in the brain. Somatostatin I prepropeptide, sstR2 and sstR3 mRNA levels were measured in (A) hypothalamus, (B) telencephalon, and (C) pituitary from dominant (open bars) and subordinate males (black bars). $^{**}P < 0.01$.

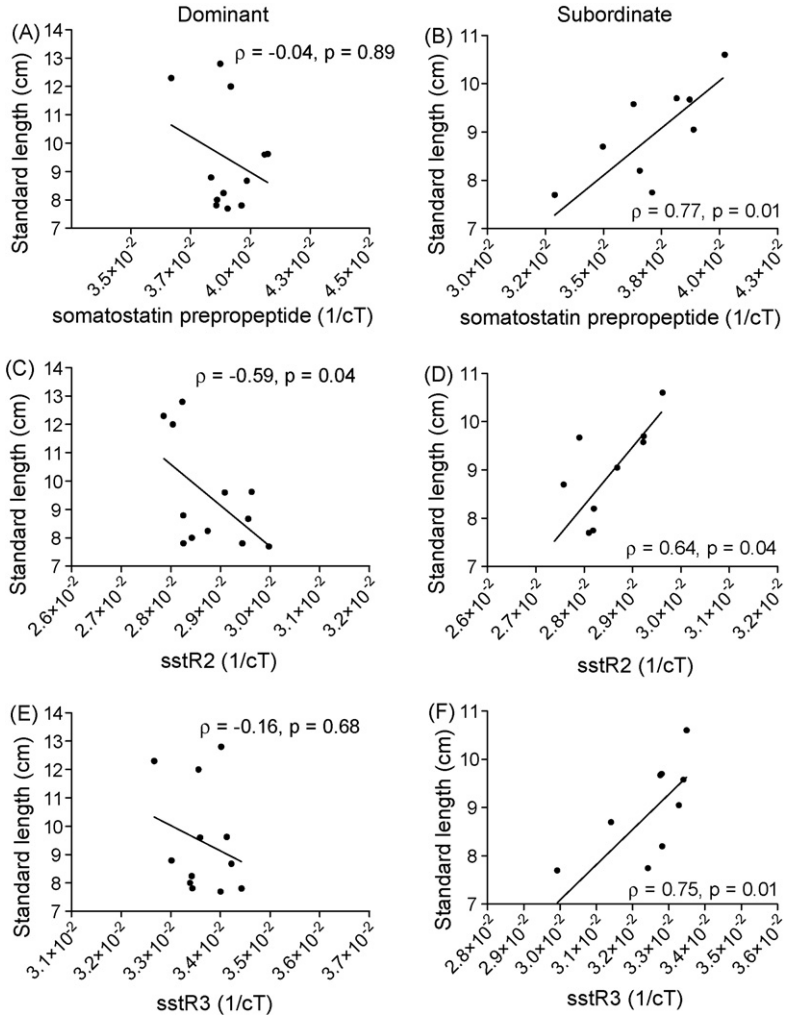

Fig. 3. Correlations between body size and hypothalamic somatostatin I prepropeptide (A and B), sstR2 (C and D), and sstR3 (E and F) in dominant and subordinate males. Spearman correlations with *p*-value are listed in each panel.

somatostatin prepropeptide (Mann–Whitney $U = 51$, $p = 0.78$), sstR2 ($U = 103$, $p = 0.43$), or sstR3 ($U = 105$, $p = 0.51$) expression (Fig. 2B). In the pituitary somatostatin prepropeptide was increased in dominant males compared to subordinates (Fig. 2C, Mann–Whitney $U = 21$, $p = 0.01$), whereas there were no differences in sstR2 ($U = 34$, $p = 0.21$), or sstR3 expression ($U = 24$, $p = 0.99$).

3.2. Somatostatin and somatostatin receptor gene expression and body size

Interestingly, hypothalamic sstR2 expression was negatively correlated with body size in dominant males (Fig. 3C, Spearman $\rho = -0.59$, $p = 0.04$). Hypothalamic sstR3 (Fig. 3E, $\rho = -0.16$, $p = 0.68$) and somatostatin prepropeptide (Fig. 3A, $\rho = -0.04$, $p = 0.89$) expression did not co-vary with body size in dominant males. In contrast, body size in subordinates was positively correlated with sstR2 (Fig. 3D, Spearman $\rho = 0.64$, $p = 0.04$), sstR3 (Fig. 3F, $\rho = 0.75$, $p = 0.01$), and somatostatin prepropeptide (Fig. 3B, $\rho = 0.77$, $p = 0.01$) expression.

3.3. Somatostatin and somatostatin receptor gene expression and social behavior

In dominant males, there were no significant correlations between social behaviors and hypothalamic sstR2 or somatostatin prepropeptide. Hypothalamic sstR3 expression was positively correlated with border threats (Fig. 4C, $\rho = 0.60$, $p = 0.04$) but not chasing ($\rho = 0.1$, $p = 0.8$) or courtship displays ($\rho = 0.22$, $p = 0.5$). There were no significant correlations between social behaviors and sstR2, sstR3, or somatostatin prepropeptide gene expression in the telencephalon or pituitary of dominant males (all p's > 0.09). There were also no significant correlations between sstR expression in hypothalamus or telencephalon and fleeing behavior in subordinate males.

4. Discussion

In the present study we have shown that in the hypothalamus the expression of somatostatin and its receptors is related to both social status and body size. Somatostatin is known to

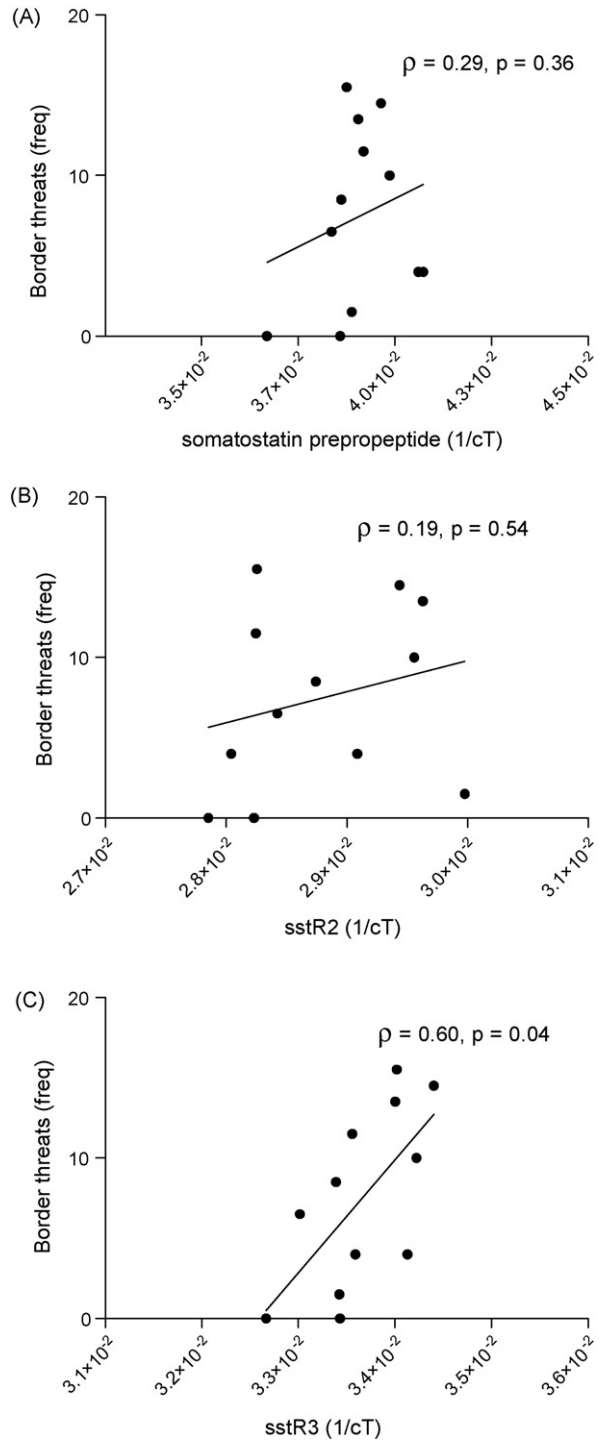

Fig. 4. Correlations between border threats and hypothalamic somatostatin I prepropeptide (A), sstR2 (B), and sstR3 (C) in dominant males. Spearman correlations with *p*-value are listed in each panel.

have an inhibitory effect on somatic growth, and we observed that dominant fish had increased expression of somatostatin prepropeptide and sstR3 gene expression in the hypothalamus. However, in subordinate fish somatostatin receptor and prepropeptide gene expression was positively correlated with body size. These results suggest that somatostatin plays a role in the intricate interplay between somatic growth and social behavior in *A. burtoni*. This complexity may explain why it has been a challenge to detect direct relationships between neural sstR2 and sstR3 expression in the brain and the previously described somatostatin-mediated regulation of social dominance.

Previous studies demonstrated that dominant *A. burtoni* males have reduced growth rates [17] and larger somatostatin immunoreactive neurons than subordinate males [21], and our results from real-time PCR experiments are consistent with these observations. Somatostatin in the preoptic area could mediate growth by directly regulating growth hormone secretion. An additional possibility is that differences in growth may be related to reduced food intake by dominant males, as subordinates have been observed to spend more time feeding than dominants [3]. Increased feeding is typically associated with increased plasma leptin levels [32], and in rats leptin has been demonstrated to decrease somatostatin release into the median eminence [33]. Somatostatin outside of the preoptic area may also be important, as somatostatin I is expressed in the ventroposterior hypothalamus [14], which is thought to control feeding behavior [34]. Somatostatin I expression in this region overlaps with expression of neuropeptide *Y* [35] and cholesystokinin [36], two peptides that are known to mediate food intake [37].

Interestingly, the relationship between somatostatin gene expression and body size differed between dominant and subordinate males, suggesting a complex interplay between the effects of somatostatin on somatic growth on the one hand and social status on the other. In dominant males, sstR2 gene expression was negatively associated with body size, whereas in subordinate fish somatostatin receptor and prepropeptide gene expression was positively correlated with body size. The negative relationship between somatostatin gene expression and growth in dominant males was expected as larger males generally have slower growth rates [17] (presumably mediated by somatostatin [21]). Previous studies indicate that subordinate males have increased activation of the hypothalamic-pituitary-interrenal axis and corticotrophin releasing hormone (CRH) can affect growth hormone secretion. Reduced somatostatin prepropeptide and receptor gene expression in subordinate males may allow for increased stimulation of growth hormone by CRH [38]. The positive relationship between somatostatin prepropeptide and body size in subordinate males was unexpected and may be due to the dual role of somatostatin affecting growth and social behavior. There were no differences between dominant and subordinate males in sstR2 and sstR3 expression in the pituitary and telencephalon. This suggests that phenotypic differences in growth may not be mediated by changes in receptor expression, or that post-transcriptional processes may be more important in altering the sensitivity of the pituitary and telencephalon to somatostatin. Future experiments will determine the effects of somatostatin on somatostatin receptor expression in the pituitary and brain.

In a previous study we demonstrated that somatostatin has robust inhibitory effects on aggressive behavior in dominant males. Based on these results, we expected that more aggressive dominant males might have reduced somatostatin prepropeptide expression in the brain. In contrast, dominant fish had more somatostatin prepropeptide and sstR3 gene expression in hypothalamus than subordinates, and sstR3 expression was pos-

itively correlated with border threats (but not chasing behavior). These results do not support the hypothesis that differences in sstR2 or sstR3 expression mediate phenotypic differences in aggressive behavior. We suggest that increased sstR3 expression in dominant males could possibly result in an autocrine inhibition of hypothalamic somatostatin release. Presumably this would result in altered signaling via other somatostatin receptor subtypes in different tissues (e.g. pituitary). An additional possibility is that subtle, anatomically specific differences in somatostatin receptor expression are present in dominant and subordinate males, with somatostatin acting in different cell populations (e.g., parvo- versus magno-cellular portions of the POA) to affect behavior versus growth. Further study at a higher spatial resolution in the brain is needed to dissect the mechanisms by which somatostatin inhibits aggression in dominant males. There may also be important phenotypic differences in somatostatin II and III expression. In goldfish somatostatin II is mostly confined to the hypothalamus including portions of the preoptic area whereas somatostatin III transcripts are less abundant in the preoptic area and more abundant in telencephalon [14]. The expression of these transcripts will be examined in future studies.

In summary we have demonstrated that hypothalamic somatostatin prepropeptide and receptor gene expression is differentially regulated in dominant and subordinate *A. burtoni*, likely as a consequence of somatostatin's dual role in regulating both dominance behavior and socially controlled growth. Further characterization of the distribution of the different somatostatin receptor subtypes and colocalization with the somatostatin prepropeptide will help in clarifying the complex interplay between a versatile neuropeptide, growth and social behavior.

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