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Immunohistochemical localization of serotonin in the brain during natural sex change in the hermaphroditic goby *Lythrypnus dalli*

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ABSTRACT

The neurotransmitter serotonin (5-HT) may play a central role in the inhibition of socially regulated sex change in fish because of its known modulation of both aggressive and reproductive behavior. This is the first study to use immunohistochemical techniques to examine the morphometry of serotonergic neurons at different times during sex change. Using a model species wherein sex change is socially regulated via agonistic social interactions (the bluebanded goby, *Lythrypnus dalli*), we sampled brains of males and females with different social status, and of females at different times during sex change. Consistent with previous studies on other teleosts, immunoreactive neurons were found in the posterior periventricular nucleus (NPPv), the nucleus of the lateral recess (NRL), the nucleus of the posterior recess (NRP) and in the raphe nucleus. We measured the total area of NPPv, NRL, NRP, and the number and mean cell area of serotonergic neurons in the raphe nucleus. There was no significant difference in any of the brain regions between males, females or sex changing fish, but there was a slight increase in the number of dorsal raphe neurons in the brain of sex changers 2 h after male removal. The results show that in *L. dalli* the serotonergic system does not present any morphological sex and status differences, nor any dramatic modifications during sex change. These data, together with previous results, do not support the hypothesis that serotonin inhibits socially regulated sex change.

1. Introduction

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) is involved in the neuroendocrine modulation of the reproductive function by acting across the hypothalamic-pituitary–gonadal (HPG) axis. In fact, serotonin stimulates the release of gonadotropins (GtH) from the pituitary gland in goldfish *Carassius auratus* [46,59] and in the Atlantic croaker *Micropogonias undulates* [19]. Serotonin can also stimulate the release of gonadotropin realeasing hormone (GnRH) from the anterior hypothalamus in goldfish [60] and in seabream *Pagrus major* [43]. In addition, 5-HT and GnRH immunoreactive elements overlap in the olfactory bulbs and preoptic anterior-hypothalamic area in the Atlantic croaker *M. undulates* [20].

Many immunohistochemical studies have examined the location and distribution of monoaminergic neurons in fishes [2,10,18,28], showing that the neuroanatomy of the serotonergic system is quite conserved across a wide range of species. Serotonin immunoreactive (5-HT-ir) cells have been found in the hypothalamic nucleus of the posterior recess (NRP) and nucleus of the lateral recess (NRL) in various species (sturgeon *Acipenser baeri* [2]; goldfish *C. auratus* [18]; Atlantic croaker *M. undulates* [20]; platyfish *Xiphophorus maculatus* [28]). The authors describe those cells as cerebrospinal fluid (CSF) contacting neurons because these cells have a cytoplasmic process contacting the brain ventricle [3,18]. Serotonergic cells are commonly present in the raphe nucleus of the brainstem (three-spine stickleback *Gasterosteus aculeatus*: [10]; platyfish: [28]) and in the pituitary [10,18,28]. 5-HT-ir cells are also found in the telencephalon of the sturgeon [2], in the nucleus dorsolateral thalami of the goldfish [18], and in the valvular portion of the cerebellum in the platyfish [28]. These immunohistochemical analyses are very important in improving our knowledge of the serotonergic system by investigating the comparative anatomy of the system and its evolution, but they do not address the system from a functional perspective, nor explore intraspecific variation (e.g., sex, ontogenetic, or contextual differences).

There is evidence of serotonergic sex differences in vertebrates. In rodents, *in vivo* telencephalic 5-HT metabolism is significantly higher in females than males in two different strains of rat [41]. There is also an area of the hypothalamus that is sexually dimorphic in rats: the medial and the central part of the medial preoptic nucleus are larger in males than females, and have very low density of serotonergic fibers, while the lateral part of the medial preoptic nucleus is larger in females, and has high 5-HT-ir fiber density [45]. In the bicolor damselfish *Pomacentrus partitus*, there

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is no effect of sex on brain serotonergic activity when comparing dominant and subordinate animals, but rates of received aggression did affect serotonin turnover [56]. To our knowledge, there are no immunohistochemical studies in fish that examined differences in serotonergic neurons between the sexes or among status classes (e.g., dominant versus subordinate animals).

Serotonin is involved in a wide variety of functions [27], but the present work is primarily focused on its association with aggression, social status, and reproductive behavior. Studying the regulation of sex change in the sequential hermaphrodite *Lythrypnus dalli* allows us to investigate how the serotonergic system might link these two important classes of behavior. In fact, in species like the bluebanded goby *L. dalli* where sex change is regulated by the social environment, dominance status is crucial in determining sexual phenotype [44,53,14]. During protogynous sex change, when the male *L. dalli* is removed from the group, the dominant female changes sex into the new male [39]. Since serotonin can modulate the HPG axis and is known to be associated with aggressive behavior and social status [1,9,24], it is a good candidate to transduce social status into a physiological change that initiates both gonadal and behavioral sex reversal.

In the protogynous bluehead wrasse *Thalassoma bifasciatum*, injecting males with the selective serotonin reuptake inhibitor (SSRI) fluoxetine decreased their aggression [32] and their expression of arginine vasotocin (AVT) mRNA, showing that low serotonergic activity might be associated with the display of dominant male aggressive behavior through its action on AVT synthesis [42]. In the protogynous saddleback wrasse *Thalassoma duperrey*, the SSRI sertraline inhibited gonadal sex change in a female providing some experimental evidence for a role of serotonin in the inhibition of sex change [22]. In tilapia, *Oreochromis mossambicus*, the feminizing effect of estradiol on sexual differentiation might be regulated by the serotonergic system [50]. In Nile tilapia, tryptophan hydroxylase (Tph), the rate-limiting enzyme in the biosynthesis of serotonin, was expressed earlier in developing male brains than in female brains but its expression showed no sex difference in adult brains [48]. Also in catfish brain, males showed significantly higher expression of Tph than females [35]. These studies suggest that sex differences exist at the level of serotonergic system during early brain development in teleosts, and therefore indicate a possible role of 5-HT in brain differentiation.

There is a large literature suggesting that elevated serotonergic activity is typically associated with subordinate status in fish [58,55,57]. In *L. dalli*, subordinate fish stay or become female, while dominant fish stay or become male [39], so we predicted that females would have higher serotonergic activity, and therefore that high 5-HT would inhibit sex change. Previous work on *L. dalli* did not support this prediction but rather showed a trend in the opposite direction: although the difference was not significant, female *L. dalli* showed lower levels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) than males [26]. We also found no differences in monoamine levels between dominant and subordinate animals in size-matched pairs of fish, and pharmacological manipulations of the serotonergic system did not affect the probability of sex change in *L. dalli* [26]. There is the possibility that the difference in monoamine levels between dominant and subordinate animals is specific to certain regions of the brain [49], and that we missed it because we analyzed whole brains. To further elucidate whether 5-HT plays a role in the regulation of sex change, we performed the present immunohistochemical study on *L. dalli* brains. The objective of this study was to localize immunoreactive 5-HT neurons in the brain of males, females and sex changing individuals and to test whether there are differences in the cell number or area based on sex and social status. It is hard to predict what the sex and status differences in 5-HT-ir neurons will be because, as mentioned above, we would expect females and subordinate animals to have higher serotonergic function, and therefore either larger or more numerous 5-HT-ir cells; while based on the results of previous work on *L. dalli* [26], we would expect the opposite to be true, with males showing a trend for larger or more numerous serotonergic neurons.

2. Material and methods

2.1. Experimental conditions

The bluebanded goby is a small benthic fish (20–45 mm adult standard length) that inhabits rocky reefs along Southern California and Baja California, Mexico [54], where it establishes social hierarchies with a dominant male that defends a nest, and spawns with multiple females over the course of the breeding season [5]. Fish used in our study were captured off the coast of Santa Catalina Island, CA (California Department of Fish & Game permit # 803024-05 to VL) during the summer of 2005. The fish were held in large seawater tables at the Wrigley Institute of Environmental Studies on Catalina Island and shipped to Georgia State University, Atlanta, where they were housed at a temperature of 19–20 °C, and a 12 h light: 12 h dark photoperiod. The experiments were run between November 2005 and March 2006. The fish were fed brine shrimp twice daily. The research conducted herein was approved by the Georgia State University IACUC protocol No. A06004.

Fish were removed from the communal tank and slightly anesthetized with tricaine methanesulfonate (MS222; Sigma). The sexes were distinguished based on genital papilla shape [6,47,54], and papilla images were captured to measure and compare papilla length-to-width ratio between the start and the end of the experiment. This ratio is a good indicator of papilla shape and therefore of sex, with female ratios being close to 1 and male being $\geqslant 1.4$ [6]. All fish that had ambiguous papilla shape were excluded from the study to avoid including fish in transitional states between the two sexes. Social groups of one large male and three females were established in individual 38 l tanks. Males were at least 3 mm larger than the largest female in the tank to ensure their dominance. In each group, the focal female (alpha) was at least 3 mm larger than the other two females in her tank (beta and gamma), to ensure that she would become the dominant female and therefore the sex changer [37]. Beta, being larger than gamma, would normally achieve an intermediate position in the social hierarchy being subordinate to alpha but dominant over gamma. A total of 60 social groups were established, and after 5 days the male was removed from 50 of these tanks. Experimental groups were terminated at different times after male removal to sample them at different stages of sex change. Ten groups were terminated for each of the following time points: 2 h, 1 day, 3 days, 7 days, and 14 days after male removal. These time points were chosen to cover previously established phases of sex change [38,39], beginning with the removal of the male (the initiation event) and continuing through 2 weeks after male removal, when gonadal sex change is completed. As controls, we had 10 stable groups where we did not remove the male. Animals were euthanized by immersion in a lethal concentration of MS222. We collected the brain of alpha females at all time points during sex change (dominants) and in stable groups with males present. We also collected brains of 10 males and 10 beta and gamma females. For each experimental fish, we measured standard length (SL) and body weight (BW), collected the gonads and recorded the genital papillae shape to assess the final sex of the animal. Brains were immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for two days, cryoprotected overnight in 30% sucrose solution, fast frozen on dry ice, embedded in HistoPrep media (Fisher Scientific), and stored at −80 °C. The tissue was sectioned in two parallel series