

Socially induced sex change regulates forebrain isotocin in *Lythrypnus dalli*

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The neurohypophyseal peptides are evolutionarily conserved and their expression can be socially modulated. Our question was what effect will socially induced sex change have on forebrain isotocin, an oxytocin homologue? We removed males from social groups to induce dominant females to change sex and become males in *Lythrypnus dalli*. Fish in the late stages of sex change had fewer forebrain isotocin-immunoreactive (-ir) cells than early stage and

unchanged females. When groups were consolidated into unequivocal males (control males and sex-changed new males) and unequivocal females (fish prior to courtship as a male), females had significantly more isotocin-ir cells than control males and recently sex-changed fish. This is the first study demonstrating the social regulation of forebrain isotocin. *NeuroReport* 15:185–189 © 2004 Lippincott Williams & Wilkins.

Key words: Dominance; Oxytocin; Preoptic area; Reproduction; Social behavior; Subordinate; Teleost; Vasopressin; Vasotocin; Vertebrate

INTRODUCTION

Social interactions can regulate the brain and reproduction. For example, social signals can cause an African cichlid fish to change from non-reproductive to reproductive status and these same social signals result in increases in the size of gonadotropin-releasing hormone cells in the brain [1]. The neuropeptide oxytocin is also modulated by social interactions. Defeated male rats release more oxytocin within the hypothalamus than those that are not defeated [2]. Social dominance also alters the behavioral response of female Syrian hamsters to centrally administered oxytocin [3].

Oxytocin has been implicated in the regulation of affiliative behavior, milk let-down, and reproductive behavior [4]. Oxytocin and the other neurohypophyseal peptides are evolutionarily conserved, and forms of this peptide family are found in all vertebrates and many invertebrates [5]. For instance, the oxytocin-related peptide annectin is implicated in egg-laying behavior in earthworms [6]. In Syrian hamsters, oxytocin promotes female receptivity by increasing the duration of lordosis in a dose-dependent manner [7]. In contrast, female prairie voles show decreased sexual receptivity, but enhanced social behavior associated with sexual receptivity, such as partner preference, with central injections of oxytocin [8]. In rats, central oxytocin administration initiates and maintains female sexual behavior [9,10]. There is a trend across species for central oxytocin to increase female sexual behavior and decrease male sexual behavior, but there are notable exceptions [4,11,12,13]. In summary, a strong case can be made for

sexual dimorphism in many aspects of the oxytocinergic system in mammals.

Isotocin, the fish homologue of oxytocin, has received little study in terms of the regulation of behavior since the early reports of Pickford and Strecker [5,14]. They reported that peripherally injected isotocin had an effect on behavior, but it was simply due to minimal binding to arginine-vasotocin (AVT) receptors [14]. Only recently has it been shown that central isotocin has different effects from vasotocin with regard to the neural activity that drives humming behavior in the plainfin midshipman fish [15]. Other recent work has shown that vasotocin and isotocin neuronal populations have distinct patterns of periodic Ca^{2+} pulses in rainbow trout, supporting separate signaling pathways for release of the two neuropeptides [16]. In addition, isotocin receptors in the teleost *Catostomus commersoni* showed dramatically lower activation by vasopressin than by isotocin [17]. These three studies suggest that isotocin function is independent of the vasotocin neuropeptide pathway, both in its release and its downstream effects. We are studying these issues in *Lythrypnus dalli*, a teleost fish that is a useful model organism for looking at the links between socially regulated behavior and neuropeptides.

The reproductive behavior of *L. dalli* changes from female to male following the removal of a dominant male from a social group. This straightforward social manipulation predictably triggers the dominant female to change sex to male under both field (Moore, Black, Canario *et al.*, unpublished) and laboratory conditions [18]. The sex

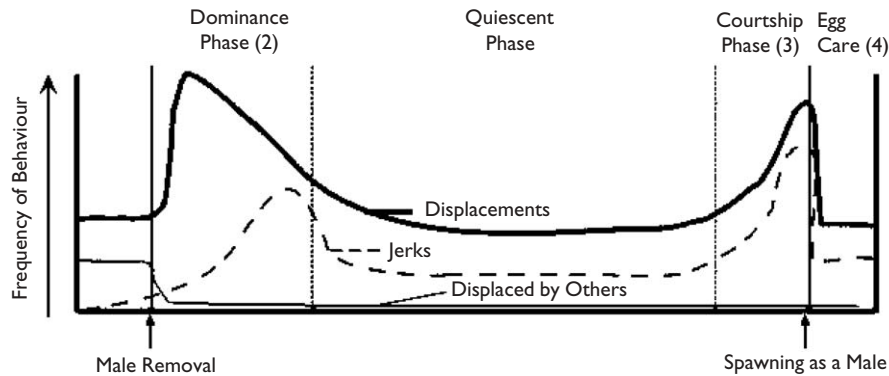


Fig. 1. A behavioral model of female to male sex change in *L. dalli* (modified from Reavis and Grober, 1999, with permission from Springer-Verlag [18]), with phases based on the jerk and displacement behavior of a focal female fish. The model represents a dominant female fish's behavior and the changes that occur over time following removal of a male. Fish with the highest initial displacement behavior were selected as dominant fish. Time from male removal to spawning as a male was 7–10 days. Phases were given integer values based on the sequence of events starting with control females (1) and ending with control males (5).

change process is characterized by a stereotyped behavior profile that allows changes in neuropeptides, such as isotocin, to be mapped onto the sex change process (Fig. 1) [18]. Using this behavioral model, we can examine the specific behavioral relevance of neurochemical changes over the course of sex change, in addition to providing basic comparisons among females, established males and new males (i.e. females that have recently changed to males). We hypothesized that (1) there is a sexual dimorphism in isotocin peptide-containing preoptic cells, and (2) social manipulation that leads to sex change would also change the isotocin system in the forebrain. We predicted that removal of male inhibition would change isotocin-like-immunoreactive (IST-ir) neurons over the course of sex reversal in *L. dalli* and, as a result, there would be a sex difference in IST-ir cells between males and females, with no difference between males and recently sex-changed fish.

MATERIALS AND METHODS

Experiments were conducted from July to August of 1997 at the Wrigley Institute for Environmental Studies at Catalina Island, California. *L. dalli* were collected from the area around Bird Rock at depths of 6–12 m with an anaesthetic solution of quinaldine sulfate (Sigma Chemical) and hand nets (California Department of Fish and Game Permit 802002-02). After capture, all fish were placed in a large tank (59.1 × 87.6 × 30.5 cm) housed outdoors, and later sorted by genital papilla shape into groups of one male and four females, with each fish identifiable by unique body markings [18]. The largest (focal) female was longer in standard length than any female in the group by ≥ 3 mm, and the male was ≥ 2 mm longer than the largest female. No individuals with a standard length of ≤ 20 mm were used. Individuals were given 5 days to adjust to their new social group in outdoor divided tanks (59.1 × 17.5 × 15.25 cm or 29.5 × 43.8 × 15.25 cm) provided with flow-through seawater. Fish in all tanks were fed fish flakes twice daily. On the fifth day following group formation, males were removed from the social group to induce sex change in the largest female. Dominance was determined by the frequency of

jerk and displacement behavior and an individual's reaction to jerks and displacements of other fish [18]. Jerk behavior is when a fish swims toward another individual in abrupt starts and stops with fins erect, alternating between moving slightly to the left and slightly to the right of the general direction of movement. Displacements are movements to within 5 cm of another fish that cause the other fish to move away. The behavior of the focal (dominant) female was recorded for 15 min twice daily before the male was removed and then each subsequent day until focal female removal at stereotyped behavioral stages in the sex change process, described by Reavis and Grober [18] and summarized below. Soon after male removal, focal females show an increase in displacement behavior and sometimes display jerk behavior. This is called the dominance phase. This is followed by dramatic decreases in jerk and displacement behavior that can last for a day or more, termed the quiescent period. The last stage is the courtship stage, where there is a second increase in jerk and displacement behavior before fertilizing eggs as a male. We sampled the brains and observed the genital papilla of focal females during the different behavioral stages of sex change: dominance, courtship, and after fertilizing eggs as a male (i.e. sex-changed; Fig. 1).

Focal females were removed following morning observations because of documented diurnal changes in plasma peptides of other fish species [19]. After a dominant female was removed from a group, the tank division was cleaned, a new group replaced the previous group in the division, and the entire process was repeated. Removed fish were euthanized with an overdose of MS-222 and immersed with their eyes removed to allow adequate diffusion into the brain for no less than one week, in 4% formaldehyde freshly prepared from paraformaldehyde. Brains were dissected out from the skull and stored overnight at 4°C in 30% sucrose in 0.1 M phosphate buffer for cryoprotection. Each brain was sliced at 20 μm on a cryostat, and every other section was labeled with oxytocin antisera (compliments of Park and Kawashima) [20]. Labeled cells in the forebrain preoptic area were quantified using NIH Scion Image1.62a (W.Rasband, NIH, Bethesda, MD). A set of control slides were double labeled for vasotocin and isotocin to assure the specificity of the oxytocin antisera for isotocin in *L. dalli*. All

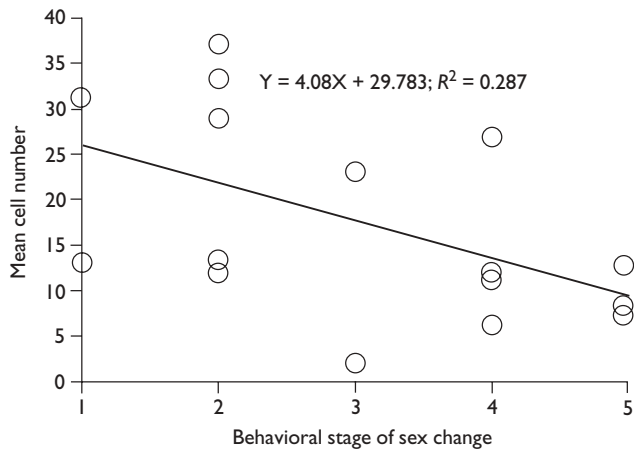


Fig. 2. Linear regression of sex change stage vs preoptic isotocin-immunoreactive cell number in *L. dalli* ($p = 0.027$). Numbers refer to the stage of sex change: 1 = control females ($n = 2$), 2 = dominance phase ($n = 5$), 3 = courtship phase ($n = 2$), 4 = sex changed females ($n = 4$), 5 = control males ($n = 4$).

slides were coded so that the individuals who quantified the size and number of the cells did not know the identity of the fish to which each slide corresponded. The IST-ir cell size and number versus stage of sex change was compared using linear regression. Each behavioral stage was assigned an integer value based on the time sequence of the stages (Fig. 2). In view of the variation revealed by the regression analysis (Fig. 2), we also compared the IST-ir cell size and number in the unequivocal males (control males and sex-changed new males) and unequivocal females (fish prior to courtship as a male) by use of a Mann-Whitney U-test. This comparison was done without correcting for body size and also correcting for body size by dividing the value by the standard length of the fish. All statistics were performed on StatView (v.5.0.1).

RESULTS

The average time for females to change sex upon removal of the male was 7.8 ± 1.3 d ($n = 5$). There was a correlation between sex change stage and preoptic area IST-ir cell number (Fig. 2, $p = 0.027$, $R^2 = 0.287$) but no statistically significant correlation between sex change stage and cell size ($p = 0.17$). When unequivocal males and females were compared, females had a larger cell number than males (Fig. 3, $p = 0.013$). Correcting for body size, the difference was even larger ($p = 0.0092$). Thus, there appears to be a robust sexual dimorphism in IST-ir cell number in *L. dalli*. No difference was found in the cell size between males and females before ($p = 0.064$) or after correction for body size ($p = 0.297$).

The correlation between sex change stage and cell number in this experiment shows a great deal of variation (Fig. 2). The least amount of variation is seen in the number of IST-ir cells in the control males. Interestingly, the male with the highest number of cells (furthest from the regression line and thus most female-like) had been seen with eggs the day before removal, but on the day of removal, this male did not have eggs in his nest tube.

Among females that had changed sex, the fish with the highest IST-ir cell number (furthest from the regression line)

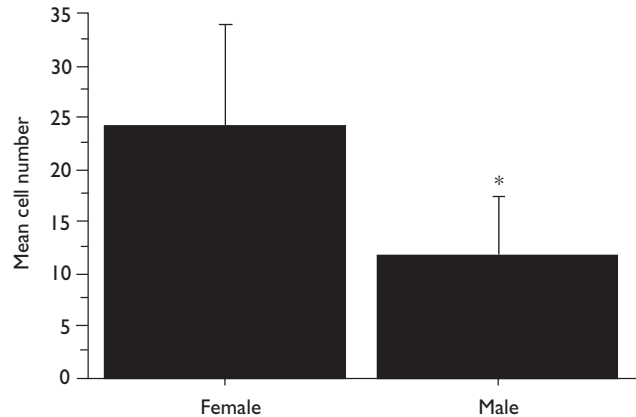


Fig. 3. Differences in preoptic isotocin-immunoreactive cell number between *L. dalli* females (stages prior to courtship phase, $n = 7$) and males (control males and sex changed females $n = 8$). Mann-Whitney U, $p = 0.013$. Error bars represent 95% confidence limits.

had a pointed but short genital papilla, while most of the others had transitional (longer) genital papillae. The sex changer with the lowest number of IST-ir cells had the only male-typical genital papilla of the group.

DISCUSSION

The removal of the male from a social group resulted in rapid sex change that was consistent in duration with previous studies in the laboratory [18] and in the field (Moore *et al.*, unpublished). This manipulation also resulted in a profound sex difference in IST-ir cell number. Females had more IST-ir cells than males, including males that had recently changed from female, yet there were no significant differences in cell size. The mammalian homologue for isotocin, oxytocin, has been reported to inhibit male sexual behavior in prairie voles [21]. Oxytocin has also been shown to promote female sexual behavior in rats [22] and other species as described above. The changes in isotocin in response to male removal may serve a similar function in *L. dalli*. The larger number of isotocin cells in females suggests that isotocin serves a modulatory function requiring more cells for repression of male function or enhancement of female function. It is interesting to note that Goodson and Bass found that inhibition of isotocin with the selective oxytocin antagonist ((des-glycinamide⁹, d(CH₂)₅¹, O-Me-Tyr², Thr⁴, Orn⁸)-vasotocin) resulted in a 300% increase in the number of fictive bursts by midshipman fish females (more male-like behavior) and infusion with isotocin decreased the number of fictive bursts and fictive burst durations in a dose-dependent manner (less male-typical behavior) [15]. These results suggest isotocin may be playing a similar behavioral role in midshipman fish.

An alternative hypothesis is that isotocin is playing a non-behavioral role, helping with normal female cycling and egg laying, that is not necessary when a fish changes to male. This would be consistent with oxytocin and related neuropeptides playing a physiological role in the production of offspring by females in both vertebrates and invertebrates [1,6].

The male-like length to width ratio of the genital papilla is dependent on androgen levels [23], suggesting that the female with more IST-ir cells and a pointed but short genital papilla was not as far along as the others in the sex change process, even though it did fertilize eggs as a male. The sex changer with the lowest number of IST-ir cells had the only male-typical genital papilla of the group, further supporting this idea. Steroids have been shown to influence the oxytocin system in mammals [24,25], so this may be indicative of an androgen effect.

Because the male class also included males that had recently changed from female, preoptic area isotocin-producing cells may die or be modified to no longer produce isotocin when a female changes to male. We have no current evidence on whether apoptosis has occurred or not. However, when males in the laboratory are artificially put together in a group with only larger males, they can revert back to females [18]. This process takes significantly longer and the social situation is unlikely to occur in the field. The longer period of time to change from male back to female could be due to changes in the body and brain that are hard to reverse, such as cell death.

Control males may have the lowest amount of variation in IST-ir cell number due to these hard-to-reverse changes. The male with the highest IST-ir number lost its eggs before removal. Since males exclusively care for the eggs, perhaps this male ate its own eggs or did a poor job in keeping females from eating its eggs because its paternal behavior was less male-like.

The decrease in IST-ir cell number as a female changes to male is different from what is found in vasotocin-producing cells, where the cell number between males and females was found to be the same, but the cells grew larger as *L. dalli* changed from female to male [18]. The difference suggests that not only are isotocin and vasotocin serving different functions, but that they may be serving different sexually dimorphic functions. This idea is supported by work on another teleost fish, the plainfin midshipman, where vasotocin and isotocin had differential effects on fictive humming in males *vs* females [15]. Further studies using *in situ* hybridization to examine mRNA expression and behavioral observations coupled with injections of AVT and IST and their antagonists are needed in *L. dalli* to examine how neuropeptide expression is changing in response to the social cue of male removal and what functions these neuropeptides may be serving.

CONCLUSION

This study provides what appears to be the first demonstration of a sexual dimorphism in the number of IST-ir cells in the preoptic area of a fish. Moreover, this is the first study to demonstrate changes in the number of isotocin neurons in response to social modulation in any vertebrate. Removal of the male from a social group resulted in preoptic area IST-ir cells decreasing from female- to male-typical numbers in the dominant female as she changed sex. We suggest that *L. dalli* provides a useful model system for investigating the role of neuropeptides in generating the sex differences that characterize most vertebrates, since these animals are

sexually plastic during adult life and easily manipulated by changing the social environment.

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