

BLOCKADE OF ARGININE VASOTOCIN SIGNALING REDUCES AGGRESSIVE BEHAVIOR AND c-FOS EXPRESSION IN THE PREOPTIC AREA AND PERIVENTRICULAR NUCLEUS OF THE POSTERIOR TUBERCULUM IN MALE *AMPHIPRION OCELLARIS*

C. YAEGER, A. M. ROS, V. CROSS, R. S. DEANGELIS,
D. J. STOBAUGH AND J. S. RHODES*

Department of Psychology, The Beckman Institute, 405 N.
Mathews Avenue, University of Illinois at
Urbana-Champaign, Urbana, IL 61801, USA

Abstract—Many marine fishes change sex in response to social cues when the dominance hierarchy is perturbed. Arginine-vasotocin (AVT) and the mammalian homolog arginine vasopressin are neuropeptides involved in social and reproductive behaviors across vertebrate taxa. The goal of this study was to determine whether AVT signaling influences aggression and expression of c-Fos, a marker of neuroplasticity, in key brain regions of the social decision circuit in *Amphiprion ocellaris* clownfish, a species where behavioral dominance precedes gonadal sex change from male to female. In experiment 1, juvenile clownfish (average mass 2.5 g) were paired together in a tank (a total of 24 pairs), matched approximately for size with one fish randomly receiving either an intraperitoneal injection of the arginine vasopressin V1a receptor antagonist (Manning compound) or saline vehicle, and evaluated for aggressive and submissive behaviors over a 10-min period. The second experiment was a repeat of the first using five pairs of mature, reproductive males, except the animals interacted for 90-min immediately followed by euthanasia for immunohistochemical detection of c-Fos protein. Numbers of c-Fos-positive cells were quantified in the preoptic area of the hypothalamus (POA), the anterior tuberal nucleus (aTn), and periventricular nucleus of the posterior tuberculum (TPp). Manning compound significantly reduced aggression and the probability of winning the contest relative to saline (vehicle) controls. In experiment 2, saline-treated fish displayed approximately twice as many c-Fos-positive cells in the POA and 25% more in the TPp than the Manning-treated fish, no differences were observed in the aTn. Taken together, results suggest AVT signaling is necessary for aggressive behavior and expression of neuroplasticity in the POA and TPp that likely contributes to behavioral

dominance and hence, sex change in *A. ocellaris*. Published by Elsevier Ltd. on behalf of IBRO.

Key words: sex change, arginine vasopressin, V1a receptor, Manning compound, clownfish, aggression.

INTRODUCTION

Social experiences (i.e., interactions between members of the same species) can have long-lasting influences on personality and behavior (e.g., Rosen, 1961). One of the most dramatic examples in nature is socially induced sex change (Robertson, 1972). Unlike mammals and birds where sex determining genes on sex chromosomes coordinate the development of the gonads and then the gonads influence sex-specific aspects of the brain and behavior, in many marine fishes there are no sex chromosomes. In these species, the brain and behavior determine gonadal development and the signal for sex change is usually a change in the social structure or hierarchy (Francis, 1992).

Some of the best studied examples include the Bluehead wrasse (*Thalassoma bifasciatum*) commonly found on coral reefs of the Caribbean Sea (e.g., Grober and Bass, 1991; Warner and Swearer, 1991; Perreault et al., 2003; Semsar and Godwin, 2004; Semsar et al., 2004; Marsh et al., 2006) and the Bluebanded goby (*Lythrypnus dalli*) found on reefs of the Eastern Pacific (Reavis and Grober, 1999; Black et al., 2005; Rodgers et al., 2007; Lorenzi et al., 2008, 2009). When a dominant male is removed from the territory and no other intermediate males are present, the largest and highest ranking female in the surrounding territory undergoes complete morphological sex change and occupies the dominant position. Remaining females move up one notch in the hierarchy. In the Bluebanded goby but not the Bluehead wrasse, the process is reversible, i.e., the male can change back into female if dominance status is lost, for example because another larger alpha male entered the territory. Sex change in these species involves significant changes in the brain, behavior, gonads, and external morphology. Ultimately, ovaries are resorbed and testes develop, and in the Bluebanded gobies testes can resorb and ovaries re-develop.

*Corresponding author. Tel: +1-217-265-0021; fax: +1-217-244-5180.

E-mail address: jrhodes@illinois.edu (J. S. Rhodes).

Abbreviations: aTn, anterior tuberal nucleus; AVT, arginine vasotocin; AVP, arginine vasopressin; C, cerebellum; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; OT, optic tectum; PT, pallium of the telencephalon; POA, preoptic area; T, telencephalon; TPp, periventricular nucleus of the posterior tuberculum; VMH, ventromedial hypothalamus; VTA, ventral tegmental area.

The physiology of socially induced sex change originates in the brain (Godwin, 2010). Fish detect a change in the social hierarchy mostly from visual information (Grosenick et al., 2007; Chen and Fernald, 2011), though input from other sensory modalities including hearing specific sounds emitted from the other fish in the social group (Colley and Parmentier, 2012; Maruska et al., 2012), detecting tactile cues from physical contact (e.g., bites, charges) and smells and tastes of various pheromones (Wyatt, 2003) likely contribute. The sensory information causes a change in behavior, specifically behaviors such as aggression which result in changes in the structure of the hierarchy.

Shifts in the dominance hierarchy in both fish that change sex and animals that do not are known to involve changes in gonadotropin-releasing hormone (GnRH) neuron signaling (Davis and Fernald, 1990; Foran and Bass, 1999; White et al., 2002; An et al., 2010; Godwin, 2010). GnRH cells in the preoptic area of the hypothalamus change in size, number, and function which affects the frequency or amplitude of GnRH released onto the pituitary gland followed by altered release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary (Foran and Bass, 1999; Maruska et al., 2011; Fernald and Maruska, 2012). Receptors for LH and FSH on the gonadal tissue then signal the gonad to change in size or tissue type (Kobayashi et al., 2009). For example, in the case of a non-sex changing African cichlid fish (*Astatotilapia burtoni*), ascending the social hierarchy from beta to alpha male is associated with enlargement of GnRH cells in the hypothalamus and subsequent enlargement of the testes (Francis et al., 1993). The neurobiology of socially influenced changes in dominance status is less well understood in species that change sex, but the pathway is thought to be generally the same. Gonads receive signals from the pituitary that cause the gonadal tissue changes. Many of the details are not known such as whether it is pulsatile release GnRH, LH or FSH, differential receptor subtypes for LH and FSH on the gonads, or whether other peptides or chemicals are involved (Godwin, 2010).

The focus of this paper is on the initial events in the brain that begin the sex change process (e.g., a beta fish starts behaving as an alpha) using the clownfish, *Amphiprion ocellaris* as an emerging model organism. Clownfish are unusual among vertebrate species in that the females are the alpha (behaviorally dominant) sex, the mating system is monogamous rather than polygynous, and they are protandrous, sex change occurs from male to female (Fricke and Fricke, 1977). Clownfish live on sea anemones for protection, with the alpha female and beta male reproductively active while the remaining fish if any on the anemone are non-breeders (Buston, 2004). Because females are the dominant sex and because they are easily bred and kept in the laboratory, *A. ocellaris* are a useful complement to the Bluehead wrasse and Bluebanded goby models in which males are dominant and subjects are usually studied in, or captured from their natural environment.

Current understanding is that the behavioral repertoires that precede sex transformation involve changes in the frequency or timing of action potentials in neurons, patterns of neurochemical release, and/or changes in distribution of receptors in brain regions comprising the social decision circuit (as reviewed in Godwin, 2010; O'Connell and Hofmann, 2011). Arginine vasotocin (AVT) signaling in the social decision circuit in particular is thought to play a key role in influencing aggression and dominance in various species of non-sex changing and sex changing fish (e.g., Foran and Bass, 1999; Semsar et al., 2001; Greenwood et al., 2008; Iwata et al., 2010). In the Bluehead wrasse, AVT messenger RNA and number of AVT immunoreactive cells increase in the magnocellular preoptic area of the hypothalamus in relation to dominance status with females displaying the lowest and territorial males the highest expression (Godwin et al., 2000; Semsar and Godwin, 2003). The arginine vasopressin (AVP) V1a receptor antagonist (Manning compound) reduced aggression, dominance and territorial defense in females that were in the process of sex change (Semsar and Godwin, 2004). Manning compound also reduced aggression and dominance in intermediate (non-territorial) and territorial terminal phase males (Semsar et al., 2001). These results suggest AVT signaling at V1a receptors is necessary for heightened aggression and dominance associated with sex change from female to male in the Bluehead wrasse.

Taken together, previous literature supports the hypothesis that AVT signaling in the brain is related to dominance and aggression across species including sex changing and non-sex changing fish with alternative male reproductive tactics and morphs (Foran and Bass, 1999). However, several major gaps in the literature remain. The effect of blocking V1a receptors on dominance-related behavior and aggression in protandrous clownfish that change sex from male to female has never been reported. In the Bluehead wrasse and the majority of other species in which AVT signaling was measured, the mating system is polygynous and males are the behaviorally dominant sex. In clownfish, the mating system is monogamous and females are the dominant, terminal sex, which could change the role of AVT. In the available Bluehead wrasse studies, AVT signaling was directly manipulated with agonists and antagonists in females that were already changing sex, and in terminal phase males without the capability to change sex (Semsar et al., 2001; Semsar and Godwin, 2004). However, no previous study that we are aware of has blocked V1a receptor signaling in males at the initial stages of a status contest, long before sex change, in which the long-term winner would eventually change gonadal sex. Finally, the effect of blocking V1a receptors on markers of neuroplasticity in key regions of the social decision circuit has never been reported. This study fills these gaps in the literature using the *A. ocellaris* model.

The goal of the present study was to determine whether AVT signaling influences aggressive and submissive behavior in non-reproductive juvenile and

mature male *A. ocellaris* and expression of the neuroplasticity marker, c-Fos, in three different regions within the social decision circuit within 90-min of exposure to the social stimulus. The three regions of focus were the preoptic area (POA) of the hypothalamus, the anterior tuberal nucleus (aTn; putative homolog of the mammalian ventromedial hypothalamus, VMH) and periventricular nucleus of the posterior tuberculum (TPp; putative homolog of the mammalian ventral tegmental area, VTA) (O'Connell and Hofmann, 2011). The POA was of interest because it is one of the only undisputed sexually dimorphic brain regions in vertebrates with functional significance for determining male versus female-specific behaviors (Gorski, 1984; Elofsson et al., 1997; Parhar et al., 2001). In addition, GnRH cells reside in the POA which are known to regulate pituitary release of FSH and LH. Moreover, FSH and LH signals are received by the gonads to regulate spermatogenesis, ovulation, release of sex-specific steroid hormones and coordinate sex-specific behaviors. Finally, AVT neurons reside in the POA, and the central hypothesis of this paper is that AVT signaling plays a role in aggression and dominance in *A. ocellaris*.

The aTn was of interest because it is interconnected with the POA, is a component of the social decision circuit and homolog of the mammalian VMH. The VMH is most well understood for its role in female-typical behavior including receptivity (Malsbury et al., 1977), and maternal behavior (Sheehan et al., 2001) but also involved in male-typical behavior including aggression (Kabelik et al., 2008; Lin et al., 2011; Yang et al., 2013). The TPp was of interest because it is the putative homolog of the VTA which in mammals is the location of the dopamine neurons that innervate the ventral striatum. These dopamine fibers are critically involved in neuroplasticity, motivation, and reward across vertebrate taxa (O'Connell and Hofmann, 2011; Salamone and Correa, 2012). The VTA is a critical component of the natural reward circuit in the brain, i.e., motivating animals to display a specific behavior (Kelley and Berridge, 2002). Finally, the reward circuit is thought to interact with the social decision circuit by allowing the circuit to change depending on experiences, i.e., selectively strengthening specific connections in the brain over others that influence patterns of behavior (Young et al., 2005).

EXPERIMENTAL PROCEDURES

Experiment 1

Subjects and husbandry. A total of 17 juvenile *A. ocellaris* were used for this experiment. None of the subjects had reached an age or size typical for reproducing. The gonads of all fish were evaluated for the presence of ootestes (see section 'Gonadal histology'). Mean body mass was 2.5 g (± 0.639 SD) at testing. The fish were purchased from ORA Farms (Fort Pierce, Florida) as juveniles, and kept in marine aquariums for one month prior to the start of the

experiment. The fish were maintained in small social groups consisting of between 2 and 6 animals, and each group was housed in its own 20 gallon aquarium (dimensions 76 cm \times 30 cm \times 30 cm) containing live rock. There were six aquariums in total and each received the same water circulation as part of the larger system consisting of an 80-gallon sump and filter. These aquariums were visually separated from each other by an opaque barrier placed between each aquarium. Instant Ocean salt mixed with reverse-osmosis or deionized water was used. The salinity (specific gravity) was maintained at 1.025 and temperature 78 degrees Fahrenheit. Water pH was kept at 8.1, and ammonia, nitrite, and nitrate were maintained at zero. The subjects were fed a mixture of frozen and dried pelleted fish food twice per day.

Experimental design. All 17 fish used in the experiment were individually marked using colored latex beads (Northwest Marine Technology, Shaw Island, WA, USA) that were inserted under the skin two weeks prior to the start of the experiment. We created 24 novel pairings of fish based on proximity in body weight (see Table 1). Fish were tested multiple times against different rivals on different test days. Fish from the same home aquarium were never paired together in order to avoid matching fish that had already established their dominance relationships.

Each pair was examined for behavioral interactions and to determine the winner and loser in a single test. A test consisted of removing the fish in the pair to be

Table 1. Juvenile clownfish contests for Experiment 1

Trial #	Date	Contest Manning vs Saline
1	9/20/11	2* (1.71) vs 18 (1.87)
2	9/20/11	5 (1.93) vs 11* (1.90)
3	9/20/11	21* (2.45) vs 1 (2.40)
4	9/27/11	21* (2.30) vs 6 (2.46)
5	9/27/11	20 (2.86) vs 17* (2.86)
6	9/27/11	16* (2.98) vs 4 (2.98)
7	9/30/11	15 (2.36) vs 7* (3.27)
8	9/30/11	6 (4.00) vs 14* (3.27)
9	10/3/11	20 (3.25) vs 16* (3.25)
10	10/3/11	4 (2.39) vs 19* (2.4)
11	10/3/11	17 (2.82) vs 22* (3.55)
12	10/4/11	19 (2.23) vs 16* (2.25)
13	10/4/11	4 (2.27) vs 20* (2.72)
14	10/11/11	18 (1.60) vs 11* (1.81)
15	10/11/11	21 (2.00) vs 17* (2.52)
16	10/18/11	6 (3.44) vs 14* (3.39)
17	10/18/11	15 (3.27) vs 20* (2.76)
18	10/18/11	22 (3.27) vs 7* (3.33)
19	10/19/11	18 (1.82) vs 4* (2.13)
20	10/19/11	22 (2.62) vs 16* (2.86)
21	10/19/11	17 (2.30) vs 21* (1.84)
22	10/20/11	16 (2.15) vs 22* (2.62)
23	10/20/11	10 (1.18) vs 18* (1.36)
24	10/20/11	19 (2.23) vs 7* (2.89)

Note: In the contest column, the fish receiving manning compound is listed first followed by the fish receiving saline. Body mass measured before the contest is shown in parentheses. The star next to an animal ID indicates the dominant individual in the pair (i.e., the outcome of the contest).

examined, and placing them in separate labeled holding buckets. Animals were then anesthetized by placing the fish in a 400 ml beaker with Tricaine (MS222, Finquel; Argent-labs, Redmond, WA, USA) at a concentration of 0.25 mg/ml for 15 s. Once the fish lost its righting reflex, it was removed from the beaker and injected intraperitoneally (using a 3/10 cc insulin syringe), between the ventral fin and urogenital orifice, with either the AVP (V1a) receptor antagonist, Manning compound (Fisher Scientific, Waltham, MA, USA) or saline vehicle (Semsar et al., 2001). Manning compound was administered at a dose of 3.2 $\mu\text{g/g}$ body weight at a volume of 10 $\mu\text{l/g}$ (following Semsar et al., 2001; Semsar and Godwin, 2004). Manning compound is a very potent V1a receptor antagonist with almost no antidiuretic activity (Guillon et al., 2004). It is known to block AVT signaling in teleosts (Mahlmann et al., 1994; Semsar et al., 2001; Semsar and Godwin, 2004). The selection of which fish in the pair received Manning and which received saline was completely randomized by a coin toss with the first fish given antagonist if the coin landed heads and saline if tails. Immediately following the injection, the fish were put in separate buckets containing fresh saltwater for a 5-min recovery period. Once the fish had recovered from the anesthesia, as indicated by swimming normally, they were placed in a tank for behavioral observation for 10-min. This tank was identical to housing tanks, and connected to the same system, except it was cleared of rock and structure that would otherwise obscure observation. Both fish were transferred at the same time to prevent prior residence from influencing the dominance outcome. Ten minutes was chosen as the duration of the test because preliminary studies suggested that 10-min was a sufficient time for the contest to become resolved and a clear winner and loser identified when two size-matched fish are introduced.

During the 10-min period of observation, episodes of biting, charging, quivering or fleeing were counted. The 10-min period was recorded using a small digital video camera. Biting was recorded any time a fish bit its opponent in the mouth, body or tail. Charging was recorded when a fish rapidly charged another with an outstretched operculum. Quivering was recorded when a fish would lie sideways and shake its body at a lowered angle from the other fish. Fleeing was recorded when a fish would respond to a charge by another fish by quickly swimming away. Biting and charging were considered aggressive behaviors whereas quivering and fleeing were considered submissive behaviors (Iwata et al., 2008). At the end of the observation period, fish were removed from the display tank and returned to their home aquariums. For each pair, the winner was identified as the fish that displayed more aggressive behaviors and tended to display fewer submissive behaviors. The winner also stayed more out in the open part of the tank after the conflict was resolved, whereas the loser stayed at the periphery or top of the aquarium and/or received a sequence of aggressive behaviors without retaliation (Rowland, 1989; Rhodes and Quinn, 1998; Benson and Basolo, 2006).

Experiment 2

Subjects and husbandry. A total of 10 adult, reproductively mature male *A. ocellaris* were used for this experiment. Mean body mass was 3.5 g (± 0.769 SD) and length from the tip of the snout to the end of the tail was 5.6 cm (± 0.435 SD) at testing. The fish were purchased from ORA Farms (Fort Pierce, FL, USA) as juveniles, and kept in marine aquariums for approximately 2 ½ years to establish breeding pairs. Aquaria consisted of twenty 25 gallon cubic tanks (45.72 cm length width height) containing live rock connected to an approximately 950 gallon main system consisting of two 200 gallon sumps and a 50 gallon filter. Originally, fish were maintained in small groups consisting of 3 or 4 fish per tank, but after several months a pair formed and then the other fish were removed as necessary to avoid fatalities from aggression. The males used for this experiment were proven breeders, and identified as the smaller individual of the pair in the aquaria. Sex was confirmed by histological analysis of the gonads (Fig. 1). Water chemistry was as described in experiment 1.

Experimental design. All 10 fish were individually marked using colored latex beads as described in experiment 1. Five pairings were created based on proximity in body weight (see Table 2). The tests proceeded similar to experiment 1 with one fish randomly receiving Manning compound (3.2 $\mu\text{g/g}$ body weight) and the other vehicle before being placed together in a display tank, except the pairs remained in the display tank for 90-min. This was done for two reasons. First, so that we could evaluate the behaviors (bites, charges, quivers, and flees) during the first 10-min and last 10-min of a 90-min test. We knew that within the first 10-min of the contest, animals would fight for dominance resulting in a victor and a loser, but we wanted to see how the types and intensity of behaviors would change at the end of the 90-min period after the

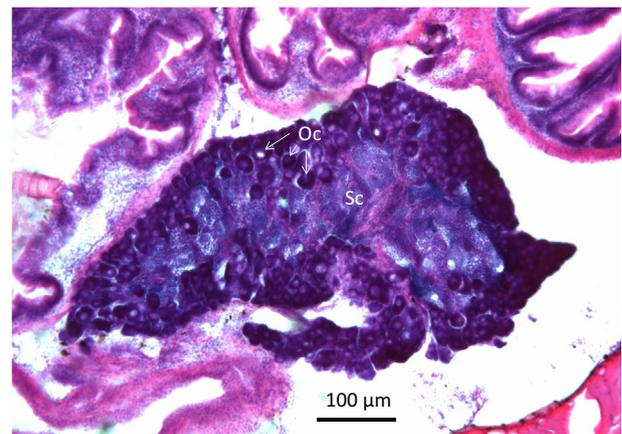


Fig. 1. Male ovotestis. Representative coronal section through the gonad stained with hematoxylin and eosin confirming male identity. "Oc" indicates non-vitellogenic oocytes, "Sc" indicates spermatozoa or spermatozoa.

Table 2. Mature male clownfish contests for Experiment 2

Trial #	Date	Contest Manning vs Saline
1	12/12/12	20B (3.22) vs 3R* (3.9)
2	12/14/12	18R (3.7) vs 14B* (2.6)
3	01/09/13	15B (5.01) vs 17R* (4.4)
4	01/10/13	1B (2.72) vs 16R* (2.76)
5	01/14/13	4B (3.56) vs 13R* (3.54)

Note: In the contest column, the fish receiving manning compound is listed first followed by the fish receiving saline. Body mass measured before the contest is shown in parentheses. The star next to an animal ID indicates the dominant individual in the pair (i.e., the outcome of the contest).

conflict was resolved. The second reason is that the brains were processed for immunohistochemical analysis of c-Fos and c-Fos reaches peak concentration in neurons approximately 90-min following a stimulus (Zangenehpour and Chaudhuri, 2002).

All five tests occurred at the same time of day, starting exactly at 9:00 AM Central Standard Time (2 h after lights turn on in the aquarium rooms). The reason we wanted to keep time of day as a controlled variable is that AVT concentrations in the plasma and pituitary gland can display a circadian rhythm (Rodríguez-Illamola et al., 2011), and we wanted to eliminate circadian variation in behavior and c-Fos expression in order to make it easier to detect main effects from the AVT antagonist treatment. After testing, all fish were anaesthetized with MS-222 (0.25 mg/ml), weighed, measured, decapitated and brain dissected. The brain and remaining body were placed into 4% paraformaldehyde for 24 h and then in 30% sucrose at 4 °C until sectioning.

c-Fos immunohistochemistry. The purpose was to identify brain regions in the social decision circuit (POA, aTn, and TPp) that display differential numbers of c-Fos-positive cells in response to the Manning compound (V1a receptor antagonist) at the initial stages of a dyadic contest. Increased numbers of c-Fos cells indicate increased genomic response and neuroplasticity in a brain region (Clayton, 2000). Brains were sectioned in the coronal plane at 40 μm and thaw-mounted onto subbed slides. Slides were dried at room temperature for 3–5 days before immunohistochemical processing following the procedure of Munchrath and Hofmann (2010) with rabbit anti-c-Fos primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; catalog #sc-253) at 1:500 dilution. c-Fos was used as the plasticity marker because it is a transcription factor that is induced by sensory stimulation in neurons and coordinates the expression of numerous genes involved in remodeling synapses, dendrites, proliferation of neurons and their survival and differentiation (Clayton, 2000). It is widely used in mammalian systems for identifying brain regions that undergo remodeling in response to a specific sensory stimulus (e.g., Rhodes et al., 2003, 2005; Zombeck et al., 2010) and has recently been adopted to study brain circuits in teleosts (Munchrath and Hofmann, 2010; O'Connell et al., 2012, 2013).

Image analysis. Sections were imaged using a Zeiss bright field light microscope interfaced to a computer via a video camera. The entire bi-lateral rostral-caudal extent of the POA, aTn and TPp was photographed at 10× (total 100×) magnification and outlined using ImageJ software to calculate area and count c-Fos-positive nuclei within the area. The regions were identified with reference to published atlases for the cichlid brain (Fernald and Shelton, 1985; Burmeister et al., 2009; O'Connell and Hofmann, 2011). Volume was estimated by multiplying the area of each section by the thickness of the sections. The total number of c-Fos-positive nuclei in the outlined area of the sections was counted by eye by an investigator blind to the experimental conditions. To avoid double counting cells, the diameter of a random subset of nuclei was measured from three sections in all 10 animals, equally represented following Clark et al. (2010). For the POA, we measured 526 nuclei, for the aTn we measured 74 nuclei, and for the TPp we measured 102 nuclei. Based on the observed average diameter of 3.32 μm, 2.76 μm and 2.89 μm, respectively, we estimated that approximately 8%, 7% and 7% of the cells we counted would have fallen in one plane of the section. Hence our estimate of total number of c-Fos cells was reduced by 8%, 7%, 7% for the POA, aTn and TPp, respectively.

Gonadal histology. The purpose was to confirm that the animals used in this study were juvenile or male *A. ocellaris*. After fixation in 4% paraformaldehyde, the bodies were sectioned anterior from the urogenital orifice in the coronal plane at 40 μm. Sections were thaw-mounted onto subbed slides. Slides were dried at room temperature for 3–5 days before dehydrating and staining with hemotoxylin and eosin following standard procedures. Gonads were examined to confirm that they were composed of ovotestes (Fig. 1), characteristic of the juvenile and male sex in *A. ocellaris*.

Statistics

The data were analyzed using SAS (<http://www.sas.com>) and R (<http://www.r-project.org>). The influence of Manning compound on the probability of winning or losing the contest was performed using a binomial test. If Manning has no effect, then the probability that a fish receiving Manning becomes the winner is 50% because the fish were assigned to the treatments randomly. Thus, the proportion of dyadic contests where the Manning fish became winner was compared to a binomial distribution to obtain an exact *p*-value. Each behavior was also analyzed separately using a paired *t*-test or paired Wilcoxon test to determine whether the mean number of times the animal displayed each of the four behaviors (bites, charges, flees and quivers) was different for the saline-treated versus Manning-treated fish. Paired tests were used because the levels of behaviors were strongly dependent on the specific pair, i.e., individuals were not statistically independent, some pairs displayed more reciprocal aggression than others, and it was the difference between Manning and saline within the pairs that was informative. Biting, chasing,

and quivering displayed normally distributed data, or data that could be transformed to normality, and therefore were analyzed using paired *t*-tests. Fleeing data were not normally distributed and therefore were analyzed using a paired Wilcoxon test. Total number of c-Fos-positive cells, volume of the preoptic area, and density of c-Fos cells were analyzed using un-paired *t*-tests. Un-paired tests rather than paired tests were used because the data indicated individuals were statistically independent, i.e., volume of the brain regions and number of c-Fos-positive cells were not related to the specific pair, they were a property of the individual. Pearson's correlations were estimated between neuroanatomical measures (i.e., number of c-Fos cells, volume of the brain regions, and density of c-Fos cells) and the behaviors (biting, charging, fleeing and quivering) collapsed across treatment group. In addition, neuroanatomical measures were evaluated using linear models with behaviors as covariates, treatments (Manning versus saline) and interactions as factors. Similar correlations and linear models were used to evaluate relationships between behaviors and body mass. $p < 0.05$ was considered statistically significant.

RESULTS

Experiment 1

In 20 out of 24 trials, the fish receiving the saline injection was declared the winner of the contest, an outcome that is significantly different from chance at $p = 0.0003$ level (see Table 1). On average, the saline-treated animals displayed significantly more bites (Fig. 2A; $t_{23} = 3.5$; $p = 0.002$) and charges (Fig. 2B; $t_{23} = 2.4$; $p = 0.02$) than the Manning-treated fish. The Manning-treated fish tended to display more quivers (Fig. 2C; $t_{23} = 1.9$; $p = 0.07$). Overall, few flees were observed and no significant differences between groups were detected (Fig. 2D). Body mass was significantly positively correlated with number of bites ($r = 0.30$, $p = 0.04$) and number of quivers ($r = 0.29$, $p = 0.04$) across all animals and all contests (i.e., more bites and quivers were displayed in contests between larger fish as

compared to smaller fish). No correlation between body mass and number of chases or flees was detected.

Experiment 2

Behavior. The mature males were significantly more aggressive than the juveniles in experiment 1. Mature males displayed approximately five times as many bites and twice as many charges as juveniles in the first 10-min of the dyadic contest (see *y*-axis for Fig. 2A, B as compared to Fig. 3A, B). Levels of submissive behaviors were relatively low and comparable in both experiments (see *y*-axis for Fig. 2C, D as compared to Fig. 3C, D).

In five out of the five trials, the fish receiving the saline injection won the contest (1-tailed p -value = 0.03; see Table 2). Combining experiments 1 and 2, a total of 29 trials, the saline group won the contest significantly more than the Manning group (25/29; 1-tailed p -value < 0.0001). Combining experiments 1 and 2, and considering only pairs where the size difference was less than 0.1 g, a total of 11 trials, the saline group won the contest significantly more than the Manning group (9/11; 1-tailed p -value = 0.03).

In experiment 2, during the first 10-min, on average, the saline-treated animals displayed significantly more bites (Fig. 3A; $t_4 = 7.5$; $p = 0.002$) and tended to show more charges (Fig. 3B; $t_4 = 2.4$; $p = 0.08$). No differences in quivers were detected between groups (Fig. 3C). Few flees were observed and no differences between groups were detected (Fig. 3D).

During the last 10-min of the 90-min test, fewer bites were observed as compared to the first 10-min, and no significant differences between groups were detected (Fig. 3E). Saline-treated fish displayed a significantly greater number of charges (square root transformed to improve homogeneity of variance between groups; Fig. 3F; $t_4 = 3.0$; $p = 0.04$) than Manning-treated fish. No differences in quivers were detected (Fig. 3G). Manning-treated fish tended to flee more than saline-treated fish (paired Wilcoxon test, $p = 0.10$; Fig. 3H).

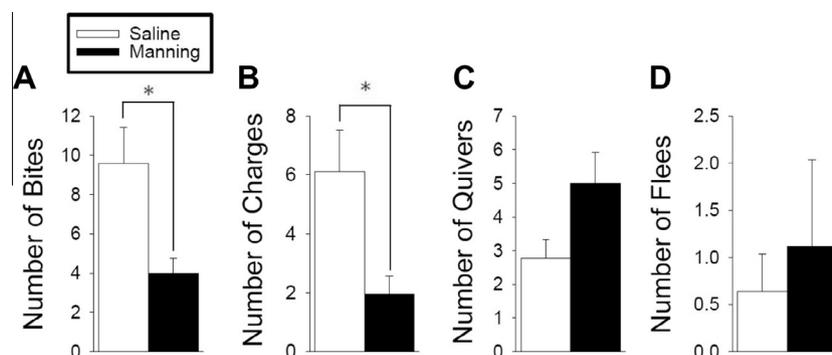


Fig. 2. Aggressive and submissive behaviors during dyadic interactions of saline- versus Manning-treated juvenile *A. ocellaris*. Bar graphs displaying the average number of bites (A), charges (B), quivers (C), and flees (D) in saline- and Manning compound-treated groups during a 10-min interaction period are shown with standard error bars. Information on the masses of the individual fish and outcome of the contests is shown in Table 1. $N = 24$ fish per bar. * indicates significant paired *t* statistic at $p < 0.05$ level.

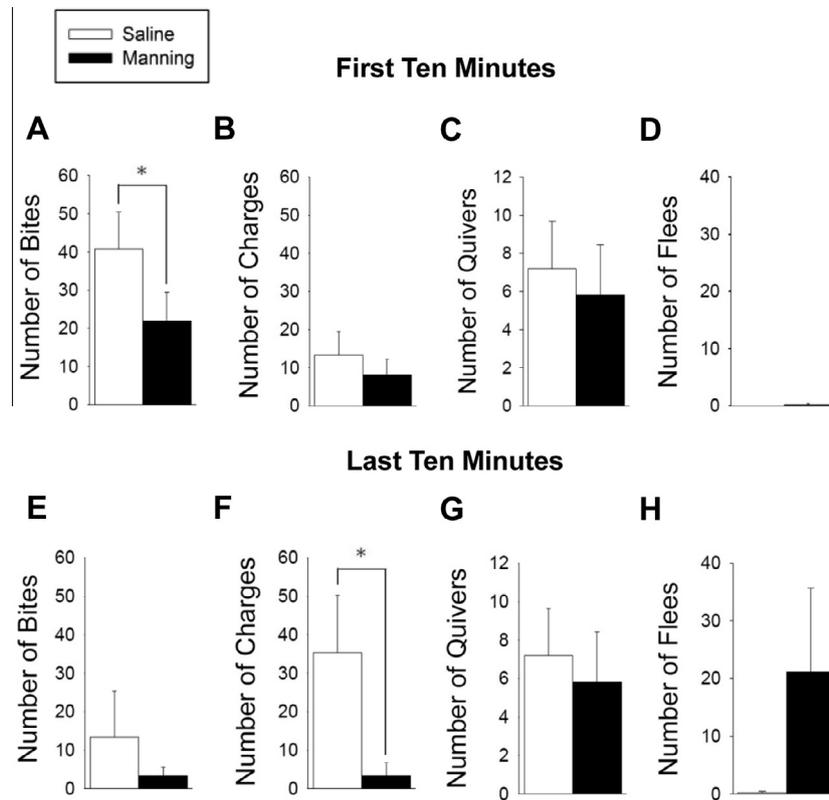


Fig. 3. Aggressive and submissive behaviors during dyadic interactions of saline- versus Manning-treated mature male *A. ocellaris*. Behavioral observations in the first 10-min and last 10-min of a 90-min interaction period are shown. Bar graphs displaying the average number of bites (A, E), charges (B, F), quivers (C, G), and flees (D, H) in the first and last 10-min, respectively, are shown with standard error bars. $N = 5$ fish per bar. * indicates significant paired t statistic at $p < 0.05$ level.

Body mass was significantly positively correlated with number of charges ($r = 0.75$, $p = 0.01$) in the first 10-min. Treatment differences for charges in first 10-min remained non-significant after including body mass as a covariate. No other behaviors (measured in the first or second 10-min) were correlated with body mass.

c-Fos in the POA, aTn and TPp. In the POA, saline-treated fish displayed approximately twice as many *c-Fos*-positive cells (Fig. 4D; $t_8 = 3.8$, $p = 0.005$) and similar volume of the POA (Fig. 4E), which resulted in approximately twice the density of *c-Fos* cells (Fig. 4F; $t_8 = 5.3$, $p = 0.0007$). In the aTn, no differences in total number of *c-Fos* cells (Fig. 5D), volume (Fig. 5E) or density (Fig. 5F) were detected. In the TPp (VTA), saline-treated fish displayed approximately 25% more *c-Fos*-positive cells (Fig. 6D; $t_8 = 2.7$, $p = 0.03$). No difference in volume (Fig. 6E) were detected and a trend for increased density of *c-Fos* cells was observed (Fig. 6F; $t_8 = 1.6$, $p = 0.16$).

A significant positive correlation was detected between number of bites in the first and last 10-min and number of *c-Fos*-positive cells in the TPp (VTA) (first 10-min, $r = 0.7$, $p = 0.03$; last 10-min, $r = 0.76$, $p = 0.01$). Including bites as a covariate in a linear model predicting *c-Fos* cells in the TPp indicated significant effects of the covariate (first 10-min, $F_{1,5} = 9.9$, $p = 0.025$; last 10-min, $F_{1,5} = 9.9$,

$p = 0.025$), significant effects of treatment for last 10-min ($F_{1,5} = 9.6$, $p = 0.027$), and marginally non-significant effects of treatment for first 10-min ($F_{1,5} = 4.4$, $p = 0.09$). No significant interactions between bites (at either time-point) and treatment were detected. No other significant correlations were detected between the neuroanatomical measures and behaviors.

DISCUSSION

The main discovery from this study is that AVT signaling at V1a receptors is necessary for *A. ocellaris* clownfish to display greater aggression than their rival and facilitates winning a dyadic contest. This is important because in *A. ocellaris*, the alpha individual in a pair of two males (or juveniles) living together will end up differentiating as a female and the beta fish will remain male (Fricke and Fricke, 1977). Hence, the result suggests AVT is critically involved in the very initial stages of behavioral interactions leading to sex change in *A. ocellaris*, because aggression and dominance precede sex change in this species.

Not only was AVT signaling necessary for heightened aggression, it also was necessary for elevated expression of *c-Fos*, a marker of neuroplasticity, in regions of the social decision circuit such as the POA known to regulate gonadal structure and function (Francis et al., 1993; O'Connell and Hofmann, 2011). The timing for the

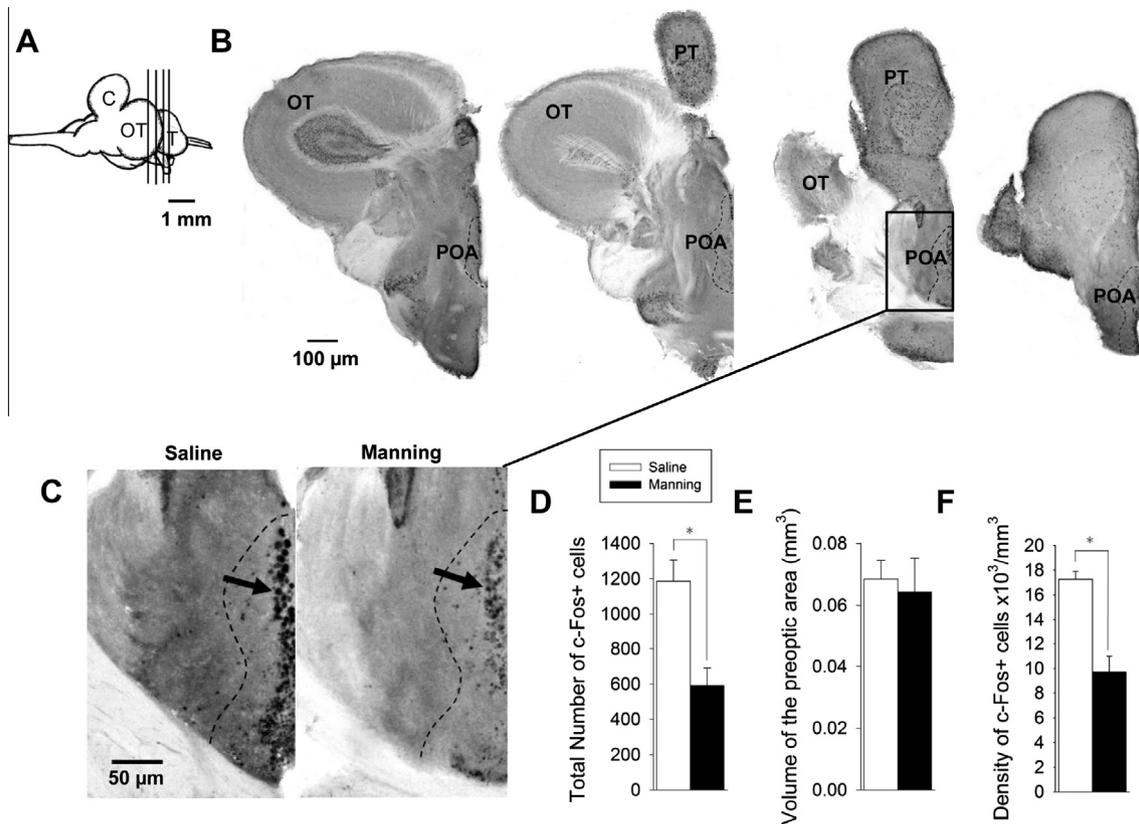


Fig. 4. Immunohistochemical detection of c-Fos in the preoptic area of saline- and Manning-treated mature male *A. ocellaris* after a 90-min dyadic interaction. (A) Sketch of the *A. ocellaris* brain, laterally oriented with the rostral portion of the brain on the right-hand side and the spinal cord extending to the left. The cerebellum 'C', optic tectum 'OT', and telencephalon 'T' are indicated, as well as the planes of coronal sections defining the rostro-caudal boundaries of the preoptic area 'POA' and the location of the representative sections shown in panel B. (B) Photographs of unilateral coronal sections from a Manning-treated *A. ocellaris* brain stained for c-Fos representing the general structure of the POA and orientation relative to other major structures in the brain (OT, pallium of the telencephalon 'PT'). Note that the entire POA, i.e., bilateral, all sections within the rostro-caudal boundaries, and not only the three representative sections shown here were analyzed. (C) A magnified image of the POA to show the c-Fos-positive cells (representative cells indicated with an arrow) that were counted in this region from representative sections of saline- and Manning-treated fish. (D) Bar graph comparing total number of c-Fos-positive cells in the POA, (E) total volume of the POA in cubic mm and (F) density of c-Fos-positive cells in the POA in saline- and Manning-treated fish. $N = 5$ fish per bar. * indicates significant unpaired t -statistic at $p < 0.05$ level.

expression of neuroplasticity is important, as results suggest that within 90-min of the social interaction between the two fish, plasticity is occurring in brain regions such as the POA that have the capability to change the gonads. Consistent with the rapid timescale of the behavioral and neuronal response, Godwin (1994) found striking increases in aggressive behavior in male sex changing *A. melanopus* within 1 day of removing the alpha female. In the *A. burtoni* cichlid model, increases in *egr-1* gene expression, another immediate early gene marker of neuronal plasticity, and *c-fos* gene expression were observed in the POA of a subordinate male 30-min after the subordinate male realized that the alpha male was removed, and hence was beginning to ascend the hierarchy (Burmeister et al., 2005; Maruska et al., 2013). Gonadal sex change in clownfish takes at least several weeks (Fricke and Fricke, 1977), although in the laboratory it may take significantly longer (Iwata et al., 2008). Hence, whether the change in c-Fos in the POA observed in our study reflects initial plasticity in neurons with a role in gonad change or whether the c-Fos expression is involved in some other function related to dominance or behavior far removed from

gonadal development is not known and will require future investigation.

Although the V1a antagonist significantly reduced aggressive behavior, there was only a trend for it to increase submissive behavior that did not reach statistical significance (see Fig. 2C, D and Fig. 3H). In many instances, the Manning-treated fish did not respond to an aggressive display from the saline fish by fleeing or quivering, hence the large variation and lack of statistical significance. Although lack of submission in response to an aggressive act could be construed as expression of aggression or dominance, we do not believe this to be the case in our experiment. In our experience with *A. ocellaris*, when two fish are paired together without any pharmacological treatment, the behavioral displays are similar to what was observed in this experiment. That is, often charges are not met with retreats or quivers. After some amount of fighting, the pairs resolve the conflict, and one fish eventually moves to the periphery of the tank, presumably trying to avoid interactions with its opponent, whereas the opponent remains in the middle of the territory. These behavioral interactions are similar to what we have observed in

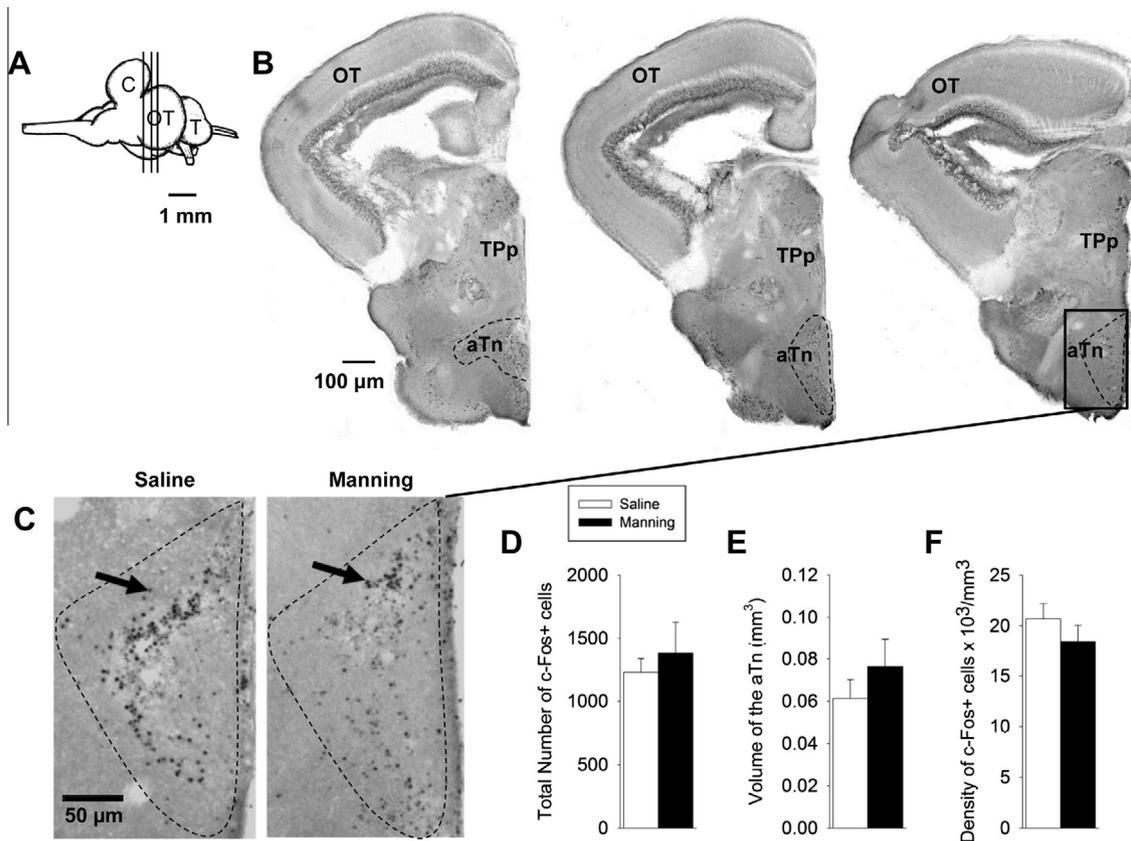


Fig. 5. Immunohistochemical detection of c-Fos in the anterior tuberal nucleus (aTn; homolog of the ventromedial hypothalamus, VMH) of saline- or Manning-treated mature male *A. ocellaris* after a 90-min dyadic interaction. Same as Fig. 4 except for focus on the aTn. No significant differences in c-Fos-positive cells were found between Manning-treated versus saline controls.

salmonid juveniles vying for territorial dominance (Rhodes and Quinn, 1998). In our experience with *A. ocellaris*, repeated aggressive displays by one fish without retaliation by the other are consistent with dominance.

Several features are known about the AVT system in fishes and other vertebrates that could influence the interpretation of our work. First, there are two types of V1 receptors: V1a and V1b in teleost fishes (Lema, 2010; Daza et al., 2012). Manning compound is reported to be a selective antagonist at V1a receptors with low affinity for V1b or V2 (Barberis et al., 1999; Guillon et al., 2004). Hence, the effect of Manning compound on aggression and c-Fos expression in this study is most likely mediated through V1a and not V1b or V2 receptors. Second, isotocin is another endogenous peptide that has a similar molecular structure as AVT, but it was found not to have agonist activity at V1a receptors expressed in *Xenopus* oocytes (Mahlmann et al., 1994). Hence, it is most likely that the behavioral and c-Fos effects observed in this study were due to blockade of AVT not isotocin signaling at V1a receptors. Third, AVT neurons are known to project throughout the brain and to the pituitary gland in teleost fishes (Saito et al., 2004) and AVT V1a receptors were found distributed throughout the brain of a sex changing grouper (Kline et al., 2011). Density of AVT fibers is relatively low in the TPp of rainbow trout (Saito et al.,

2004), though V1a receptors are clearly present in grouper (Kline et al., 2011). These data suggest that the effect of the antagonist treatment on c-Fos in the TPp could be direct or indirect, i.e., the antagonist might affect activation of neurons in the TPp or in a different brain area that subsequently projects to the TPp. Finally, in mammals, AVP is released from dendrites in addition to axonal terminals, and often AVP neurons contain other classical neurotransmitters such as GABA or glutamate in addition to AVP (Ludwig and Leng, 2006). More research is needed to determine the relative contribution of axonal versus somatodendritic release of AVT in different regions of the clownfish brain, whether other neurotransmitters are colocalized in these AVT neurons and their relative influence on aggression, dominance and c-Fos expression.

One line of research that would help clarify the functional significance of the c-Fos data would be to identify the main neurotransmitters or peptide hormones being released from the c-Fos-positive neurons. In the POA, logical candidates include AVT and GnRH, as neurons containing these neuropeptides are located in the POA (Foran and Bass, 1999). AVT is a logical candidate because the Manning compound blocked AVT signaling and the blockade could have affected presynaptic (i.e., the AVT neuron) as well as postsynaptic cells (i.e., target cells in the pituitary or

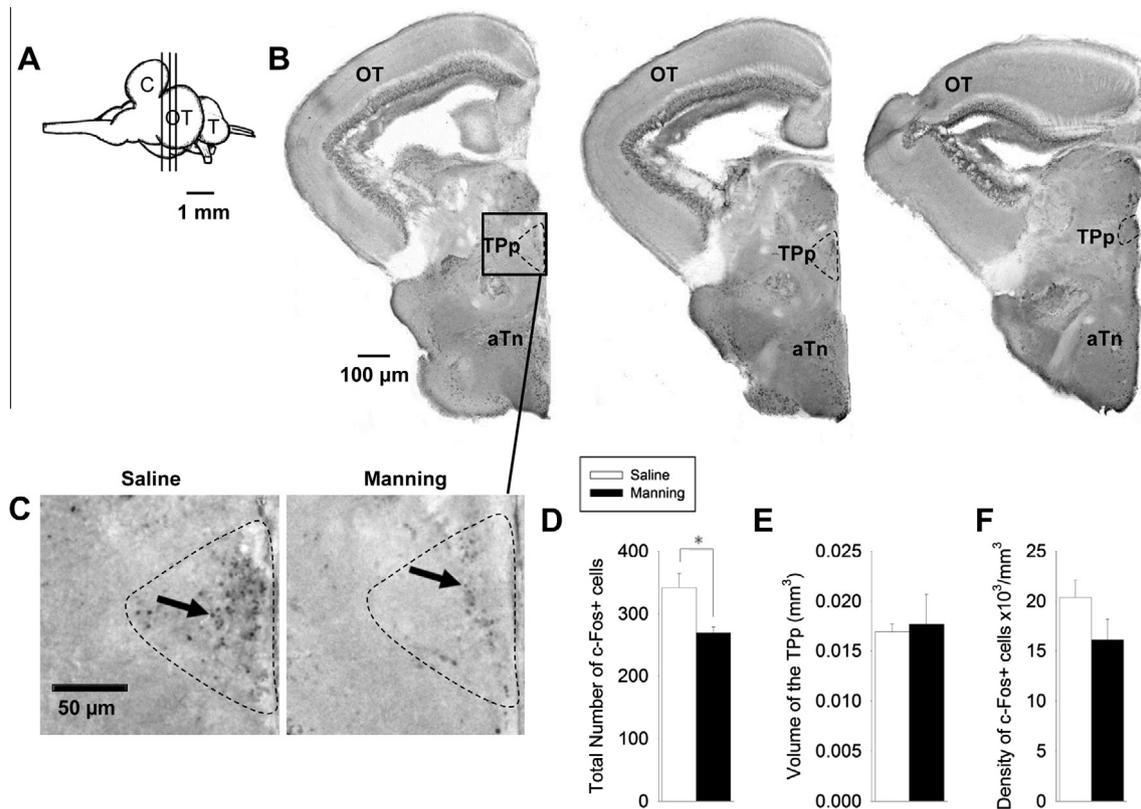


Fig. 6. Immunohistochemical detection of c-Fos in the periventricular nucleus of the posterior tuberculum (TPp; homolog of the ventral tegmental area, VTA) of saline- or manning-treated mature male *A. ocellaris* after a 90-min dyadic interaction. Same as Fig. 4 except for focus on the TPp. The total number of c-Fos-positive cells was significantly greater in saline than the Manning group ($*p < 0.05$).

neurons in the brain). For example, blockade of V1a receptors could block feedback to the AVT-releasing neuron and thereby reduce the neuron's responsiveness (Ludwig and Leng, 1997, 1998; Gillard et al., 2007).

GnRH is another logical candidate. Previous studies in *Amphiprion melanopus* have established that females display fewer GnRH neuron cell bodies in the POA as compared to males (Elofsson et al., 1997). One intriguing possibility is that the increased c-Fos observed in the POA of Saline-treated relative to Manning-treated fish within 90-min of the dyadic contest was coordinating changes in gene expression in GnRH cells to begin the process of apoptosis (Preston et al., 1996) or down-regulation of GnRH expression in cells (Ofir et al., 1990). The functional significance of fewer GnRH cells is still not known, and would have to be confirmed in *A. ocellaris*. One idea is that changes in GnRH cell numbers, size or function affect the quantity or pattern of GnRH signaling which affects the types or frequency of peptides released from the pituitary, and finally steroidogenesis and the coordination of gonadal sex change (Godwin, 2010).

In the TPp the obvious candidate cell type for displaying increased c-Fos in response to elevated social status is dopamine, but other candidates include GABA interneurons and glutamate (Yamaguchi et al., 2007). The current state of knowledge posits that dopamine neuron projections from the VTA (putative mammalian homolog of the TPp) are modulated by

GABA interneurons and play a critical role in learning and memory circuits and setting habitual patterns of goal directed behaviors (Young et al., 2005; Salamone and Correa, 2012). Dopamine modulation during the initial stages of re-modeling of the brain in response to a change in the social hierarchy could help coordinate the changes in brain circuits necessary to support elevated rank and female-typical behaviors.

In the majority of studies evaluating the effect of AVT signaling on aggression and dominance, the males were the dominant sex, hence in these analyses dominance status is confounded with male sexual phenotype (Grober and Sunobe, 1996; Foran and Bass, 1999; Reavis and Grober, 1999; Godwin et al., 2000; Semsar et al., 2001; Grober et al., 2002; Semsar and Godwin, 2003; Greenwood et al., 2008). Results here support the idea that AVT signaling is necessary for aggression and facilitates winning a contest regardless of which sex is behaviorally dominant. Hence, despite the unusual life history of the clownfish, the role of AVT appears conserved, with AVT signaling necessary for elevated rank (Semsar et al., 2001; Semsar and Godwin, 2004).

We observed that blockade of V1a receptors with the Manning compound reduced aggressive behavior in both the juveniles in experiment 1 and the adult mature males in experiment 2. Taken together, our results suggest that similar AVT-related mechanisms are involved in the regulation of social behavior in juveniles that have not spawned yet and mature males. It is interesting that

during the last 10-min of the 90-min contest in experiment 2, relatively more charges and fewer bites were observed from the winner of the contest as compared to the first 10-min (compare Fig. 3A–D with Fig. 3E–H). We interpret this shift in display of aggression as evidence that the dominance relationship between the pair has matured from the initial contest where the pair was vying for dominance to a point closer to an established hierarchy, where the alpha individual merely enforces their position primarily via charges rather than bites.

Relatively few studies have directly manipulated AVT signaling using receptor agonists and antagonists in sex changing fishes to evaluate effects on aggression and dominance (Semsar et al., 2001; Semsar and Godwin, 2004). However, several studies have measured AVT mRNA expression and number and size of AVT-immunoreactive neurons in the POA (Grober and Sunobe, 1996; Reavis and Grober, 1999; Godwin et al., 2000; Grober et al., 2002; Semsar and Godwin, 2003; Iwata et al., 2010). In most of these species, the aggressive phenotype was reported to have more AVT mRNA expression, larger AVT cells and/or more numerous AVT cells. One study in *A. ocellaris* found increased numbers and larger cell sizes of AVT-immunoreactive neurons in the POA of alpha individuals over beta, and gamma, 90 days after placing three immature fish together (Iwata et al., 2010). In this study and most of the others, dominance status was confounded by body mass, since larger fish were dominant. Correcting for body mass by entering it as a covariate in linear regression or as a ratio relative to body mass may not be appropriate under these conditions. Moreover, the relationship between number of cells (or size) and release of AVT or signaling at receptors is not clear. Putting these issues aside, our data that suggest increased AVT signaling at V1a receptors is necessary for dominance are consistent with the broader literature showing increased AVT mRNA, number and/or size of AVT neurons in dominant individuals.

Our study strongly implicates AVT signaling in the initial establishment of dominance in clownfish, but that does not mean that other neurotransmitters and hormones are not involved. Many other molecules likely contribute, including serotonin (Larson et al., 2003; Perreault et al., 2003; Lorenzi et al., 2009), dopamine, glutamate, GABA, acetylcholine, aromatase (Lee et al., 2001, 2002; Black et al., 2005; Marsh et al., 2006; Zhang et al., 2007; Kobayashi et al., 2010; Iwata et al., 2012), isotocin (Pickford and Strecker, 1977; Black et al., 2004; O'Connell et al., 2012), steroid hormones (Godwin and Thomas, 1993; Godwin, 2009), among others. Moreover, it is exceedingly likely that combinations of these chemicals converge on neurons to induce plasticity necessary to support behavioral changes associated with shifts in social status (Foran and Bass, 1999; Perry and Grober, 2003; Godwin, 2010).

With the current data, it is not possible to decipher whether the c-Fos differences are due to the Manning compound *per se* or the resulting effect on behavior and

dominance status because these variables were confounded. In all five pairs in experiment 2, the saline-treated fish were more aggressive and ended up the victor. We observed a positive correlation between number of bites and number of c-Fos-positive cells in the TPp, which is consistent with the idea that the c-Fos results in the TPp reflect behavior rather than direct pharmacological effect of Manning compound. While it might be possible to tease apart Manning effects from dominance on c-Fos levels in the POA with additional experiments and treatment groups, this is beyond the scope of the present study. Regardless, the outcome of additional studies will not change the main conclusion that V1a receptor signaling is necessary for elevated c-Fos expression in the POA and TPp during dyadic contests.

The entire sequence of biological events that are necessary to produce socially influenced sex change in marine fishes remains a mystery. Progress over the years has established that the process begins with changes in the social hierarchy that are perceived in the brain. *A. ocellaris* represent a useful model organism for discovering the neuroendocrinology of post-maturational sex change because the species is easily maintained and bred in the laboratory. Results from this study demonstrate that AVT signaling at V1a receptors is necessary for *A. ocellaris* male clownfish to display heightened aggression associated with winning dyadic contests and necessary for elevated expression of markers of neuroplasticity in key brain regions of the social decision circuit. Many questions are left unanswered including which neurons are undergoing plasticity within a 90-min social interaction, and how they might begin the process of altering signaling to the pituitary and gonad. Future experiments using the *A. ocellaris* model hold promise for advancing our understanding of the neuroendocrine mechanisms of post-maturational sex change in fishes.

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