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Testosterone-related behavioral and neural mechanisms associated with location preferences: A model for territorial establishment

Xin Zhao*, Frank R. Castelli[1], Ruyi Wang, Anthony P. Auger, Catherine A. Marler

Department of Psychology, University of Wisconsin, Madison, WI 53706, USA

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ABSTRACT

Territoriality is an adaptive behavioral trait that is important for animal's fitness and there still remains much to learn about the proximate mechanisms underlying the development of territoriality. We speculate that the formation of a conditioned place preference (CPP), an increased time allocation to the environment where a rewarding experience occurred, contributes to territoriality. Testosterone (T) plays an important role in modulating territorial behaviors and T pulses can induce a CPP. We confirmed previous findings in California mice (*Peromyscus californicus*) that T pulses can induce a CPP in singly-housed, but not group-housed males. Housing singly may be similar enough to dispersal in nature to initiate similar hormonal and neuroanatomical changes needed for the development of territoriality. We further revealed that T pulses interact with the single housing experience and appear to enhance the motivation to be aggressive towards a stimulus male. On a neural level, being singly housed upregulated levels of androgen receptors in the preoptic area, which positively correlated with the strength of the CPP. We speculate that this change in androgen sensitivity in the preoptic area is characteristic of males that have dispersed, making them more sensitive to T pulses. Also, single housing increased markers of synaptic plasticity in the nucleus accumbens, ventral and dorsal hippocampus, neural changes that may be associated with dispersal, reproduction and territory establishment. These behavioral and neural changes may reflect the life history transition from residing in the natal territory to dispersing and establishing a new territory.

1. Introduction

Territoriality is one of the most widespread behaviors among animal species and is generally defined as the defense or maintenance of an area to the exclusion of others (Maher and Lott, 1995). For many monogamous or solitary territorial species, the establishment of a territory can be achieved by leaving the natal area and settling in a new site (Getz et al., 1994; Ribble, 1992; Selonen and Hanski, 2010; Waser and Jones, 1983), the process of which is usually accompanied or even driven by a change in the social environment such as leaving the natal group (Eikenaar et al., 2007; Howard, 1960; Lambin et al., 2001; Lamhin, 1994). While several theoretical models have been proposed to explain how social interactions contribute to the emergence of territoriality (Giuggioli et al., 2011; Potts and Lewis, 2014), it remains largely unclear how the changed social environment influences the neuroendocrine system, which could be a proximate mechanism that contributes to the development of territoriality.

To explain how learning from social experience contributes to the process of territory establishment, Stamps and Krishnan (1999) proposed the hypothesis that a territory is gradually formed by animals returning to areas in which they previously had rewarding experiences (see also Tirpak, 2007; White and Harris, 1994). This hypothesis is consistent with our speculation that the formation of a conditioned place preference (CPP) is likely to be an important mechanism contributing to the establishment of a territory (Marler and Trainor, 2020; Zhao and Marler, 2016). CPP refers to the development of a preference for the location in which an animal is exposed to a stimulus that activates the internal reward systems (Tzschentke, 1998). Stimuli related to territoriality that might induce CPPs include both social interactions and testosterone (T). For instance, winning a male-male antagonistic contest in green anole lizards (*Anolis carolinensis*) (Farrell and Wilczynski, 2006) and OF1 mice (Martínez et al., 1995) and engaging in male-female sexual encounters in Syrian Hamsters (*Mesocricetus auratus*) (Bell et al., 2010; Meisel and Joppa, 1994) can induce CPPs. Moreover, T injections can induce CPPs in laboratory rats and mice (Alexander et al., 1994; Arnedo et al., 2002; Packard et al., 1997), as

* Corresponding author.
E-mail address: xzhao49@wisc.edu (X. Zhao).
[1] Present Address: Department of Neurobiology and Behavior, Cornell University, Ithaca, NY 14853, USA.

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well as in the California mouse (*Peromyscus californicus*), a territorial and strictly monogamous species (Zhao and Marler, 2014, 2016). Evidence from African striped mice (*Rhabdomys pumilio*), yellow baboons (*Papio cynocephalus*), and European badgers (*Meles meles*) has also demonstrated that males that dispersed from the natal area or became solitary roamers experienced increased levels of T (Alberts et al., 1992; Raynaud and Schradin, 2014; Schradin and Yuen, 2011; Woodroffe et al., 1995), suggesting the potential role of T in initiating the process of dispersal and establishing a new territory and that dispersal is a key time point at which sensitivity to T might be especially important. Additionally, experimentally elevating T levels leads to occupation of larger home areas in male African striped mice (*Rhabdomys pumilio*) and male dark-eyed juncos (*Junco hyemalis*) (Chandler et al., 1994; Raynaud et al., 2012).

Working with California mice, we previously induced CPPs by administering T injections (Zhao and Marler, 2014) designed to mimic natural T pulses found in intact male California mice after winning a male-male aggressive encounter (Marler et al., 2005; Oyegbile and Marler, 2005) or a male-female sexual encounter (Zhao and Marler, unpublished data). Furthermore, we revealed plasticity in the development of T-induced place preferences: CPPs to a novel environment were only produced when males were singly housed, but not when males were housed with peers since weaning (Zhao and Marler, 2016). We suggested that this enhanced CPP development in males after being singly housed parallels the increased motivation to establish a territory after dispersing from the natal area, which is consistent with arguments made by Chabout et al. (2012). We specifically hypothesized that single housing may increase territoriality by facilitating T-induction of CPPs in the appropriate environment. What remains relatively unexplored is (a) whether a T-induced CPP contributes not only to a preference for a location, but also to increases in aggressive motivation at the preferred location and (b) what the neuroendocrine mechanisms are that could underlie the T-pulse induction of territorial behavior. The current study aims to explore these areas.

Here, we used a previously established CPP paradigm in which a T-induced CPP was produced in singly-housed male California mice that were compared to group-housed male mice that did not form a T-induced CPP (Zhao and Marler, 2016). To test the effect of the CPP on aggressive motivation in the current study, we measured the latency to approach and the amount of time interacting with a novel male stimulus across a mesh barrier. We predicted that in the area where the T-induced CPP was formed, the focal male would approach the novel stimulus male faster and spend more time interacting with the stimulus male across a wire mesh, two measures that reflect aggressive motivation to a novel stimulus male in male mice (Kudryavtseva, 2003; Smagin et al., 2015).

We also made several measurements to explore neural changes that might occur in a male during the transition from living in the natal group to being temporarily solitary and establishing a new territory prior to pair-bonding with a female. Androgen receptor (AR) levels were measured because we speculate that upregulation of AR levels may be one of the mechanisms through which housing singly enhances T's ability to induce the formation of a CPP. Furthermore, the regulation of AR densities has been implicated in mediating experience-dependent plasticity in aggression of California mice (Trainor et al., 2004). We also measured synaptic plasticity at a broader level by measuring levels of synapsin and its activated form, phosphorylated synapsin (phosphosynapsin), which are useful protein markers of synaptogenesis and the activity of neurotransmitter release (Cesca et al., 2010).

Levels of AR and synapsin proteins were measured in steroid-sensitive brain regions that are part of the social neural network (O'Connell and Hofmann, 2011) and are implicated in behaviors associated with territorial establishment. Specifically, we focused our neuroanatomical investigations on the preoptic area (POA), the nucleus accumbens (NAc), the amygdala (AMY), the bed nucleus of the stria terminalis (BNST), and the dorsal and ventral hippocampus (dHIP and vHIP). The POA and NAc may have potential roles in mediating T's reinforcing effects on territoriality as suggested by previous studies in rats in which microinjections of T into the POA (King et al., 1999) and the NAc (Frye et al., 2002b; Packard et al., 1997) were sufficient to induce a CPP. The AMY has a high density of neurons containing ARs in rats (Bingaman et al., 1994; Wood and Newman, 1995b) and its activities in response to social threat are subject to the modulation of T in humans (Hermans et al., 2008; Radke et al., 2015). Winning male-male encounters and experiencing natural T pulses increases AR levels in the BNST and brain regions associated with reward (Fuxjager et al., 2010a). Finally, the hippocampus has been implicated in facilitating CPP formation in rats (Ferbinteanu and McDonald, 2001) and administration of T into the hippocampus influences spatial learning and memory abilities in rats and great tits (*Parus major*) (Babanejad et al., 2012; Hodgson et al., 2008).

2. Methods

2.1. Animals

We used 48 sexually mature and sexually naïve male California mice (*Peromyscus californicus*) aged 4–10 months as subjects and 48 sexually mature and sexually naïve males as stimuli in social interaction tests. They were reared in a laboratory colony at the University of Wisconsin-Madison (Madison, WI, USA). Approximately four weeks after birth, males were weaned by removing them from their parents' home cage (48.3 × 26.7 × 15.6 cm, L × W × H) and housing them in a cage (29.2 × 19 × 12.7 cm, L × W × H) with two other recently-weaned males that sometimes included their brothers. After one month, all occupants of a cage were transferred to a larger cage (48.3 × 26.7 × 15.6 cm, L × W × H). The animals were kept under a 10 L:14D light cycle with lights off at 01:30 PM and received free access to Purina 5001 mouse chow and water. All procedures were approved by the University of Wisconsin-Madison Animal Care and Use Committee (L0021-0-03-10) and adhered to the standards of the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. Subjects were randomly assigned to either the singly-housed (n = 24) or group-housed (n = 24) conditions. Males in the singly-housed condition were separated from their same-sex cage mates and housed alone (48.3 × 26.7 × 15.6 cm, L × W × H) for three days before the start of CPP conditioning. Males in the group-housed condition continued to live with the males they were housed with since weaning throughout the experiment, never having experienced living alone.

2.2. CPP apparatus and conditioning procedure

The CPP apparatuses were six large polycarbonate testing cages (90 × 46 × 43 cm, L × W × H) divided into three equal chambers (30 × 46 × 43 cm, L × W × H; Fig. 1a). The two side chambers were connected to the middle chamber by a hole (diameter = 5 cm) in the dividing walls that could be blocked with manually controlled, sliding guillotine doors. The outer walls of each side chamber were decorated with horizontal or vertical black-and-white stripes and the decorations were counterbalanced between the two side chambers (left vs. right) across the six CPP apparatuses. Within each side chamber, a mesh wall (30 × 43 cm, L × H) extending from the floor to the ceiling formed a small enclosure (30 × 13 × 43 cm, L × W × H) behind which a stimulus male could be placed, thereby limiting physical interaction with the subject but presumably not inhibiting auditory or olfactory communication.

The conditioning procedure was conducted over eight days (see Zhao and Marler, 2014; Fig. 1b). On day 1, we determined each subject's initial chamber preference during the pre-conditioning test. For this test, each subject was removed from his home cage, placed in the middle chamber of the CPP apparatus and allowed to habituate for

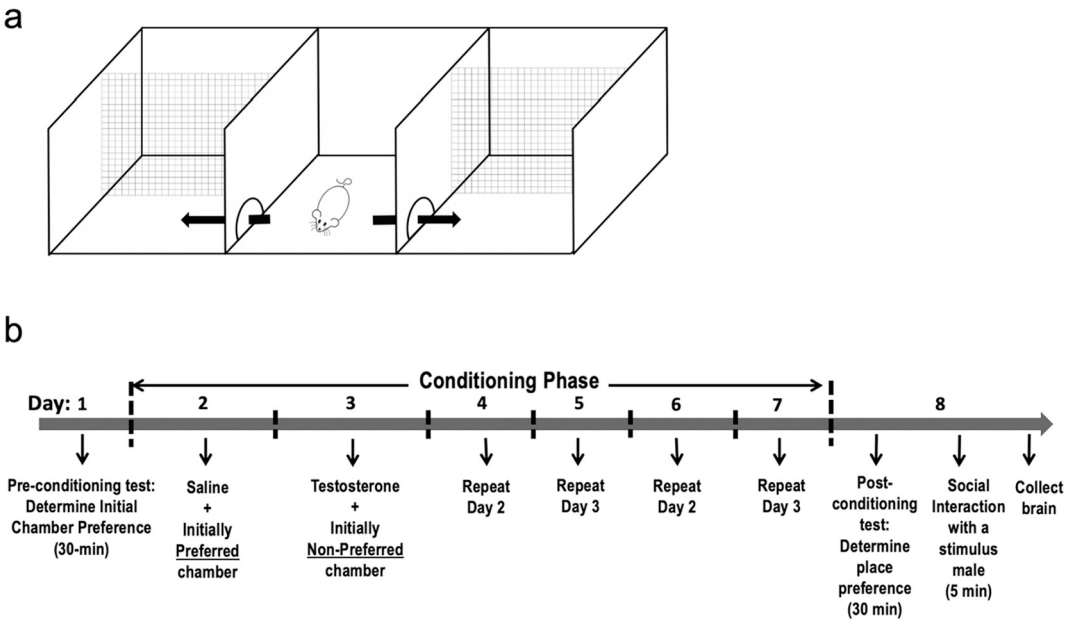

Fig. 1. (a) Diagram of the CPP apparatus. (b) Timeline of experimental procedures for subjects in the testosterone treatment group. Subjects in the control group followed the same timeline but received saline instead of testosterone on days 3, 5, and 7.

5 min. Then, the doors were opened to allow the male to move throughout all three chambers for 30 min. The side chamber in which the male spent the most time was defined as the initially preferred side chamber. To reduce the likelihood of a chamber being preferred because of a side preference, we chose the non-preferred chamber as the location for conditioning.

After the pre-conditioning test, subjects were returned to their home cage. On days 2–7, males received a series of 45-min conditioning sessions, one session per day for six consecutive days. In order to avoid aversion to the CS+ chamber, on days 2, 4, and 6, each male was removed from his home cage, administered a saline injection, placed into the chamber that he initially preferred during the pre-conditioning test (CS− chamber) and allowed to explore the chamber alone for 45 min. On days 3, 5, and 7, males were removed from their home cages, placed in the initially non-preferred compartments (CS+ chamber), and administered either T injections (T group) or vehicle control injections (saline group).

We administered T injections (T-cyclodextrin dissolved in saline) with a dose of 36 μg/kg for several reasons. First, this dose previously produced an increase in T levels approximately 3–5 times higher than the baseline, reaching a maximum of 4–5 ng/ml and lasting for approximately 10 min (Trainor et al., 2004). Second, this dose produces CPPs in male California mice (Zhao and Marler, 2014, 2016). Third, it mimics natural changes in T found in male California mice after winning a male-male aggressive encounter (Marler et al., 2005; Oyegbile and Marler, 2005), such as would be needed for establishing and defending a territory, and after male-female sexual encounters (Zhao and Marler, unpublished data). Moreover, this dose influences behavioral responses such as acoustic communication (Pultorak et al., 2015; Timonin et al., 2018) and future winning ability in California mice (Fuxjager et al., 2011a; Fuxjager et al., 2011b; Gleason et al., 2009; Trainor et al., 2004; Zhao et al., 2019). Half of the subjects in each drug treatment were randomly selected from either the group-housed or singly-housed condition. Twenty-four hours after the last conditioning session, on day 8, subjects were tested for their place preference in a 30-min post-conditioning test using the same procedure as in the pre-conditioning test.

2.3. Social interaction test

Immediately after the post-conditioning test concluded, the entrances to the two side chambers were closed and the activity of the focal male was restricted to the middle chamber. A novel age-matched conspecific stimulus male was then placed in the small enclosure of the CS+ chamber behind a wire mesh. All stimulus males were group-housed. A 5-min interaction test was started by lifting the door to the side chamber. We recorded the latency to approach the stimulus male (starting from lifting the door to CS+ chamber to the first time the focal male sniff the stimulus male) and the duration of interacting with the stimulus male (nose-to-nose and nose-to-anogenital sniffing). Although we did not directly measure aggressive behavior here, we measured behaviors that we view as a proxy for aggressive motivation. Adult male California mice readily fight with other males (Fuxjager and Marler, 2010; Fuxjager et al., 2010b; Fuxjager et al., 2011a; Oyegbile and Marler, 2005; Trainor et al., 2004). Similarly, so do adult male laboratory mice, and the time that male laboratory mice spend near a conspecific male across a perforated transparent partition has been shown to correlate positively with aggressive behavior in subsequent physical confrontations (Kudryavtseva, 2003; Smagin et al., 2015).

2.4. Western immunoblotting

After all behavior tests were concluded, brains were collected within 5 min and freshly frozen on dry ice before being stored at −80 °C. Brain tissue was cut into 300-μm coronal sections using a cryostat at −11 °C onto glass slides. For each brain, micropunches of each region were made using the Allen Mouse Brain Atlas (https://mouse.brain-map.org) as a guide (Fig. 2). The NAc (including core and shell) micropunches were collected in a range from 1.2 mm to 2.2 mm from bregma, and the BNST was collected in a range from −0.26 mm to −0.3 mm from bregma. The NAc, POA, and AMY were collected using a gauge#11 sample corer (Fine Science Tools, item No. 18035-02; diameter = 2 mm; 2 punches for each region), and the BNST, dHIP and vHIP were collected using a gauge#15 sample corer (Fine Science Tools, item No. 18035-01, diameter = 1 mm; 4 punches for each region). All samples were then stored at −80 °C.

Micropunches were homogenized after adding 100 μl of ice-cold lysis buffer consisting of 50 mM Tris-HCl, 1% Na-deoxycholate, 0.25%

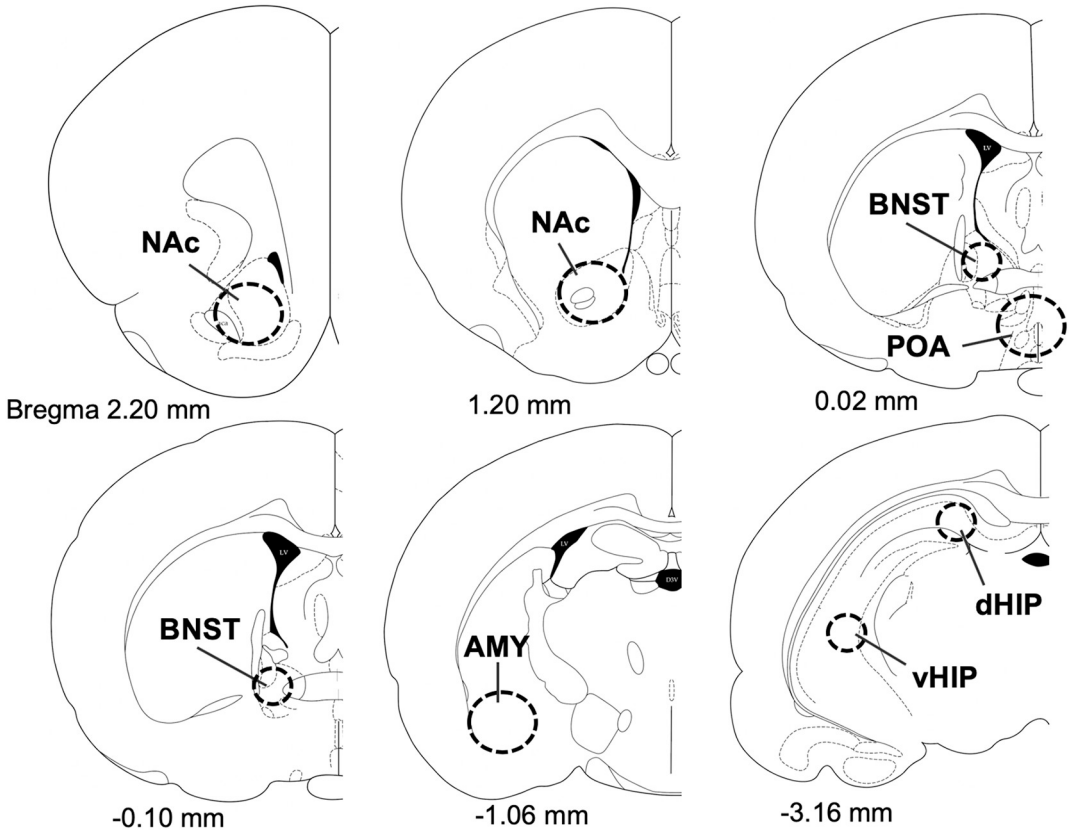

Fig. 2. Coronal brain sections from which nucleus accumbens (NAc), bed nucleus of the stria terminalis (BNST), preoptic area (POA), amygdala (AMY), dorsal and ventral hippocampus (dHIP and vHIP) were collected. Stereotaxic coordinate of each section (bregma) is provided below each illustration. Circles depict areas of punches. Section images are modified from the Web-based Allen Mouse Brain Atlas (https://mouse.brain-map.org).

Nonidet P-40, 150 mM NaCl, 1 mM EDTA, Protease Inhibitor Cocktail (P8340, as directed; Sigma-Aldrich, St. Louis, MO, USA), and Phosphatase Inhibitor Cocktail (P2850, as directed; Sigma-Aldrich, St. Louis, MO, USA). To remove cellular debris and nuclei, the samples were centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant was collected, and protein concentrations were measured using a Bradford assay. Thirty micrograms of total protein from each animal were gel electrophoresed using a 7.5% precast polyacrylamide gel (catalog #456-1026; Bio-Rad, Hercules, CA, USA) and transferred to a polyvinyl difluoride membrane (Immobilon-P; catalog #IPVH000010; Millipore, Burlington, MA, USA). Membranes were blocked for 2 h in 0.1 M TBS containing 5% nonfat dry milk (catalog #170-6404; Bio-Rad, Hercules, CA, USA) with constant agitation at room temperature. Membranes were then incubated overnight at 4 °C in TBS containing 2% nonfat dry milk with the following primary antibodies: AR (1:500 dilution; mouse IgG κ; catalog#sc-7305; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), synapsin (1:1000 dilution; catalog#2312S; cell signaling technology, Danvers, MA, USA), phospho-synapsin (Ser9) (1:1000 dilution; catalog#2311S; cell signaling technology, Danvers, MA, USA) and β-actin (1:3000 dilution; mouse IgG_1; catalog#sc-47778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The phospho-synapsin (Ser9) antibody detects endogenous levels of synapsin when phosphorylated at serine 9. Both the synapsin and phospho-synapsin (Ser9) antibodies recognize all synapsin isoforms and detect endogenous levels of total synapsin protein. The next day, the membranes were given three 5-min washes in TBS. After being washed, membranes were incubated in a secondary antibody (Anti-rabbit IgG, catalog#7074S; Anti-mouse IgG, 7076S) (1:3000 dilution) and horseradish-peroxidase-conjugated anti-biotin antibody (1:3000 dilution; catalog#7075P5; cell signaling technology, Danvers, MA, USA) for 2 h at room temperature with agitation and washed three times for 5 min each with TBS. Immunoreactive bands were detected using a chemiluminescence kit (LumiGLO® Reagent; cell signaling technology, Danvers, MA, USA) and exposed to x-ray film (CL-XPosure Film; Thermo Scientific, Waltham, MA, USA). The developed x-ray films were digitized using a scanner and analyzed using the Western blot macro gel plot 2 within the Scion Image program (Scion Corporation, Frederick, MD, USA).

2.5. Data analysis

Eight mice were excluded from the statistical analysis because they did not explore all three CPP chambers during either the pre-conditioning or post-conditioning tests. To assess whether the T injection induced a CPP, we used a preference score, computed as the time spent in the CS+ chamber divided by the sum of the time spent in the CS+ and CS− chambers (Bell et al., 2010; Martínez and Paredes, 2001; Meerts and Clark, 2007). The formation of a CPP was defined as a significant increase in the preference score from pre-conditioning test to post-conditioning test by an independent-sample *t*-test. For testing aggressive motivation, we used the latency to approach the stimulus male and duration of interaction with a stimulus male. Results of the western immunoblots are expressed as ratios of the blot optical densities of AR, synapsin, and phospho-synapsin each to β-actin, a cytoskeletal protein that shows consistent production across tissue types regardless of treatment and is commonly used as a control in western immunoblotting. The results for synapsin and phospho-synapsin levels were measured by combining both visible isoforms on the western immunoblots. Shapiro-Wilk test was used to test assumptions of normality. Two-way ANOVAs (2 × 2) were conducted to analyze the interaction between drug treatment (T vs. saline) and housing condition (singly-housed vs. group-housed). When the interaction was not significant, main effects of the ANOVA were reported. Regardless of whether the interaction was

significant, we used independent *t*-tests as planned contrasts (Hager, 2002) to further analyze a priori predictions regarding the effects of each drug treatment (T or saline) at each level of housing condition (singly- or group-housed). When a significant effect was detected by the ANOVA or planned contrasts for either the levels of synapsin or phospho-synapsin, the ratio of phospho-synapsin to synapsin was also analyzed to determine if differences in phospho-synapsin levels were due to differences in overall levels of synapsin. The Pearson correlation test was used to analyze the correlation between neural changes (AR, synapsin and phospho-synapsin levels) and behavioral responses (preference score for the chamber location of drug administration, latency to approach the stimulus male and the duration of interacting with the stimulus male). All data were analyzed using SPSS (Version 16.0.1, SPSS Inc., Chicago, IL). Effect sizes are reported as partial eta-squared (η_p^2) for ANOVA effects and as Cohen's d for planned contrasts between groups using independent *t*-tests.

3. Results

3.1. Behavioral patterns

We found effects of T on the formation of a CPP and aggressive motivation in single-housed, but not group-housed males. Specifically, T treatment induced a CPP in the single-housed condition but not the group-housed condition while saline did not induce a CPP in either housing condition. Preference scores during pre-conditioning tests and post-conditioning tests were not significantly different for the control group receiving saline (group-housed: $t_{10} = 1.18$, $p = 0.262$, Cohen's $d = 0.41$; singly-housed: $t_9 = 0.62$, $p = 0.55$, Cohen's $d = 0.25$), but significantly increased for the singly-housed T group after conditioning ($t_{11} = 2.76$, $p = 0.02$, Cohen's $d = 1.37$). In contrast, group-housed males did not show a CPP for the side chamber where they received T injections (CS+ chamber) ($t_{11} = 1.44$, $p = 0.18$, Cohen's $d = 0.56$; Fig. 3). As for aggressive motivation, singly-housed males that received T showed shorter latencies to approach the stimulus male and longer durations of interaction across a mesh barrier. Specifically, for the latency to approach the stimulus male during the social interaction test, a two-way ANOVA revealed a significant interaction between drug treatment and housing condition ($F_{1,38} = 4.23$, $p = 0.047$, $\eta_p^2 = 0.10$). Further planned contrasts revealed that the singly-housed males that received T, which was also the only group that formed a CPP, displayed

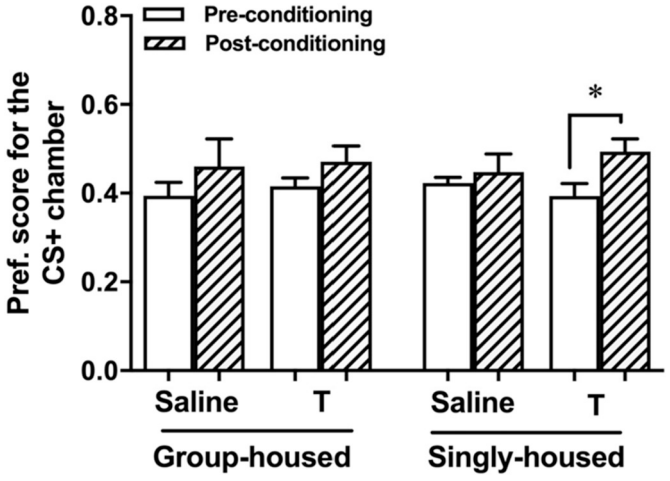

Fig. 3. Preference score of male California mice during CPP pre-conditioning tests (open bars) and post-conditioning tests (hatched bars) for the initially non-preferred chamber where either testosterone (T) or a vehicle control (Saline) was administered. Males were either group-housed since weaning or singly-housed for 3 days before testing. Data are mean ± SEM; * indicates a significant difference at $p = 0.02$.

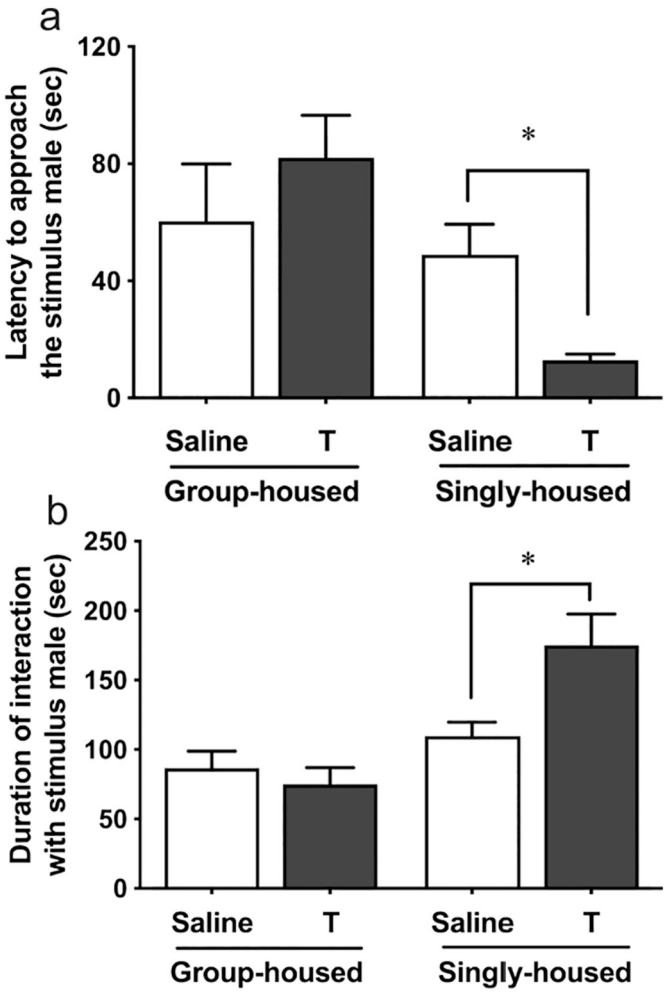

Fig. 4. (a) The latency of male California mice to approach the stimulus male during the social interaction test, and (b) the duration of interaction with the stimulus male for males that were either group-housed since weaning or singly-housed for 3 days before testing and received either saline (open bars) or T (solid bars) during conditioning. Data are mean ± SEM; * indicates significant differences (latency: $p = 0.03$; duration: $p = 0.01$).

significantly shorter latencies to approach the stimulus male ($t_{16} = 2.33$, $p = 0.03$, Cohen's $d = 1.39$) than singly-housed males that received saline (Fig. 4a; a result of either T, CPP, or a combination of the two). For the duration of interaction with the stimulus male during the social interaction test, a two-way ANOVA revealed a significant effect of the interaction between drug treatment and housing condition ($F_{1,39} = 7.703$, $p < 0.01$, $\eta_p^2 = 0.17$). Further planned contrasts revealed that the singly-housed males that received T, which is also the only group that formed a CPP, interacted with the stimulus male for longer periods of time, compared to the singly-housed males that received saline ($t_{10} = 2.757$, $p = 0.01$, Cohen's $d = 1.27$; Fig. 4b).

3.2. Protein levels

3.2.1. AR levels

Our results only revealed a significant effect on AR levels in the POA, and statistics on the expression of AR in all regions are shown in Table S1. In the POA, singly-housed males showed higher levels of AR than group-housed males while no differences in AR levels were found between groups in the NAc, AMY, BNST, dHIP, or vHIP. The ANOVA did not detect a significant interaction nor main effect and planned contrasts did not find any significant differences in AR levels in the NAc, AMY, BNST, dHIP, or vHIP ($p > 0.37$). For AR levels in the POA,

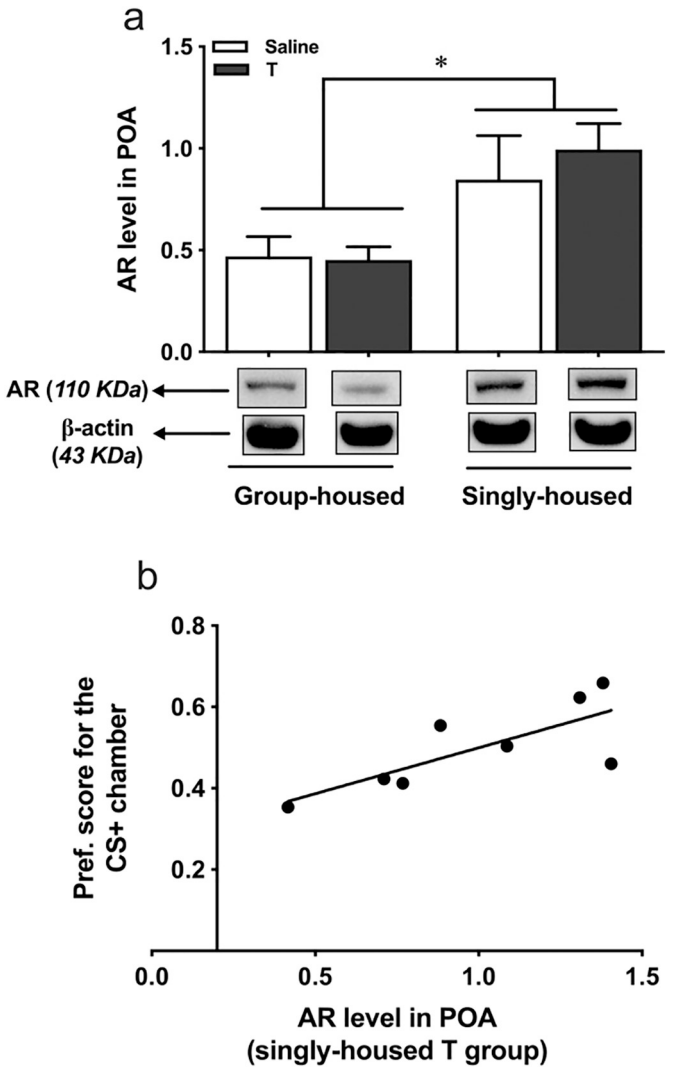

Fig. 5. (a) AR levels (AR/β-actin) in the POA for male California mice that experienced conditioning procedures with either testosterone (T) or a vehicle control (Saline) and were either group-housed since weaning or singly-housed for 3 days before testing. Data are mean ± SEM; * indicates significant difference at $p < 0.05$. Images below the x-axis are representative western immunoblots. (b) Relationship between AR level in the POA and the post-conditioning preference score of the CS+ chamber in singly-housed males that received T (Pearson's correlation: $r = 0.760$, $p = 0.028$).

the two-way ANOVA did not reveal a significant interaction between drug treatment (T or saline) and housing condition (singly- or group-housed) ($F_{1,22} = 0.353$, $p = 0.56$, $\eta_p^2 = 0.016$) or main effect of drug treatment ($F_{1,22} = 0.223$, $p = 0.64$, $\eta_p^2 = 0.01$) in the POA, but did reveal a significant main effect of housing condition, such that the singly-housed males expressed significantly higher AR levels than the group-housed males ($F_{1,22} = 10.953$, $p = 0.003$, $\eta_p^2 = 0.332$; Fig. 5a). Using planned contrasts, we did not find significant differences between drug treatments in either housing condition in the POA ($p > 0.24$).

The results above reveal a change in androgen sensitivity in the POA in response to housing conditions, but not in response to T-injections. However, in the singly-housed males that received T, the group that formed a CPP, AR levels were positively correlated with the preference scores for the location where T was administered, the CS+ chamber ($r = 0.760$, $p = 0.028$, $n = 8$; Fig. 5b). AR levels in the POA were not correlated with preference scores for the other three groups ($p > 0.65$; Fig. S1). Furthermore, neither the latency to approach the stimulus male nor the duration of interaction with the stimulus male were correlated with AR levels in the POA in the singly-housed males that received T. No significant correlation was found for each of the other three groups between AR levels and each of the two behavioral measures ($p > 0.56$). No significant correlation was revealed between the AR levels in other brain regions and the behavioral measures ($p > 0.41$).

3.2.2. *Synapsin and phospho-synapsin levels*

Mean levels of synapsin, phospho-synapsin, and their relative ratio can be viewed for each combination of housing condition and drug treatment group for each brain region in Table S2. Statistically significant differences were found in the NAc, dHIP, and vHIP, but not in the POA, AMY or BNST ($p > 0.16$). In the NAc, singly-housed males showed higher levels of synapsin and phospho-synapsin than group-housed males. In the dHIP, singly-housed males showed higher levels of phospho-synapsin and the ratio of synapsin to phospho-synapsin than group-housed males. In the vHIP, singly-housed males that received T showed higher levels of phospho-synapsin and the ratio of synapsin to phospho-synapsin than singly-housed males that received saline.

In more detail, the two-way ANOVA did not reveal a significant interaction or main effect of drug treatment for either the ratio of NAc synapsin/β-actin or phospho-synapsin/β-actin ($p > 0.28$). A significant main effect of housing condition, however, was found, with the singly-housed males showing significantly higher levels of both NAc synapsin/β-actin ($F_{1,19} = 18.978$, $p < 0.001$, $\eta_p^2 = 0.50$; Fig. 6a) and phospho-synapsin/β-actin ($F_{1,20} = 5.466$, $p = 0.03$, $\eta_p^2 = 0.22$; Fig. 6b) than the group-housed males. No statistically significant effects were detected in the ratio of NAc phospho-synapsin/synapsin ($p > 0.71$). Planned contrasts did not find any significant differences between T and saline on each level of the housing condition for the levels of NAc synapsin and phospho-synapsin ($p > 0.47$).

In the dHIP, the two-way ANOVA did not reveal any statistically significant effects in the levels of dHIP synapsin/β-actin ($p > 0.66$). For phospho-synapsin/β-actin, the interaction between drug treatment and housing condition and the main effect of drug treatment was not statistically significant ($p > 0.37$), but the main effect of housing condition was significant, with the singly-housed males showing higher levels than the group-housed males ($F_{1,23} = 6.52$, $p = 0.02$, $\eta_p^2 = 0.22$; Fig. 6c). A similar significant effect was found for housing condition in the ratio of phospho-synapsin/synapsin ($F_{1,23} = 7.55$, $p = 0.01$, $\eta_p^2 = 0.25$; Table S2). Planned contrasts did not find any significant differences in dHIP synapsin, phospho-synapsin between T and saline on each level of the housing condition ($p > 0.42$).

In the vHIP, the two-way ANOVA did not reveal a significant interaction nor main effects ($p > 0.05$). Planned contrasts did not reveal any statistically significant differences in the levels of vHIP synapsin/β-actin ($p > 0.42$). However, planned contrasts showed that the singly-housed males that received T, again the group that formed the CPP, expressed a significantly higher level of phospho-synapsin/β-actin in the vHIP compared to the singly-housed males that received the saline control ($t_{10} = 2.262$, $p = 0.04$, Cohen's $d = 1.32$; Fig. 6d). Such a significant difference was also detected in the ratio of vHIP phospho-synapsin/synapsin ($t_{10} = 2.238$, $p = 0.04$, Cohen's $d = 1.30$). However, no significant differences were found between drug treatments in the group-housed males ($p > 0.53$).

No significant correlations were found between synapsin or phospho-synapsin levels and behavioral measures in the brain regions examined ($p > 0.27$).

4. Discussion

The current study fleshes out a model for the mechanisms underlying the emergence of territoriality that is partially based on a rewarding/reinforcing mechanism that is reflected by the formation of CPPs through transient increases or pulses of T. Our results reaffirm the findings of Zhao and Marler (2014, 2016) that single housing facilitates

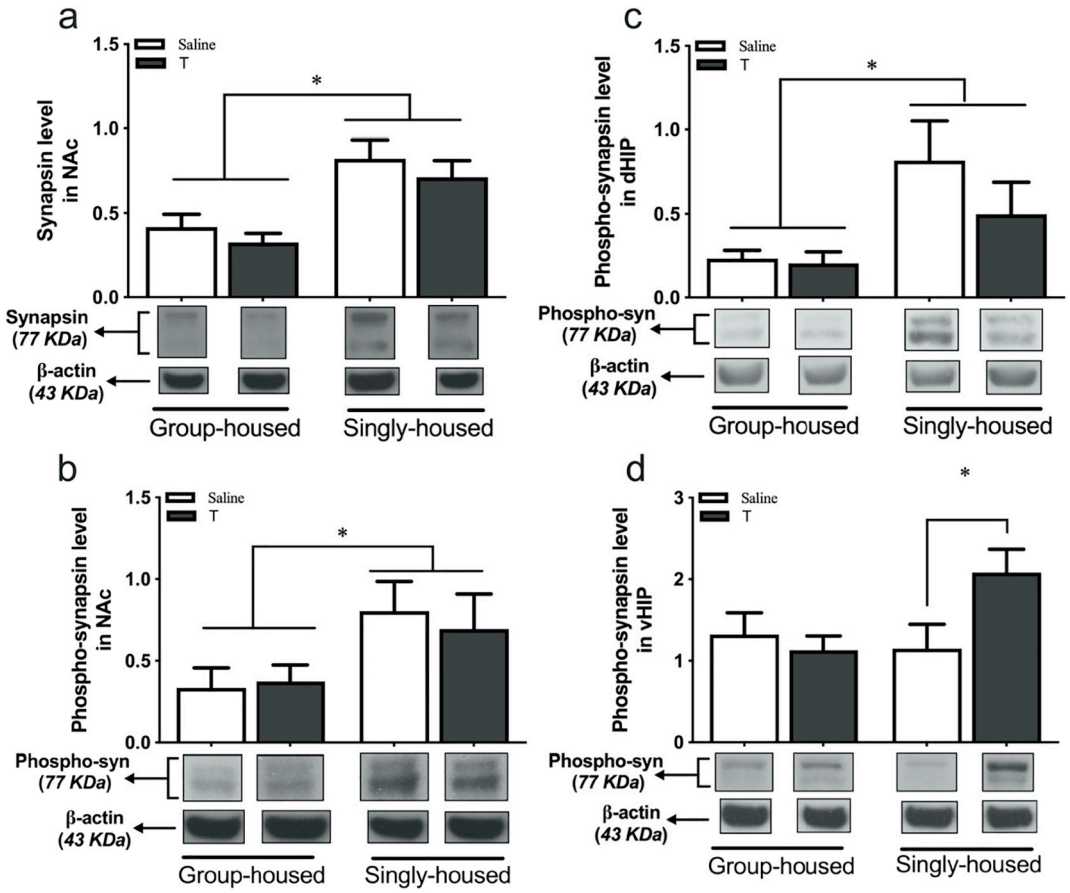

Fig. 6. (a) Synapsin levels (synapsin/β-actin) in the NAc and phospho-synapsin levels (phospho-synapsin/β-actin) in the (b) NAc, (c) dHIP, (d) vHIP for male California mice that experienced conditioning procedures with either testosterone (T) or a vehicle control (Saline) and were either group-housed since weaning or singly-housed for 3 days before testing. Data are mean ± SEM; * indicates significant difference at $p < 0.05$ (see text). Images below the x-axes are representative western immunoblots.

T-induced CPPs to an unfamiliar environment in sexually naïve males. In these and the current study, sexually naive, singly housed males formed a CPP for an initially unfamiliar apparatus chamber when treated with T but not saline. The comparison animals were group-housed males that did not form a CPP via saline or testosterone. We speculate that temporarily housing males singly following group housing since weaning may be similar enough to dispersal in nature to initiate similar hormonal and neuroanatomical changes needed for the development of territoriality. The T pulses further increased motivation to approach a stimulus male in singly-housed but not group-housed males (Zhao et al., 2019). We then revealed for the first time that single housing can change the levels of ARs in the POA and alter the correlations between ARs and the time spent in the T-induced preferred CS+ chamber such that a positive correlation was only found in the group of singly-housed males that received T pulses, but not in group-housed males receiving saline or testosterone. Housing conditions and T pulses may therefore reveal a link between the intensity of a CPP and AR density in the POA, which may play multiple roles in the formation of a territory. We further found an increase in markers of synaptic plasticity in the NAc, vHIP and dHIP in these same sexually naive, singly-housed males that formed a CPP for an initially unfamiliar apparatus chamber when treated with T but not saline. These behavioral and neural changes may be involved in the life history transition from residing in the natal territory to dispersing and establishing a new territory. We develop a model below (summarized in Fig. 7) and place our findings in the context of previous behavioral and neural research.

The facilitated CPP in singly-housed males may reflect the natural functions of T's rewarding effects at different life history stages. Field studies in California mice have shown that male mice usually settle first in the mated pair's home range and that the males may gain access to females indirectly by monopolizing critical resources (Ribble, 1992; Ribble and Salvioni, 1990). Therefore, the ability to establish a territory and monopolize resources are likely critical for the reproductive fitness of dispersed sexually naïve males. Evidence across taxa points to T playing an important role in modulating this ability. Field studies in several territorial mammalian species have shown that the experience of dispersal or becoming solitary is associated with elevated baseline T levels (Schoepf and Schradin, 2013; Schradin and Yuen, 2011; Woodroffe et al., 1995). In nature, endogenous pulses of T could be elicited by either winning male–male antagonistic encounters or engaging in male–female sexual encounters (review by Gleason et al., 2009). Furthermore, both aggressive and sexual encounters can induce CPPs (Bell et al., 2010; Martínez et al., 1995). We speculate that these rewarding social experiences may facilitate territory establishment by reinforcing the monopolization of resources and that CPPs induced by the T-pulses following these social experiences might influence territoriality by adjusting site preferences. Site preferences would be adjusted during territory settlement and may drive the male to allocate more time to a location where he is more likely to encounter a female or drive off an intruding male. In comparison, the dampened CPP in group-housed males may reflect an inhibited motivation to establish a territory, which parallels laboratory findings that group-housed male laboratory mice are less aggressive compared to singly-housed males (Siegfried et al., 1981; Yang et al., 2017). Previous studies have shown that compared to group-housed mice, singly-housed mice spent significantly less time at home, reduced neophobic reaction and increased

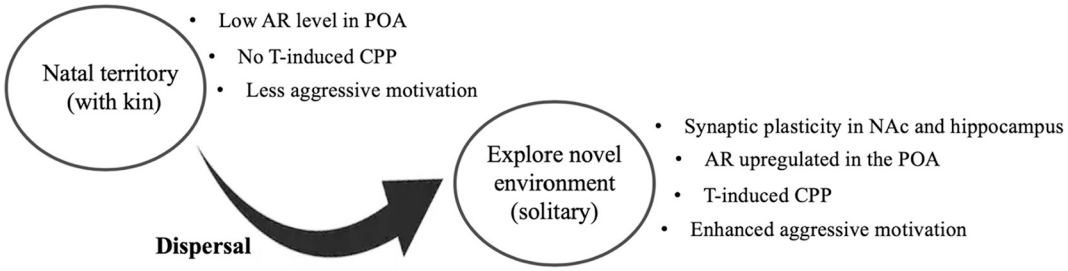

Fig. 7. For territorial species like California mice, the development of territoriality likely involves dispersing from the familiar natal environment, learning a new spatial environment, forming a preference for that environment, and aggressively defending it against intruders. We speculate that temporarily housing singly following group housing since weaning may be similar enough to dispersal in nature to initiate similar hormonal and neuroanatomical changes needed for the development of territoriality. Single housing can upregulate the levels of ARs in the POA, which may contribute to the formation of a conditioned place preference (CPP) to the new territory. Additionally, single housing may cause synaptic plasticity in the NAc, dHIP and vHIP, neural changes likely associated with dispersal, reproduction and/or territory establishment. These behavioral and neural changes may be involved in the life history transition from residing in the natal territory to dispersing and establishing a new territory.

levels of locomotor activity and exploration-related behaviors (Bartolomucci et al., 2003; Goldsmith et al., 1978; Griebeii et al., 1993; Rilke et al., 1998).

The switch from group housing to single housing appears to facilitate the expression of T-induced aggressive motivation. In the current study, we did not directly measure the physical aggressive behaviors between the focal male and the novel stimulus male, but we previously demonstrated that resident males that received T injections also spent more time interacting with a stimulus male and initiated attack more quickly once the mesh barrier between the two males was removed (Zhao et al., 2019). Furthermore, other researchers using a similar "partition test" have shown that time spent near a transparent perforated barrier separating male laboratory mice correlates positively with attacks once the barrier is removed (Kudryavtseva, 2003; Smagin et al., 2015). Our results demonstrated that only the singly-housed males that received T, but not saline-injected singly-housed males or either set of group-housed males, exhibited decreased latencies to approach the stimulus male and increased duration of interaction with the stimulus male, indicating a T-induced enhancement of aggressive motivation. One question that remains unanswered, however, is whether the T-induced aggression is amplified by the formation of a T-induced CPP. At the very least, however, it is likely that the T-induced CPP increases the time allocated to the location where the rewarding drug was experienced. Whether enhanced aggressive motivation relies on the place of preference requires further testing in which the behavioral responses to a stimulus in CS+ versus CS− is compared. It is also possible that alternatively or in addition to enhanced aggression, the latency to approach and duration of interaction with the stimulus male may reflect T-induced enhanced investigatory behavior and/or reduced fearfulness/anxiety (Aikey et al., 2002; Frye and Seliga, 2001). Regardless of which of these behaviors is affected by T, each of them would likely contribute towards dispersal and establishing a territory.

Along with the formation of T-induced CPPs in the single housing condition, male California mice also experienced neuroanatomical changes in the POA that may contribute to the establishment of a territory. Singly housed males showed upregulated AR levels in the POA, a brain area that responds to sex steroids in the regulation of reproductive and aggressive behaviors (Ball and Balthazart, 2002; Hutchison et al., 1996; Panzica et al., 1996). The projections from the medial POA to the ventral tegmental area contains estrogen receptors and have been implicated in facilitating a CPP to the chamber that was previously paired with an opposite-sex conspecific odor (McHenry et al., 2017). One potential mechanism through which T pulses can enhance location preferences is through enhancement of aromatase activity, which facilitates the production of estrogen from T and then activates the estrogen receptors expressed on the projections from the medial POA to the ventral tegmental area (Roselli and Resko, 1984). Previous studies in rats have shown that microinjections of T into the preoptic area (POA) are sufficient to induce a CPP (King et al., 1999). It is unlikely that the interaction with the stimulus male upregulated the AR levels because the brain tissue was collected immediately after the 5-min social interaction test. We were not able to test the direct relationship between AR levels in the POA and aggressive behavior, but our results indicate a potential role for AR activity in the POA in mediating aggressive motivation, which may be a valuable direction for future research. The functions of the POA have been mainly investigated in the context of sexual behaviors (Dominguez and Hull, 2005; Edwards and Einhorn, 1986; Wood and Newman, 1995b) and it was previously hypothesized that medial POA belongs to a steroid-responsive neural network that controls mating behaviors (Newman, 1999). Later evidence also revealed that the caudal part of the medial preoptic nucleus showed similar Fos-immunoreactivity following sexual and aggressive encounters (Veening et al., 2005). Beyond that, the POA has been implicated in male-male aggression (Albert et al., 1986) and maternal care (Lee and Brown, 2007). Adding to these studies, our findings further revealed the potential role of the POA in mediating the establishment of a territory.

The induction of synaptic plasticity in the NAc, vHIP and dHIP may lead to a series of behaviors or cognitive changes that help animals adapt to a new environment. It is well established that the NAc plays an important role in processing sensorimotor information that motivate animals to seek out a conditioned stimulus that was previous associated with an unconditioned reward (Day et al., 2006; Parkinson et al., 2002). Microinjections of T into the nucleus accumbens (NAc) are sufficient to induce a CPP in rats (Frye et al., 2002a; Packard et al., 1997) and our recent study showed that T injections can rapidly modulate decision-making in male California mice's and promote approach to a potential threat (Zhao et al., 2019). In the NAc, being singly-housed upregulated the levels of synapsin and phospho-synapsin, suggesting an increased reserved pool of synaptic vesicles within the terminal and an increased pool of vesicles ready to be released at the synapse, respectively. The increase in synapsin may also account for the increase in phospho-synapsin in singly-housed subjects, as more synapsin can be phosphorylated if more of it is initially present. The fact that housing condition had a significant effect on synapsin and phospho-synapsin but not the ratio of phospho-synapsin/synapsin suggests that the relative levels of both proteins remained similar across housing conditions despite increases in the individual proteins. We speculate that the transition from group living in the natal area to single living in a new territory may involve the formation or rearrangements of synaptic contacts in the NAc as well as the ability of modulating neurotransmitter release.

Dispersal and the establishment of a territory may require an increased ability to navigate an unfamiliar environment that might be accomplished through changes in the hippocampus. In the dHIP, the level of phospho-synapsin, but not synapsin, increased after being singly housed. This effect is independent of the drug treatment and

suggests that the experience of being singly housed may sensitize the activity of neurotransmitter release in response to the CPP test. The dHIP plays an important role in spatial memory (Ferbinteanu and McDonald, 2001), and therefore, the activity of neurotransmitter release in this region may function to prepare animals' spatial navigation after dispersal from the natal area. In the vHIP, we found the increased level of phospho-synapsin, but not the level of synapsin, in the singly-housed males that received T, the only group that showed CPP. However, our correlation analysis did not reveal any significant relationship between the phospho-synapsin level in the vHIP and the behavioral measures. This suggests that the vHIP may not be directly involved in modulating the formation of CPP or aggressive motivation. In future studies, it will be especially interesting to elucidate the role that the vHIP plays in territory establishment.

The mechanisms underlying these isolation-induced changes in response to T are not well understood. We speculate that dispersal likely leads to changes in the brain and how it responds to T; changes include AR expression levels and the restructuring of synaptic pathways. Temporary isolation is a natural segue for species that disperse to establish territories (Eikenaar et al., 2007; Howard, 1960; Lambin X, 2001; Lamhin, 1994). Isolation may reduce chemosensory input from familiar same-sex cage mates, which may also influence animals' reaction to multiple T injections. A recent study in male Mongolian gerbils (*Meriones unguiculatus*) demonstrated that male-male cohabitation dampens the T-induced territorial aggression, while male-female cohabitation enhances it (Piña-Andrade et al., 2020). Studies of male Syrian hamsters have shown that AR-containing and androgen concentrating neurons are rich along the chemosensory pathways including the POA (Wood et al., 1992; Wood and Newman, 1993) and exposure to conspecific olfactory stimuli has been shown to modulate AR-immunoreactive cells in the medial amygdala in hamsters (Blake and Meredith, 2011). Future experiments could be conducted to further investigate how the deprivation of chemosensory input influences the neural AR expression. Finally, the possibility that the density of cage occupants (higher density in group-housed males and lower density in singly-housed males) affect the T's actions cannot be excluded based on the current design. Studies in mice have shown that cage density did not affect basal levels of T (Ortiz et al., 1984), but can influence aggressive behavior, territoriality, and the social hierarchy, with territoriality being more likely at lower densities and dominance hierarchies more likely at higher densities (Anderson and Hill, 1965; Davis, 1958; Van Loo et al., 2001).

In the current study, we did not find any significant changes in the levels of AR in the NAc, which initially seems to conflict with a previous study showing that winning male-male encounters (and experiencing natural T pulses) increases levels of AR in the NAc when the encounters occurred in a subject's home cage and not in an unfamiliar cage (Fuxjager et al., 2010a). Such a discrepancy may be accounted for by two possible factors. First, the upregulated AR in the NAc may be specific to a winning experience that involved physical contact; in the current study males were separated by a wire mesh. Second, the male subjects of the current study were sexually naïve, whereas the winner effect study used pair-bonded males (Zhao and Marler, 2014). Thus, the upregulated AR in the NAc might be specific to pair-bond formation, which is a marker for an important life history stage in monogamous animals and affects several T-related social behaviors such as aggression and partner preferences (Insel et al., 1995). Furthermore, pair-bonding also dampened the T-induced CPP to an unfamiliar environment, but enhanced the CPP to the home environment (Zhao and Marler, 2014). It would be interesting to test the effects of T-induced CPP to the home environment on the regulation of AR in the NAc in pair-bonded males. Taken together, our findings did not support the role of AR in the NAc in facilitating the production of CPP in singly-housed males. In the current study, we cannot rule out the role of estrogen receptors in mediating the effects of T pulse and it would be interesting to examine the level of the aromatase activity and estrogen receptors in the POA in future studies.

In conclusion, our controlled laboratory findings suggest that in nature, the experience of dispersing from the natal area may upregulate the androgen receptors in the preoptic area, which could enhance the sensitivity to the rewarding effects of T pulses that result from winning a male-male contest or encountering a female. Animals may therefore associate the rewarding experience with the environment and then allocate more time to that specific location. Also, single housing increased synaptic plasticity in the NAc, dHIP and vHIP, neural changes likely associated with dispersal, reproduction and/or territory establishment. All these behavioral and neural changes may contribute to the establishment of a territory. Besides social encounters, other factors such as exploring a novel resource-rich environment may also influence T pulses and remain unexplored research directions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2020.104709.

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