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Social and physical environments as a source of individual variation in the rewarding effects of testosterone in male California mice (*Peromyscus californicus*)

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ABSTRACT

Despite extensive research revealing the occurrence of testosterone (T) pulses following social encounters, it is unclear how they lead to varied behavioral responses. We investigated the influence of residency (home versus unfamiliar environment) and social/sexual experience (pair-bonded, isolated or housed with siblings) on the plasticity of T's rewarding effects by measuring the development of conditioned place preferences (CPPs), a classical paradigm used to measure the rewarding properties of drugs. For pair-bonded males, T-induced CPPs were only produced in the environment wherein the social/sexual experience was accrued and residency status had been achieved. For isolated males, the T-induced CPPs only occurred when the environment was unfamiliar. For males housed with a male sibling, the T-induced CPPs were prevented in both the home and unfamiliar chambers. Our results reveal the plasticity of T's rewarding effects, and suggest that the behavioral functions of T-pulses can vary based on social/sexual experience and the environment in which residency was established. The formation of CPPs or reward-like properties of drugs and natural compounds can therefore exhibit malleability based on past experience and the current environment.

1. Introduction

The reciprocal relationship between the steroid hormone testosterone (T) and territorial aggression occurs in a wide variety of species (review see Hirschenhauser and Oliveira, 2006). Plasma T can be positively correlated with the expression of particular forms of aggression such as territorial and dominance aggression (Gesquiere et al., 2011; Wingfield and Wada, 1989) (but see Apfelbeck and Goymann, 2011), and experimental manipulations of circulating T levels can alter aggressiveness (Fuxjager et al., 2011b; Monaghan and Glickman, 1992; Trainor et al., 2004). Alternatively, T-pulses can be elicited following male-male agonistic encounters as well as male-female sexual encounters (review see Gleason et al., 2009). Post-encounter T-pulses occur in males of many vertebrate species; but the function of these T-pulses remains largely unclear. Within the context of social behavior, however, the post-encounter T-surges may reinforce learning associated with an aggressive encounter (Marler et al., 2005). The rewarding effect of T in rodents have been revealed (Packard et al., 1997; Rosellini et al., 2001; Sato et al., 2010; Zhao and Marler, 2014), but researchers have ignored the impact of the physical and social environment on the rewarding effects of T. The current study explores the plasticity of T's rewarding effects, which may provide individuals with a mechanism for altering their behavioral responses to the environment.

One important source of plasticity in T's rewarding effects may be the environmental context in which T is released, such as the location where residency has been established. Territories are critical for maintaining access to resources in many species, and the primary behavioral mechanism for retaining a territory is aggression; territorial context significantly modulates aggressive behavior and the outcome of a contest (Fuxjager et al., 2010b; Snell-Rood and Cristol, 2005). The best example of this is the phenomenon called the 'home advantage' (Schwartz and Barsky, 1977) or 'residence effect' (Kemp and Wiklund, 2004), whereby the resident has an advantage over an intruder in a territorial dispute. Manipulations of and correlations with the environmental context support this notion and in many species, males behave more aggressively toward intrusion in the home cage when residency has been established, but less aggressively in an unfamiliar environment (Fayed et al., 2008; Krebs, 1982; McGuire et al., 1992; Waage, 1988). Given the important role of T in modulating territorial aggression, if the rewarding effect of T contributes to such context-dependent behavioral responses, the rewarding effect may also rely on the environmental context.

Besides context, the social/sexual experience may also influence animals' responses to the rewarding properties of T. We previously found that pair-bonding experience dampens the rewarding effect of T in male California mice (*Peromyscus californicus*) (Zhao and Marler, 2014).

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Social interactions can also decrease the rewarding properties of amphetamine in prairie voles (*Microtus ochrogaster*) (Liu et al., 2011) and influence drug intake and susceptibility to drug abuse (review see Young et al., 2011). Moreover, the social/sexual experience accrued within the home area can shape the salience of the home environment when coupled with residency. Studies in mice (Martínez et al., 1995; Popik et al., 2003), rats (Ma et al., 2006), hamsters (Bell et al., 2010; Meisel and Joppa, 1994), European starlings (*Sturnus vulgaris*) (Kelm-Nelson et al., 2012; Riters et al., 2014), green anole lizards (*Anolis carolinensis*) (Farrell and Wilczynski, 2006) and gilthead sea bream (*Sparus aurata*) (Millot et al., 2014) demonstrate that animals can associate natural rewards such as the social/sexual experience and foods with the physical environment in which the experience was acquired, and produce conditioned place preferences (CPPs). The social/sexual experience may therefore interact with residency to modulate animals' responses to T's rewarding effect.

We investigated the plasticity of T's rewarding effects by examining the influence of residency combined with the social/sexual experience on T-induced CPPs. CPP is a classical paradigm used in examining rewarding properties of drugs (Tzschentke, 2007). Although widely used in the laboratory, exploration of its adaptive function has been neglected. Here, we hypothesized that T-induced CPPs are plastic in response to residency and social/sexual experience, thereby contributing to the behavioral flexibility in different environments; to test this, we used the California mouse because of the extensive research on interactions among residency, T and aggression in this highly territorial and monogamous species (Fuxjager et al., 2010a; Fuxjager et al., 2009; Oyegbile and Marler, 2005; Trainor et al., 2004; Trainor and Marler, 2001). According to laboratory and field studies, males appear to experience three types of home environments that differ in terms of the social/sexual experience (Ribble, 1992). First, in the natal home, the young of one litter can occupy the nest with the parents during the rearing of a second litter (Eisenberg, 1962). In this natal environment, the male response to T in the form of CPPs may be inhibited because of both the social presence of siblings/family (e.g. Bennett et al., 1999) and residency in the natal territory. Second, males then typically disperse approximately the distance of one home range (1161 m^2) (Ribble, 1992; Ribble and Salvioni, 1990) and establish their own territories. Third, a female may then disperse to the male's territory (Ribble, 1992) and a pair bond ensues. These three types of home environments may be salient to animals in different respects as the natal home could include interactions with siblings; sexually naive males need to monopolize critical resources in their own home ranges to attract females; home is imperative for pair-bonded mice to maintain bonding and breed. In the current study, we indirectly mimic aspects of the above three types of home environments and study how residency interacts with social/sexual experience to influence the T-induced CPPs.

2. Methods

2.1. Subjects

We used 120 male *P. californicus* aged 6–12 months. They were group-housed (2–3 per cage; 48 × 27 × 16 cm) under a 10L: 14D light cycle with lights off at 01:30 pm. Animals were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Males were randomly assigned to one of three groups: male-male (MM) group ($n = 40$), male-single (MS) ($n = 40$) and pair-bonded (PB) group ($n = 40$). For the MM group, two males were weaned, housed together (no aggression or injuries were observed) and moved to the middle chamber of the CPP apparatus three days prior to the CPP trial; one of the two males was randomly selected as the focal animal. For the MS group, a randomly selected sexually naive male was separated from its cage mate and moved to the middle chamber three days prior to the CPP trial. For the PB group, each male was paired with a female 1-week before being housed in the middle chamber for three days before the CPP trial. The paired male and female mice were huddling side-by-side after 24 h of pairing, which is a well-accepted indicator of partner preference in monogamous prairie voles (Ahern et al., 2009; Liu et al., 2001; Williams et al., 1992). We did not record the mating behavior of paired animals, but typically a majority has mated within 10 days (Gleason and Marler, 2010). Pairs were observed for compatibility and if fighting occurred then the pair was separated and excluded from the experiment ($n = 3$).

2.2. Testosterone dose

We used 36 μg/kg T-injections (T-cyclodextrin inclusion complex) because in a previous study this dose 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). Moreover this dose produces CPPs in male California mice (Zhao and Marler, 2014). While the dose in the current study is lower than those used to identify CPPs in rats and mice, it mimics natural changes in male T-levels found in intact California mice after winning an aggressive encounter (Oyegbile and Marler, 2005) and male-female encounters (Zhao and Marler, 2014); in keeping with this, the same dosage enhances aggression and future winning ability (Fuxjager et al., 2011a; Fuxjager et al., 2011b; Gleason et al., 2009; Trainor et al., 2004). In the current study, half of the males were randomly selected to receive T-injections during the conditioning phase (T-group). As T-cyclodextrin was dissolved in saline, the other half of the males constituted the controls and received injections of saline (saline group).

2.3. CPP apparatus and procedure

Conditioning took place in large polycarbonate testing cages (91 cm long × 46 cm wide × 43 cm high) divided into three equal chambers. The two side chambers were connected to the middle chamber by manually controlled, sliding guillotine doors. To elicit the residence effect, we housed animals in the middle chamber for three days. We have used three days and fewer (24–48 h) to establish residency status (Fuxjager et al., 2010a; Oyegbile and Marler, 2005). For the unfamiliar environment, the middle chamber was again used but without prior residency, and therefore no odor cues. The focal males in each of the three groups were randomly assigned to be conditioned to either the home environment (home group) or the unfamiliar environment (unfamiliar group) (Fig. 1).

The CPP procedure was conducted over eight days (see Zhao and Marler, 2014). On day 1, a male was allowed to explore all three chambers for 30-min and was excluded if all three were not investigated. During this 30-min period, the female partner or the male cage mate was removed from the apparatus. The training phase occurred from days 2–7. On days 2, 4 and 6, each male received a T-injection and was placed into the home or unfamiliar middle chamber for 45-min. On days 3, 5 and 7, the male explored all three chambers for 45-min. The order of injections (T or saline) and exploration were also randomly assigned to the focal males but counterbalanced to reduce the chances of the order of treatment or other factors adversely influencing the results. On day 8, we tested the male's preference by recording time spent in each chamber as males explored all three chambers for 30-min. The female partner or the male cage mate was temporarily removed from the apparatus during the 30-min adaptation, the 30-min test and each 45-min conditioning session (Fig. 2).

2.4. Data analysis

Time spent in the middle chamber was used for analysis. The normality of the data was determined by the Shapiro-Wilk test. Three

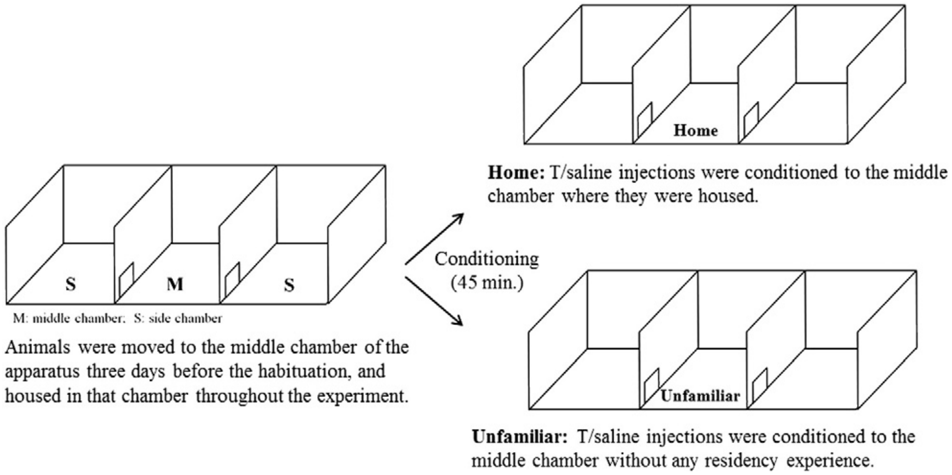

Fig. 1. The focal males in each of the three groups were randomly assigned to be conditioned to either the home environment (home group) or the unfamiliar environment (unfamiliar group).

factors, experience (PB, MM and MS), environment (home and unfamiliar) and treatment (T and saline), were included in three-way ANOVA. When the three-way ANOVA revealed a significant interaction, two-way ANOVAs (2×2) were conducted for analyzing the interaction between treatment and environment in PB, MS and MM groups. After a significant two-way interaction, we used independent *t*-tests to compare the two treatments (T vs. saline) because of our criteria that the production of CPP via T occurs when the T-group spends significantly more time than the saline group in the middle chamber. Two-way ANOVAs (2×3) were also conducted to analyze interactions between the environment and experience in T- and saline-group. Again, after a significant two-way interaction, an independent *t*-test was used to compare the two environments (home vs. unfamiliar); while the Newman–Keuls post hoc test was used to compare the three groups with different social experience (PB, MS and MM). The effect size of *t*-test and ANOVA was estimated by Cohen's d and eta-squared respectively. Two outliers (one from MS and one from MM) were removed based on the ESD outlier test. Seven males were excluded from the statistical analysis because they either did not explore all three CPP chambers or were not compatible with their female partners.

3. Results

The three-way ANOVA analysis revealed a significant interaction ($F_{(2,96)} = 7.12$, $p < 0.01$, eta-squared $= 0.09$) between experience (PB, MM and MS), environment (home and unfamiliar) and treatment (T and saline). Thereafter, two-way ANOVAs were used to analyze the interaction between treatment and environment in PB, MS and MM groups. For PB males, there was a significant interaction between environment and treatment ($F_{(1,34)} = 7.98$, $p < 0.01$, eta-squared $= 0.18$). *t*-tests revealed that T-treated males allocated significantly more time in the home middle chamber than the saline injected males ($t_{17} = 3.06$, $p < 0.01$, Cohen's $d = 1.39$), suggesting the formation of a CPP in the home middle chamber; but no significant difference between T and saline groups in the unfamiliar environment ($t_{17} = -1.02$, $p = 0.32$, Cohen's $d = -0.47$), suggesting no T-induced CPP in the unfamiliar middle chamber. In addition to analyzing the formation of CPPs, the independent *t*-test was also used to compare the two environments (home vs. environment) in saline and T groups. Specifically, the saline group spent more time in the unfamiliar middle chamber than the home chamber ($t_{17} = 2.34$, $p < 0.05$, Cohen's $d = 1.07$), potentially reflecting the novelty-seeking tendency of males (further supported by the ANOVA results below). In the T group, there was only a nonsignificant trend in the opposite direction ($t_{17} = 1.79$, $p = 0.09$, Cohen's $d = 0.82$). According to Ruscio (2008), such value of Cohen's d (0.82) indicates 79% of the home group was above the mean of the unfamiliar-environment group, and there was a 71% chance that a male from the home group would have a higher score than a male selected from the control group. The results suggest that T may overcome PB animals' novelty-seeking tendency and reinforce them to allocate more time to the home.

For the MS group, there was also a significant interaction between environment and treatment ($F_{(1,29)} = 7.63$, $p < 0.05$, eta-squared $= 0.12$). CPPs, however, were formed only in the unfamiliar environment; T-injected males spent significantly more time than saline-injected in the unfamiliar middle chamber ($t_{15} = 2.79$, $p < 0.05$, Cohen's $d = 1.37$), but not in the familiar home environment ($t_{14} = 0.68$, $p = 0.51$, Cohen's $d = 0.34$). Such environment-dependent effects of T were further supported by the independent *t*-test results for the comparison between the two environments in T and saline groups; the time spent in

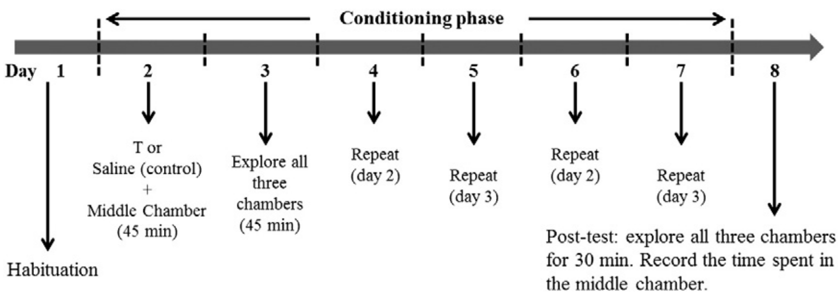

Fig. 2. Experimental timeline. The CPP procedure consists of three phases: habituation (day 1), conditioning (days 2–7) and post-test (day 8). During the conditioning phase, the focal males received either T or saline (control), and were then conditioned to either the home or unfamiliar middle chamber for 45 min (days 2, 4 and 6). On the alternate days (3, 5 and 7), animals were allowed to explore all three chambers for 45 min.

the unfamiliar middle chamber was significantly higher than that in the home chamber ($t_{15} = 4.71$, $p < 0.01$, Cohen's $d = 2.37$) in the T group, but not in the saline group ($t_{15} = 1.29$, $p = 0.21$, Cohen's $d = 0.64$).

For the MM group, there were main effects for both environment ($F_{(1,33)} = 14.12$, $p < 0.01$, eta-squared $= 0.27$) and treatment ($F_{(1,33)} = 4.44$, $p < 0.05$, eta-squared $= 0.08$), but no interaction ($F_{(1,33)} = 0.65$, $p = 0.43$, eta-squared $= 0.01$). Specifically, regardless of the treatment, males spent more time in the unfamiliar middle chamber than in the home middle chamber. Moreover, regardless of the environment, the T group spent less time in the middle chamber (more time in the novel side chambers) than the saline group. The results further indicate T's function in driving animals to explore novel areas. However, the independent *t*-test showed no significant difference between T and saline (both $p > 0.05$), suggesting no CPPs were produced in the MM group. (Fig. 3).

Two-way ANOVA did not reveal a significant interaction between experience and environment in animals that received saline ($F_{(2,47)} = 0.38$, $p = 0.69$, eta-squared $= 0.01$), but a main effect was found for the environment ($F_{(1,47)} = 8.45$, $p < 0.01$, eta-squared $= 0.12$). Specifically, regardless of experience, animals in the saline group spent more time in the unfamiliar chamber than in the home, further supported the baseline novelty-seeking tendency of control animals that was revealed in the previous paragraph. In males that received T, the two-way ANOVA revealed a significant interaction between experience and environment ($F_{(2,49)} = 13.24$, $p < 0.01$, eta-squared $= 0.28$). The Newman–Keuls post hoc test revealed that PB group spent more time in the home middle chamber than MS and MM groups (both $p < 0.05$); no significant difference was revealed in terms of the time allocation in the unfamiliar middle chamber. The results for the comparisons between the two environments in PB, MS and MM groups can be found above and are shown in Fig. 3.

4. Discussion

Our study revealed that the rewarding effects of T-pulses that elicit location preferences (CPPs) in male California mice can be modulated by the physical environment (home versus unfamiliar environment) and whether males are bonded, housed with siblings or isolated. We previously demonstrated that pair-bond formation can dampen males' responses to the rewarding effect of T by preventing T-induced CPPs to an unfamiliar environment (Zhao and Marler, 2014). Our current study reinforces this notion, and adds a new dimension showing that presence or absence of residency further modulates the ability of T to produce CPPs. These findings help us to understand the natural functions of the reinforcing effects of rapid pulses of T and how experience can induce plasticity in the development of a behavior. The variation in CPP development in response to T may reflect the natural functions of T's rewarding effects in different development stages and environments and the value of resources associated with the environment. In PB males, T-induced CPPs only occurred in the environment wherein the social/sexual experience was accrued and residency status achieved, but not in an unfamiliar environment. Through the location preferences, T may further promote site-specific social behaviors such as territorial defense, mate-guarding behavior and parental care. In comparison, the absence of cues associated with residency and the female mate may make the unfamiliar middle chamber less salient and inhibit male responses to the rewarding properties of T in areas without an immediate link to male reproduction success. In contrast, CPPs to the home environment did not develop in resident males without pair bonds (MM and MS groups); residency alone is therefore not sufficient for development of T-induced CPPs to the home site. Such experience-dependent effects of T are also supported by the finding that the novelty-seeking tendency is reversed by T in the PB males, whereas it is amplified in MS and MM males. Pair bonding may therefore alter physiological and/or neurobiological substrates controlling responsiveness, specifically, the ability of T to produce CPPs to a familiar environment. Our results parallel a study in Titi Monkey (*Callicebus cupreus*), in which males paired with females in the home cage formed a greater preference for a rewarding sweet substance compared to males living alone or housed with their natal groups (Ragen et al., 2012). Pair bonding can therefore change the rewarding attributes of a resource, allowing the home chamber to become the focal point of attention and defense.

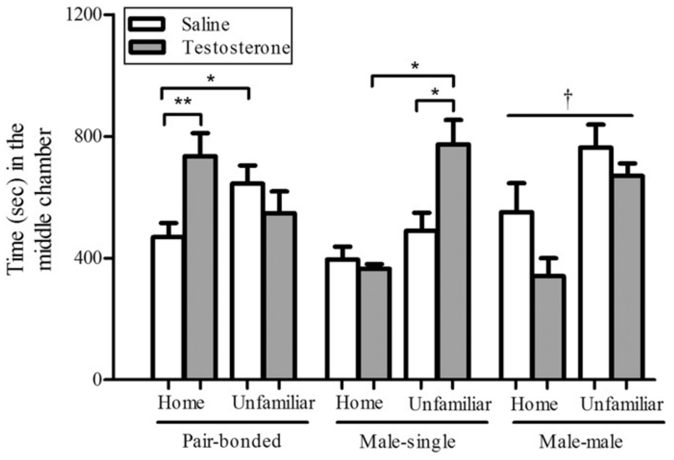

Fig. 3. Mean time (±SEM) spent in the middle chamber (home vs. unfamiliar) of the CPP apparatus for pair-bonded, sexually naive single males (male-single) and sexually naive males with another male cage mate (male-male). $^*p < 0.05$ and $^{**}p < 0.01$ independent *t*-test. $^\dagger p < 0.05$ main effects for two-way (environment × treatment) ANOVA. $N = 8–10$ in each group.

Resident males without a mate (MS) but separated from their natal environment needed an unfamiliar environment for T to induce CPPs. We speculate that T-induced CPPs may function to expand a territory by reinforcing the monopolization of novel resources, instead of defending the unchallenged resources they currently control. In nature, the ability to find resources is critical for survival and access to potential female mates (Ribble, 1992); T-induced CPPs may reinforce such ability. In males housed with a male sibling (MM), the T-induced CPPs were prevented in both the home and unfamiliar middle chambers. We speculate that association of the home with same-sex siblings inhibited males' responses to the rewarding properties of T in the familiar middle chamber, leading to a different outcome from the MS group.

The lack of conditioning by MM males to the unfamiliar middle chamber was unexpected because we previously found that T produced CPPs to unfamiliar environments in males also housed with other same-sex siblings (Zhao and Marler, 2014). The previous study differs from the current study in three primary ways: (1) males were housed in a standard cage (48 × 27 × 16 cm) rather than in the middle chamber of the CPP apparatus, (2) males were conditioned to a side instead of a middle chamber in the same three-chambered apparatus and (3) there was a greater level of novelty and complexity in the side chamber, including a wire mesh wall and black and white stripes on the walls. In the current study, the middle chamber (where T-induced conditioning was tested) had the same three-dimensional configuration to their home cage even when it was "unfamiliar" because of the lack of their own odor and residency. In the previous study, the T-conditioning occurred in side chambers with the novel wire mesh wall and contrasting stripes thereby changing more novel visual cues and adding a three-dimensional active space (ability to climb up mesh). We speculate that in dispersing male California mice, T induces exploration of more novel environments. In the current study, MM males that received T compared to saline spent more time in the side chambers containing the novel items. In rats, T drives the exploration of novel environments (Hawley et al., 2013). Moreover, male spotted hyenas (*Crocuta crocuta*) (Holekamp and Smale, 1998) and European badgers (*Meles meles*) (Woodroffe et al., 1995) immigrants have higher T levels compared to males in the natal areas, suggesting that T may modulate dispersal behavior. Overall MM males may condition to unfamiliar environments only when there is a greater level of novelty or perhaps additional resources.

Both baseline changes and pulses of T can mediate social behaviors. For example, in birds, the long-term elevation of T increases behaviors associated with reproductive success, such as song rate (Silverin, 1980), mate guarding (Saino and Møller, 1995), territorial extension (Chandler et al., 1994) and defense (Hegner and Wingfield, 1987). In lizards, T-implanted males can spend more time patrolling their territories (Marler and Moore, 1989) and increase frequencies of male-female interactions following territorial encounters (Marler and Moore, 1991). As described by Wingfield and colleagues, baseline levels may be used to change seasonal shifts in behavior while pulses occur in response to current social interactions (Goymann et al., 2007). Such pulsatile T releases are hypothesized to help animals cope with the immediate situation that stimulate release (Nyby, 2008). In male California mice, T-pulses can rapidly influence social behavior in the form of ultrasonic vocalizations (Pultorak et al., 2015) but, in concert with winning experience, can also have longer-term, cumulative effects on behavior in the form of future aggression and winning behavior (Fuxjager et al., 2011b; Trainor et al., 2004), and as we show here, the development of CPPs. There are endogenous pulses of T that occur in response to male–male antagonistic encounters and male-female sexual encounters (review see Gleason et al., 2009). These behavioral and hormonal changes can induce males to differentially allocate time based on location of the encounter (Gleason et al., 2009); specifically, both aggressive and sexual encounters can induce CPPs (Bell et al., 2010; Martínez et al., 1995), and the T-pulses following these social encounters might mediate the CPPs for the location in which an encounter occurs (Marler et al., 2005). Therefore, the T-induced CPPs might influence territoriality by adjusting site preferences during instances of territory settlement or drive the male to allocate more time in an environment he is more likely to encounter a female.

Using the framework of the current study, T influenced the development of CPPs in males at least over two days. Whether there was a cumulative or rapid effect of T on CPPs is unknown. Repeated T pulses may allow males to temporarily adapt to changing social conditions that last more than one social interaction and to associate relevant stimuli in their environment. The current study reinforces this notion since T pulses can elicit CPPs that may then influence future social behaviors. At this stage the advantages of altering T pulses versus baseline levels are unclear but some costs of increase T and aggression may be avoided (Wingfield et al., 2001) and more transient changes in behavior may be supported.

Other studies provide insight into potential mechanisms underlying the plasticity in T's ability to induce CPPs. In California mice, winning male-male encounters (and experiencing natural T pulses) increases expression of androgen receptors (ARs) in brain areas associated with reward, the nucleus accumbens (NAcc) and ventral tegmental area (VTA), but only when males are in their home cage and not an unfamiliar cage (Fuxjager et al., 2010a). This links changes in ARs to both social experience and residency. We speculate that during the conditioning sessions in the current study, PB males experience elevated AR expression in the NAcc and VTA, which increases androgen sensitivity, and could thereby enhance the rewarding properties of T. Testosterone alone can increase AR receptors in other species (Burgess and Handa, 1993; Handa et al., 1996). Moreover, pair bonding experience can alter the neural dopamine system (Bell and Sisk, 2013; Packard et al., 1997) that can mediate the rewarding effects of T. In prairie voles, pair-bonding experience decreases NAcc D1 receptor binding which inhibits amphetamine-induced CPPs (Liu et al., 2011). Dopamine may also influence the presence or absence of CPP development. In addition, the mechanisms underlying the effects of T on CPPs in the current study could be functioning through either rapid effects or a cumulatively additive effect over the week-long injection phase. Likewise, the reinforcing effects of T may be mediated through its metabolites that can affect the NAcc dopamine system through effects on γ-aminobutyric acid (GABA)A/benzodiazepine receptor complexes (Rosellini et al., 2001). Moreover, in rats, mutations of classical ARs does not inhibit the development of dihydrotestosterone self-administration (Sato et al., 2010), suggesting some reinforcing effects of T can be mediated through the activation of non-classical receptors. Also, estrogen can be self-administered in male hamster and produce CPP in female rats, indicating the potential for involvement of aromatization in the development of CPP.

In summary, we demonstrated extensive plasticity in the ability of a steroid hormone to induce a classical behavior associated with addicting drugs, that of the formation of CPPs. Importantly both the social environment and establishment of residency can significantly impact CPPs in a manner that can be associated with natural variations in behavioral and physical contexts. It can further be extended into the natural functions of the reward system such as how changes in CPPs can influence social behaviors.

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