The Potential Role of Testosterone Pulses on Territoriality Establishment and Related Behavioral Decision-Making

By

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Abstract

Perhaps no animal behavior is more intuitive to humans than the territoriality which describes the inward compulsion in animate beings to possess and defend an exclusive area. Most species inhabit a fixed area for at least some portion of their lives and the ability to establish and defend such an area can be crucial for an individual's fitness. Territoriality is usually defined as the defense or maintenance of an area to the exclusion of others, typically same-sex conspecifics. Despite the wealth of empirical studies based on observations of animals that live in established territories, little is known about the processes by which animals establish territories. In my dissertation, I examined the novel hypothesis that conditioned place preference (CPP) induced by testosterone (T) pulses, which occurred in males in different species across taxa after male-male competitive encounters and male-female sexual encounters contribute to the establishment and modification of territoriality. The T-induced CPP may naturally motivate animals to allocate more time to specific locations in the environment where the T pulses occurred. Moreover, I proposed that this ability to form T-induced CPPs varies depending on social experience and location in the physical environment. To test this hypothesis, I used California mice (*Peromyscus californicus*), a monogamous species in which territoriality varies based on reproductive stages and environments. In Study 1, I created a behavioral paradigm showing that the CPP can be developed through T injections that mimic natural changes in T found in intact California mice after winning an aggressive encounter. In Study 2, I used this paradigm to examine the plasticity in the T-induced CPP. I found that the CPP to a novel environment was only produced when the male mouse was singly housed, but not in a male housed with a sibling or paired with a female; the pair-bonded male can form a CPP to an environment where the residency has been established. This plasticity in the T-induced CPP and potential aggressive motivation parallels the variation in natural territorial behaviors, such as the diminished territoriality in the natal area (group housed with siblings), increased motivation of territory establishment after dispersing from the natal area (singly housed) and enhanced territory defense after bonding with a female partner. By comparing group housed with singly housed unpaired male mice in Study 3, I further examined the

effects of CPP on aggressive motivation toward a novel male intruder and the associated neural changes. I found that through interactions with the T pulses, the experience of singly housed (mimic dispersing) not only facilitated the development of CPP but also enhanced the aggressive motivation towards a novel male intruder. On a neural level, I found that upregulation of androgen receptors in the preoptic area (POA) occurred after animals were singly housed, suggesting that preparation for responsiveness to T pulse after dispersal may be reflected by increased POA AR. Single housing also increased the synapsin/phosphorylated synapsin in the nucleus accumbens, ventral and dorsal hippocampus, suggesting that the synaptic plasticity in these regions may prepare males for reproduction, likely including the establishment of a territory. Altogether, these results suggest that the territoriality establishment/enhancement may be achieved by developing T-induced CPPs in appropriate contexts, and such a process may require neural changes that are associated with the social experience.

General Introduction

Territoriality, winner effect and testosterone

Perhaps no animal behavior is more intuitive to humans than the territoriality which describes the inward compulsion in animate beings to possess and defend an exclusive area. Most species inhabit a fixed area for at least some portion of their lives and the ability to establish and defend such an area can be immensely important to the overall fitness for many species (Greenwood 1980). Territoriality is usually defined as the defense or maintenance of an area to the exclusion of others, typically same-sex conspecifics (Maher and Lott 1995). The importance of territoriality is exemplified by its extensive emergence across a wide variety of animal taxa ranging from bacteria (Gibbs and Greenberg 2011) to humans (Dyson-Hudson and Smith 1978). A widely known and classical example is the domesticated dog (*Canis lupus familiaris*), as this species inherited a highly territorial disposition from its wild relative, the wolf (*Canis lupus*). Dogs show instantly recognizable hallmarks of territorial behavior, including scent marking around a home turf and barking at intruders that threaten to enter that area.

To date, mechanisms of territorial behaviors such as the indirect self-advertisement and direct, interactive aggressive behaviors, have been well demonstrated based on observations of animals that live in established territories (Maher and Lott 1995). However, as Maynard Smith (1982) stated in his seminal work *Evolution and the Theory of Games*, ‘‘Although much is known about how animals behave once territories are established, we know little about how those territories are established in the first place’’. For territorial species, territory is often acquired through either inheriting the natal territory, or dispersing and searching for new territories. On the behavioral level, most theoretical and empirical studies have assumed that a primary mechanism by which territoriality occurs is through winning an aggression over a space, so that the winner gains possession of the contested area, while the contest loser relinquishes it (Huntingford and Turner 1987; Krebs 1982; Smith and Parker 1976; Ydenberg et al. 1988).

One interesting behavioral phenomenon that reflect this notion is the winner effect, which is defined as an ability to win fights following the acquisition of prior social victories. Winner effects are

found in a wide variety of taxa, including mammals (Oyegbile and Marler 2005; Schwartzer et al. 2013), reptiles (Schuett 1997), birds (Apfelbeck et al. 2011), fish (Hsu and Wolf 1999), and invertebrates (Bergman and Moore 2003). The functional significance of the winner effects is currently still poorly understood. One potential function of the formation of the winner effects in nature may be to facilitate the establishment/enhancement of territoriality.

Given that territorial behavior is the expression of aggression to defend or maintain a physical space, it is unsurprising that context plays an enormous role in determining how and when territorial aggression is produced. The best example of this is the resident advantage, whereby the owner of a territory has an advantage over an intruder when competing in a territorial dispute. Noble (1939) observed that familiarity with an area gives residents a decided competitive boost over a newcomer. Braddock (1949) conducted the first empirical study with the Mexican platy fish (*Platypoecilus maculates*) to test the effect of residency versus body size on aggressive output; the resident experiences an advantage over the intruder, even if the resident is at a size disadvantage. Later studies using field observations and lab manipulations found that the residency advantage exists in a wide variety of taxa, including invertebrates (Takeuchi and Honda 2009), amphibians (Baugh and Forester 1994), reptiles (Olsson and Shine 2000), birds (Snell-Rood and Cristol 2005), and mammals (Corlatti et al. 2013). The development of the winner effects in pair-bonded male California mice is actually also relying on the context with the full winner effects only occurred in the place where the residency has established (Fuxjager and Marler 2010).

Testosterone (T) is the primary male sex hormone that is mainly synthesized and released from testicles in males. It is vital for development, maintaining sexual characteristics as well as expression of social behaviors. Several studies have revealed 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).

Short-term T-pulses can not only influence social behaviors, but also can be elicited following male-male agonistic encounter as well as male-female sexually encounter (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. 2005a).

Winning a male-male agonistic encounter elicits an increase in T in individuals across species and taxa (Elekonich and Wingfield 2000; Jasnow et al. 2000; Sperry et al. 2010; Trainor and Marler 2001). Most notably, individual male California mice that win fights in their home cage display a transient T pulse 45 minutes following their winning experience (Marler et al. 2005a; Oyegbile and Marler 2005). Such T-pulses are required to see an increase in aggressive behaviors in later fights following winning experiences. Castrated males that received T implants to maintain baseline levels of T but received post-victory saline showed no changes in future aggressive behavior. In addition, animals that received aromatase inhibitors, which prevent the conversion of T to estrogen, still display increased aggression in later fights, suggesting that this experience dependent system is also androgen dependent (Trainor et al. 2004). Moreover, if an individual does not win, or wins in an unfamiliar environment, the T-pulses following the agonistic encounters do not occur (Fuxjager and Marler 2010), suggesting that experience, environment, and hormonal mechanisms are required for the full formation of a winner effect.

Interestingly, in the non-territorial and polygamous white-footed mouse, winning experiences do not alter levels of T. This species difference in post-victory hormone changes accounts for the formation of the winner effect in California mice but not white-footed mice (*Peromyscus leucopus*) (Fuxjager and Marler 2010; Oyegbile and Marler 2006). However, exogenous post-victory T-pulses in white-footed mice induces a winner effect, thereby eliminating species differences in winner effect formation (Fuxjager et al. 2011b). This provides evidence that pulsatile testosterone cements the winning experience and creates the neural and psychological changes necessary for the winner effect to take hold. As such, one key to the formation of the winner effect may be the release or response to post-victory T.

Testosterone and the conditioned place preference

Conditioned place preference (CPP) is a form of stimulus-outcome learning that is commonly used to draw inferences about the motivational effects of psychoactive drugs (Tzschentke 1998). It is based on the observation that animals will learn to approach spatially distinct environmental cues that have previously been associated with rewarding drug effects. In general, place conditioning paradigm is widely used to measure the rewarding properties of drugs, such as psychostimulants and opiates, electrical self-stimulation of the brain and also natural rewards, such as sweet solutions or a sexual partner (Tzschentke 2007).

The general CPP procedure consists of three phases: Habituation, conditioning trials and post-conditioning test. One purpose of a habituation session is to familiarize animals with nonspecific stressful aspects of the procedure such as handling, transportation and the novelty of the apparatus in order to reduce the likelihood that such events will interfere with later learning about the cue-drug relationship. The second purpose of habituation is to assess animal's initial preference for the CS (e.g. side preference). During the conditioning phase, animals are given one or more exposure to each of the stimulus alternatives ('place'), preferably in a counterbalanced order. Based on the design (unbiased or biased), the initially non-preferred place or a randomly selected place is consistently paired with the rewarding drug (US), while the other place is consistently paired with vehicle. Consistent with the principles of Pavlovian conditioning, the strength of place conditioning is positively related to the total number of conditioning trials (Cunningham et al. 2002). In most conditioning studies, the preference test is conducted 24-hour or later after the last conditioning trial by offering the animal a choice between the 'places' that contain the drug-paired and vehicle-paired CSs. In the post-conditioning phase, animals are given the opportunity to choose between the two sets of environmental cues by moving between locations that contain those cues, typically in the absence of the conditioning drug. Greater approach and contact with the drug-paired cue are seen as evidence for a rewarding drug effect. Such preference has relevance for understanding the general phenomenon of "drug seeking".

The general assumption about the acquired preference is that it is based on the classical conditioning that derived from 'incentive motivation'. Huston et al. (2013) argued that this assumption may be an oversimplification of the multiple learning processes involved, and they proposed three potential mechanisms that may account for the development of CPP. The first hypothesis is the CPP is an incentive-driven behavior. After repeated associations with US, the CS (e.g. neutral environmental cues) could assume 'incentive value' of their own, which leads the organism to seek these out, or to prefer them. The second hypothesis is the operant conditioning of behavior prevailed at the conditioning site and caused preference. Although drugs are administered before CPP conditioning, their behavioral effects are usually expressed and perceived during and after place conditioning. When placed into the same environment, the animal is likely to re-engage in whatever behavior in that context. Consequently, it will increase the probability that the animal will remain in that place. The third hypothesis is the CPP is resulted from the conditioned treatment effect, which is that the stimulus paired with a drug-induced behavior can serve to elicit behavior very similar to that induced by the drug itself. My interpretation about this hypothesis is the CS is paired with the drug-induced behavior rather than the rewarding properties of the drug. Also, the animal's conditioned response may simply prevent it from leaving this particular environment. Presumably, all three processes could operate concurrently in a CPP paradigm and, thus, contribute additively or counteract one another.

De Beun et al. (1992) first used the conditioned place preference paradigm to demonstrate that 4-androsten-17β-ol-3-one-testosterone (subcutaneous injection) has rewarding effects. Later, a group led by Packard conducted a series of studies to demonstrate that in Long-Evans rats CPP can be developed via subcutaneous (Alexander et al. 1994) and intra-nucleus accumbens (Packard et al. 1997) injections of testosterone-hydroxypropyl-B-cyclodextrin inclusion complex, which mimics pulsatile release of the hormone; the T-induced CPP can be blocked by peripheral or intra-accumbens injection of dopmaine receptor antagonist, a-flupenthixol, suggesting the rewarding effects of T are mediated through the dopamine system (Packard et al. 1998). However, to question the associative effects of T in conditioning learning. Showing that the increased time spent in the putative conditioning side was also occurred in

Sprague-Dawley rats that received testosterone in the home cage 3 hours after removal from the conditioning context, which should preclude the formation of an association between context and drug exposure (Caldarone et al. 1996). Both dopamine type 1 and 2 receptor subtypes are involved in the acquisition of testosterone CPP (Schroeder and Packard 2000). Intra-medial preoptic area (MPOA) injections of T (0.1 μg) produced a CPP, while injections of a higher dose (0.2 μg) produced a conditioned place aversion (King et al. 1999). The rewarding effects of intra-MPOA T may in part modulate the motivation of sexual behavior. Frye et al. (2002) further investigated the affective hedonic properties of T metabolites, dihydrotestosterone (DHT) and 3-α diol in rats, and revealed that T as well as its metabolites, DHT and 3a -diol implants that administered to the shell of the nucleus accumbens (NAc), but not the core, conditioned a place preference to the nonpreferred side of the testing chamber and vehicle administration had no effect. Arnedo et al. (2000; 2002) investigated the CPP can only be produced in male OF-1 mice by pairing the 4-androsten-17β-ol-3-one-testosterone (dissolved in peanut oil; sc injection) with a black compartment, but not the white one. It worth note that the sample size is fairly small (two in each group) for the animals received T in black compartment, resulting in lower statistical power and effect size. Therefore, it is still unclear about the T-induced CPP in mice.

In the context of social behaviors, hormone systems typically work by acting within the central nervous system. In the brain, for instance, there is a relatively conserved group of interconnected nuclei consisting of overlapping social behavior and mesolimbic reward system neural networks that are highly sensitive to hormonal action, with some nuclei even releasing neurohormones on other parts of the brain to influence behavior (Goodson 2005; Goodson et al. 2005). These brain nuclei including NAc, ventral tegmental area (VTA), amygdala (AMY), hippocampus, bed nucleus of the stria terminalis (BNST), preoptic area (POA), anterior hypothalamus, ventromedial hypothalamus, and periaqueductal gray/central gray lateral septum, together referred to as social decision-making network, play important roles in evaluating the salience and valence of stimuli and controlling the output of most social behavior,

including adaptive aggression and territoriality (Goodson 2005; Newman 1999; O'Connell and Hofmann 2011).

It is thought that the relative activation of these network nodes influences the type of social behavior a given animal exhibits (i.e., social affiliation vs. territorial aggression). Research by Goodson et al. (2005) supports this idea by comparing activation patterns of regions within the social behavior network across avian species with different social and aggressive propensities. When placed with a same-sex conspecific, the territorial species shows a unique activational pattern throughout the network compared to a moderately gregarious species and a highly social (colonial) species. Indeed, these latter species also show their own unique patterns of activation in the network nodes according to their level of sociality. Hormone action comes into play with the social behavior network because it is thought to fine-tune these activation patterns (Maney et al. 2008).

Generally, T can influence behavior through multiple pathways. The first is through activation of androgen receptor (AR) which is described in mammals as a 110 kDa nuclear receptor (Chang 2012). Testosterone stimulation causes the release of AR from heat shock proteins in the cytoplasm after which the liganded AR translocates to the nucleus and binds to specific DNA sequences called androgen response elements (AREs). The binding of the steroid to its receptor produces conformational changes that result in the formation of a "transformed" or activated receptor that has high affinity for specific DNA-binding sites (Tsai and O'Malley 1994). When in the proper context, the AR bound to DNA recruits co-activators or co- repressors of gene expression (Bagchi et al. 1992). The second is through the activation of estrogen receptors, as T is converted to estradiol via the enzyme aromatase. Estrogen receptors can similarly behave as transcription factors, but can also induce rapid effects by initiating second messenger systems within the cellular apparatus (Cornil et al. 2012; Mangiamele and Thompson 2012). Androgen and estrogen receptors are linked to the control of territorial aggression in a variety of ways. Activation of ARs, for example, is often related to the execution of aggressive behavior, in that it influences variables such as attack frequency and attack duration (Juntti et al. 2010). These factors ultimately influence who wins the fight, meaning that ARs play an important role in modulating

aggression in adaptive contexts. Some of the brain regions known to respond to androgenic action via ARs and contribute to aggressive output include the BNST and the NAc: These brain areas are involved in the social behavior network and behavioral reward or reinforcement (Fuxjager et al. 2010a; O'Connell and Hofmann 2012).

Testosterone-pulses following winning experiences lead to changes in androgen receptor patterns across the brain. In the BNST androgen receptors are increased following victories either in the home cage or a novel cage (Fuxjager et al. 2010a). This indicates that the BNST is related to aggression and winning in general but not necessarily to territoriality. Androgen receptors in the NAc and the VTA increase following wins that occur in the home territory only (Fuxjager et al. 2010a), indicating that these brain areas are likely vital to forming the winner effect in the context of residency. Changes in progesterone receptors following aggression in male California mice were not seen, suggesting that progesterone receptors do not play a role in the formation of the winner effect in males. We speculate that, because the NAc and VTA are tied to functions in reward and reinforcement, increased androgen receptors in these two areas may act to increase the intrinsic reward of fighting, thus increasing the motivation to fight in the future following winning experiences.

For the neural substrate of CPPs, the mesolimbic system plays a critical role in evaluating the salience of external stimuli and processing reward. Earlier studies mainly used lesioning methods to investigate the brain regions involved in the place conditioning. As early as 1956, Weiskrantz suggested that the behavioral changes following amygdala lesions may indicate a role for the AMY in the association of environmental stimuli with a variety of biologically important aspects of events, thus mediating the impact of their reinforcing value (Weiskrantz 1956). A later study by Spiegler and Mishkin (1981) demonstrated a marked impairment in remembering stimulus-reward associations over short delays by monkeys with AMY lesions, which was not due to difficulties in object recognition. Everitt et al. (1991) further found that the lesions of the basolateral AMY and ventral striatum abolish the sucrose-induced CPP in rats. However, the two areas may serve different functions. They suggested that the two areas may form a limbic-motor interface with amygdala involved in the association of environmental

stimuli with reward, while the ventral striatum is involved in motor response output directed towards such stimuli.

It has been well established that the neural circuits involved with conditioning to context is dependent on the hippocampus. Holahan (2005) used a conditioned cue preference task in an open field to study the roles of the AMY and the hippocampus during different phases of appetitive information processing by transiently inactivating these structures by local infusion of muscimol ($GABA_A$ agonist) prior to training, after training and prior to testing. Different arms of a radial 8-arm maze were paired with food or no food, and untreated rats subsequently showed a preference for the food-paired over the non-food-paired arm. Muscimol injected into the hippocampus immediately after training, but not before training or test, blocked the conditioned preference. In comparison, muscimol injected into the amygdala before training or testing, but not after training, blocked the conditioned preference. A subsequent study by McDonald et al. (2010) found that inactivations of the dorsal hippocampus impaired expression of a learned response required for the, accurate performance of a spatial navigation (water maze) task but did not impair the learned response required for, the expression of a CPP. In contrast, inactivation of the AMY impaired the expression of a, previously acquired CPP but did not impair the expression of a learned spatial response required for, accurate performance of a spatial navigation task. Thus, the blocked conditioned preference observed in the 8-arm maze may be resulted from the impaired spatial navigation ability after the inactivation of hippocampus (Holahan 2005). These findings also suggest that AMY and hippocampus may have independent and complementary roles in appetitive information processing.

Hypothesis

Territoriality is immensely important to the overall fitness for many species; yet, we have not touched on the most immediate proximate mechanisms through which individuals gain and maintain territories. The goal of this work is to elucidate a potential role for T pulses in the territoriality establishment/enhancement. **My overarching hypothesis is the formation of a T-induced CPP**

facilitate the establishment/enhancement of territoriality. To test this hypothesis, I used California mice (*Peromyscus californicus*), a monogamous and territorial species.

In Chapter 1, I examined the development of a CPP that is induced by T injections, which produces a transient increase in plasma testosterone level that mimics natural changes in T found in intact California mice after winning an aggressive encounter (Oyegbile and Marler 2005; Trainor et al. 2004). To explore the effects of pair-bonding experience on animal's responses to T pulses, I also compared the development of CPP in both sexually naïve and pair-bonded male California mice.

In Chapter 2, I used the CPP paradigm created in Chapter 1 and further investigated the plasticity of T-induced CPPs based on the influence of residency combined with the social/sexual experience. According to findings of field studies and observations in laboratory setting, territoriality of male California mice is varied in different environment and reproductive stages. If the T-induced CPP contribute to the territoriality, it is speculated to show plasticity that parallels the variation in the territoriality.

In Chapter 3, I further explored the functions of the T-induced CPP in territory establishment and its underlying neural mechanisms. I replicated a previous CPP paradigm in which the T-induced CPP was produced in singly-housed male California mice and group-housed mice that did not form a CPP act as a comparison. After the CPP test, I measured the aggressive motivation toward a novel male intruder that was placed. I also measured the expression of androgen receptors and synaptic plasticity in the POA, NAc, BNST, AMY, dorsal and ventral hippocampus.

In Chapter 4, as a first step of understanding the functions of T pulses in influencing social decision-making, I measured the sexually naïve and pair-bonded males' approach behavior in a challenge/opportunity situation (a novel male versus a female in estrus stage) in Experiment 1. I speculated that T would enhance the approach to the novel male competitor and that such an effect would rely on the pair-bonding experience. In Experiment 2, I examined the change of social decision-making along repeated exposures to the challenge/opportunity situations. I speculated that the resident animals will initially approach the challenge (male side) which will predict future aggression. I also speculated

that acquiring the knowledge about the restrained male intruder behind a wire mesh will decrease the approach to the already controlled challenge when later exposure to the same situations.

Chapter 1

Pair bonding prevents reinforcing effects of testosterone in male California mice in an unfamiliar environment

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Abstract

Testosterone (T) can be released by stimuli such as social interactions, and thereby influence future social behaviors. Because the reinforcing effects of T can induce preferences for specific environmental locations, T has the potential to alter behavior through space use. In a monogamous species, this T pulse may contribute differently to space use in sexually naïve (SN) and pair-bonded (PB) males as SN males may be more likely to explore new areas to set up a territory than PB males that are more likely to defend an existing, established territory. In this study, we test for variation in T-driven space use by examining variation in the formation of conditioned place preferences (CPPs) in SN and PB male California mice. In the three-chambered CPP apparatus, subcutaneous administrations of physiological levels of T were used to repeatedly condition SN and PB males to a side chamber, which is an unfamiliar/neutral environment. The final tests revealed that T-induced CPPs in the side chamber are developed in SN, but not PB males. This study fills a gap in our knowledge about plasticity in the rewarding nature of T pulses, based on past social experience.

Key words: testosterone, reinforcing effects, conditioned place preference, pair bonding

Introduction

Reinforcing effects are elicited by the incentive properties of a stimulus and may have been evolutionarily important for survival, reproduction, and fitness. One mechanism through which reinforcing effects can be measured is the development of a preference for the location in which an

animal was exposed to a stimulus that, in turn, activates the internal reward systems. The strength of addicting and rewarding drugs can be measured by the development and strength of conditioned place preferences (CPPs). Testosterone is a natural hormone that elicits CPPs in rats (Alexander et al. 1994; Packard et al. 1997) and mice (Arnedo et al. 2000). In hamsters, T is voluntarily consumed through oral, intravenous, and intracerebroventricular self-administration (Johnson and Wood 2001; Wood 2002; Wood et al. 2004). Together these studies suggest that T has reinforcing effects. However, the natural functions of T's reinforcing effects are not well understood.

Testosterone release in a male animal usually occurs under two social contexts: male–male aggressive encounters and male–female sexual encounters (Gleason et al. 2009). Post-encounter T surges modulate many reproductively-related behaviors such as territorial defense, mate guarding and exploratory behaviors. The modulation of these behaviors can require an animal to adjust the space use by allocating more time to one location and less time to another. We therefore speculate a potential natural function of T's reinforcing effects is to influence an animals' space use. Specifically, the reinforcing effects of T facilitate the association of the environmental context with the stimulus that elicits the T pulses.

The pair bond 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 (Aragona et al. 2005; Insel et al. 1995). Pair-bonded (PB) male California mice (*Peromyscus californicus*), a monogamous and territorial species, dampen their scent-marking responses towards novel females (Becker et al. 2012). While some neural changes (i.e. dopamine system) associated with pair bond formation have been uncovered (Aragona et al. 2003; Gingrich et al. 2000), less is known about how the pair bonding experiences affect the reinforcing properties of hormones or neurochemicals. Formation of a pair bond in male prairie voles (*Microtus ochrogaster*) decreases the effect of the drug amphetamine on the formation of a CPP (Liu et al. 2011). This finding suggests that social experience can alter the reinforcing effects of other external and internal stimuli.

The present study investigated the influence of pair bonding experience on the reinforcing effects of T by testing T's ability to produce CPPs in PB and SN male California mice. Studies on wild California mice showed that SN males are more likely to explore unfamiliar areas to set up a territory than pair-bonded (PB) males, that are more likely to defend an existing, established territory (Ribble 1991; Ribble 1992). We predicted that the reinforcing effects of T pulses in an unfamiliar/neutral environment would be greater in SN males than in PB males, illustrating how pair bonding can alter the reinforcing effects of T.

Material and methods

Subjects

We used 48 male and 24 female *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 either the PB group (n =24) or the SN group (n = 24). For the PB group, each male was paired with a female 10 days before the CPP experiment. The paired male and female mice were huddling side-by-side after 24 hours of pairing, which is a well-accepted indicator of partner preference in monogamous prairie voles (*Microtus ochrogaster*) (Ahern et al. 2009; Liu et al. 2001; Williams et al. 1992). We did not record the mating behavior of paired animals, but under laboratory conditions, 50% of animals have mated after 10 days of pairing with a range from the first few days after introduction to at least 35 days (Gleason and Marler 2010). Pairs were always observed for compatibility and if fighting occurred then both the male and female were excluded from the experiment. The other 24 males (SN group) were sexually naive and housed in male-male groups (including two siblings) established after weaning (no wounds were observed on any of the males and no aggression was observed).

Testosterone dose

We used 36 μg/kg T-injections (T-cyclodextrin inclusion complex) because a previous study found that 36 μg /kg subcutaneous testosterone injection produces a transient increase in plasma T levels that is approximately 3-5 times higher than the baseline, and lasts for about 10 min (Trainor et al. 2004). While the dose in the current study is lower than those used to identify CPPs in rats and mice, it mimics natural changes in T found in intact California mice after winning an aggressive encounter (Oyegbile and Marler 2005). Intraperitoneal T injections of 36 μg/kg are enough to elicit an intermediate winner effect, and necessary for a full winner effect (Fuxjager et al. 2011a; Fuxjager et al. 2011b; Gleason et al. 2009; Trainor et al. 2004).

CPP apparatus and procedure

Conditioning took place in a large polycarbonate testing cage (91cm long x 46 cm wide x 43 cm high) divided into three equal chambers. The two side chambers were connected to the middle chamber by manually controlled, sliding guillotine doors. The walls of one side chamber were decorated by horizontal black-and-white stripes, whereas vertical black-and-white stripes were used for the other side chamber.

Our CPP procedure was modeled after a previous study (Bardo et al. 2001). On day 1, each male's initial compartment preference was tested (pretest). Following a 5 min habituation period in the middle chamber, we raised the doors 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. From days 2-7, males received a series of 45 min conditioning sessions, one session per day on six consecutive days. Saline or T conditioning sessions occurred on alternating days, beginning with the saline conditioning session on day 2. During the saline conditioning sessions (days 2, 4 and 6), all males were taken from their home cages and placed into their initially preferred compartments, where they were given a subcutaneous saline injection and isolated for 45 min. Animals were randomly assigned to either the T-group or the control group. During T-conditioning sessions (days 3, 5, and 7), mice in the T-group

were removed from their home cages, placed in the initially non-preferred compartments and administered subcutaneous T-injections (saline for the controls). The CPP apparatus was cleaned thoroughly with 50% ethanol and left to dry following each conditioning session and test. Twenty-four hours after the last conditioning session, mice were tested for their place preferences using the same procedure as in the pretest. During all conditioning sessions and tests, the female partner or male cage mate of the focal male was moved out of the testing room.

Data analysis

Two types of scores were used to assess whether the T-injection induced a CPP. The *preference score* was the time spent in the T-paired chamber divided by the sum of the time in the T-paired chamber plus the saline-paired chamber. The *difference score* was the time spent in the saline-paired chamber subtracted by time in the T-paired chamber. The difference score was used to confirm the production of CPP because the preference score may increase solely due to decreased time spent in the non-reinforced chamber (meanwhile more time spent in the middle chamber). Therefore, the formation of a CPP was defined as significant changes in both preference and difference scores. These two scores have been widely used in CPP studies (Bell et al. 2010; Martínez and Paredes 2001; Meerts and Clark 2007; Tenk et al. 2009). We tested normality using the Kolmogorov-Smirnov test. Groups were compared using paired t-tests to evaluate the changes in pre- and post-conditioning scores. Three mice were excluded from the statistical analysis because they either did not explore all three CPP chambers or were not compatible with the female partners.

Results

SN male California mice receiving saline injections did not form a CPP, whereas T-treated mice formed a CPP for the side chamber associated with the T-injections (Fig. 1 a). Preference scores during pretests and tests were not significantly different for the control group ($t_{(10)} = 0.873$, $p = 0.40$), but increased significantly for the T-group ($t_{(10)} = 2.229$, $p = 0.049$). Likewise, the difference scores of pretest

and test did not change significantly for the control group ($t_{(10)} = 1.634$, p = 0.13), but decreased significantly for the T-group ($t_{(10)} = 2.797$, p = 0.02).

In contrast, PB males did not show a CPP for the side chamber associated with the T-injections (Fig. 1 b). Paired t-tests showed that the preference scores did not change significantly for either the control group ($t_{(10)} = 0.504$, p = 0.63) or the T-group ($t_{(11)} = 0.349$, p = 0.73). Likewise, the difference scores did not decrease significantly for either the control ($t_{(10)} = 0.934$, p = 0.37) group or the T-group ($t_{(11)} = 1.386$, p = 0.19).

Discussion

Using a classical design for examining reinforcing effects of drugs, we demonstrate that subcutaneous administration of T (36 µg /kg) can produce CPPs to an unfamiliar/neutral environment in male California mice. This finding is consistent with previous studies showing that transient T pulses are reinforcing in rats, mice and hamsters (Alexander et al. 1994; Arnedo et al. 2000; Johnson and Wood 2001; Packard et al. 1997; Wood 2002; Wood et al. 2004). More importantly, we find that the reinforcing effects of T rely on the pair bonding status; T created CPPs for a previously neutral environment in SN males, but not PB males. To our knowledge, this is the first study demonstrating variation in T-induced CPPs based on social experience. The variation in T-induced CPPs in SN and PB males may reflect plasticity in the strength of the reinforcing effects of T based on social experience. This plasticity may further affect an animal's space use, which is related to different reproductive priorities. Before forming pair bonds, most SN males are usually motivated to disperse up to 80 meters and establish ownership of a territory (Ribble 1992). The T-induced CPPs observed in SN males may reinforce the allocation of time towards exploration of an unfamiliar environment (Hawley et al. 2013) and/or help to initiate territoriality in SN mice. In contrast, PB males have already established their own territories, where the interactions with the partner and familiarity with the environment may increase the salience of the territory. The importance of the territory to PB California mice has been revealed in other studies; the winner effect (increased ability to win based on previous wins) developed in the home cage is later expressed in the

home cage but not in an unfamiliar cage (Fuxjager and Marler 2010; Fuxjager et al. 2009). Owing to the higher salience of the territory over a neutral environment, PB males may decrease time spent in a neutral environment but increase the time spent close to their mates, which might be the mechanism of maintaining sexual fidelity and reducing the risk of extra-pair copulations (Gubernick and Nordby 1993). Moreover, the T pulses induced by the interactions with female partners may also help enhance the proximity between pair-bonded animals (Gleason and Marler 2012) and further block formation of CPPs in an unfamiliar/neutral environment. The variation in T functions between SN and PB males may underlie the plasticity of behavioral reinforcement in the neutral environment and orient males to allocate more time in the salient environmental cues in which the display of T-related behaviors are important for individual fitness.

The potential of T to influence the space use to reproductive effort appears to be mediated by long term changes in T as well as short term T pulsatile changes as described in our study. In birds, the long-term elevation of T increase 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). Male dark-eyed juncos (*Junco hyemalis*) implanted with T tend to increase the allocation of time to locomotion and foraging, and decrease the time allocated to sleeping and preening (Lynn et al. 2000). In spiny lizards (*Sceloporus jarrovi*), T-implanted males spend more time in territorial defense during the day (Marler and Moore 1989) and increased frequencies of male-female interactions following a territorial encounter with an introduced male (Marler and Moore 1991). The change of the above reproductively associated-behaviors requires animals to allocate more time to salient locations such as the territory and the nest.

As a complement to previous studies using T implants, our result indicates that a few T pulses may also impact time allocation to different environments. Such pulsatile T releases are hypothesized to help animals cope with the immediate situation that stimulated release (Nyby 2008). The T pulse that occurs after either male–male antagonistic encounters or male–female sexual encounters may induce males to differentially allocate time based on location of the encounter (Gleason et al. 2009). Aggressive

encounters can induce CPPs *per se* (Farrell and Wilczynski 2006; Martínez et al. 1995), and the T pulses following a fight might mediate the CPPs for the location in which a given encounter occurs (Marler et al. 2005b). Therefore, the T-induced CPPs might influence territoriality by adjusting site preferences during instances of territory settlement. Moreover, the T pulses after the sexual encounters may drive the male to allocate more time in the environment in which the possibility of encountering a female is higher.

The focus in this study is on pulses of T, however, we cannot rule out the possibility that the baseline T levels are dissimilar between sexually naïve and pair-bonded males since levels were not measured, but consider it unlikely because the baseline levels of T are not significantly different between sexually naïve and pair-bonded male California mice that have not had pups (Gubernick and Nelson 1989). Also, in California mice, the T levels are characterized by being high during the first 24 hours after a pair is introduced and fall back to baseline by three weeks (Gleason and Marler 2010).

On a neurophysiological level, pair bonding experience may affect T's ability to produce CPPs by altering the dopamine system. In monogamous prairie voles, the pair bonding experience itself elevates nucleus accumbens dopamine receptor binding (Aragona et al. 2005). Further, such experience decreased the reinforcing properties of amphetamine by decreasing the effect of amphetamine on dopamine-1 receptor binding but not amphetamine-induced dopamine release or metabolism (Liu et al. 2011). The same effects of pair bond formation on amphetamine may be applied to T, which also activates the dopamine system to induce its reinforcing effects. While it is not possible to identify whether these effects are occurring rapidly via the final T injection or a cumulatively additive effect over the week long injection, it is suggested that the reinforcing effects of T can be mediated through some of its metabolites that can affect the nucleus accumbens dopamine system through effects on γ-aminobutyric acid (GABA)A/benzodiazepine receptor complexes (Nyby 2008). Also, a study in rats showed that the mutation of classical ARs does not inhibit the development of dihydrotestosterone self-administration (Sato et al. 2010), suggesting the reinforcing effects of T may be mediated through the activation of non-classical receptors. Future research will be needed to reveal the expression changes of ARs and also other

neurophysiological alterations that occurred after forming the pair bond, and how these alterations contribute to the decreased reinforcing effects of T in the neutral environment.

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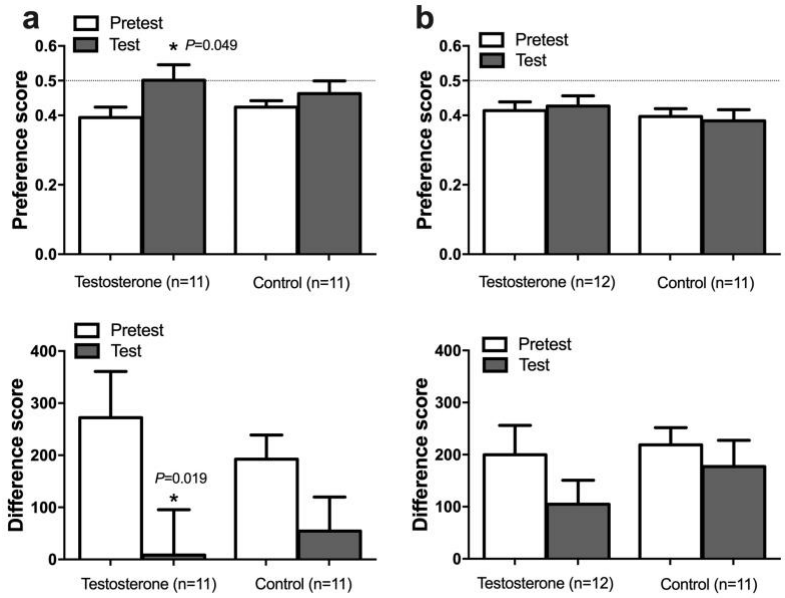

Figure.1 Preference (top) and difference scores (bottom) on pretests (white bars) and tests (gray bars) for control and testosterone groups of sexual naïve males (a) and pair-bonded males (b). Asterisks indicate a significant change in preference and difference scores between pretest and test for the testosterone group ($p< 0.05$). Data are mean ± SEM.

Chapter 2

Social and Physical Environments as a Source of Individual Variation in the Rewarding Effects of Testosterone

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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.

Key words: testosterone, reinforcing effects, territory, conditioned place preference, pair bonding

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 encounter as well as male-female sexually encounter (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. 2005a). 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). 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 (Kelm-Nelson et al. 2012; Riters et al. 2014), green anole lizards (Farrell and Wilczynski 2006) and gilthead sea bream (*Sparus aurata*) (Millot et al. 2014) demonstrate that animals can associate natural rewards such as foods and the social/sexual experience 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.

Methods

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 hours 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).

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 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 T found in intact California mice after winning an aggressive encounter (Oyegbile and Marler 2005) and male-female encounters (X. Zhao & CA. Marler, unpublished data); 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 animals 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 animals constituted the controls and received injections of saline (saline group).

CPP apparatus and procedure

Conditioning took place in large polycarbonate testing cages (91cm long x 46 cm wide x 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 hours) 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).

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 mice 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.

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 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). Two outliers (one from MS and one from MM) were removed based on the ESD outlier test. Seven mice were excluded from the statistical analysis because they either did not explore all three CPP chambers or were not compatible with their female partners.

Results

The three-way ANOVA analysis revealed a significant interaction ($F_{(2,96)}$=7.12, p<0.01) with a large effect size (partial eta-squared = 0.13) 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) with a large effect size (partial eta-squared = 0.19). 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; large effect size, 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; large effect size, cohen's d=1.07), potentially reflecting the novelty-seeking tendency of animals (further supported by the ANOVA results in the next paragraph). In the T group, there was only a nonsignificant trend in the opposite direction (t_{17}=1.79, p=0.09), but a large effect size (cohen's d=0.82) was revealed (Cohen 1977); 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 (Ruscio 2008). 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) with a large effect size (partial eta-squared = 0.21). CPPs, however, were formed only in the unfamiliar environment; T-injected males spent significantly more time than saline-injected in the middle chamber in the unfamiliar environment (t_{15}=2.79, p<0.05, cohen's d=1.37), but not in the familiar environment (p=0.51, cohen's d=0.34). Such environment-dependent effects of T were further

supported by the independent t-test results described above for the comparison between the two environments in T and saline groups; the time spent in 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; large effect size, partial eta-squared = 0.3) and treatment ($F_{(1,33)}$=4.44, p<0.05; large effect size, partial eta-squared = 0.12), but no interaction (p=0.43; small effect size, partial eta-squared = 0.02). 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, 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. 1).

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, partial eta-squared = 0.01), but a main effect was found for the environment ($F_{(1,47)}$=8.45, p<0.01; large effect size, partial eta-squared = 0.15). 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; large effect size, partial eta-squared = 0.35). 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 figure 3.

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 animals' 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 mice, 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.

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 are critical for survival and access to potential female mates (Ribble 1992); T-induced CPPs may reinforce such an 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. 2005a). 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 at least over two days. Whether there was a cumulative or rapid effect of T on CPPs is unknown. Repeated T pulses may

allow animals 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 (NAc) 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 NAc 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). In addition, pair bonding experience can alter the neural dopamine system that can mediate the rewarding effects of T (Bell and Sisk 2013; Packard et al. 1997). In prairie voles, pair-bonding experience decreases NAc D1 receptor binding which inhibits amphetamine-induced CPPs (Liu et al. 2011). Dopamine may also influence the presence or absence of CPP development. The mechanisms underlying the effects of T on CPPs in the current study could also be functioning through either rapid effects or a cumulatively additive effect over the week-long injection phase. The reinforcing effects of T also may be mediated through its metabolites that can affect the NAc 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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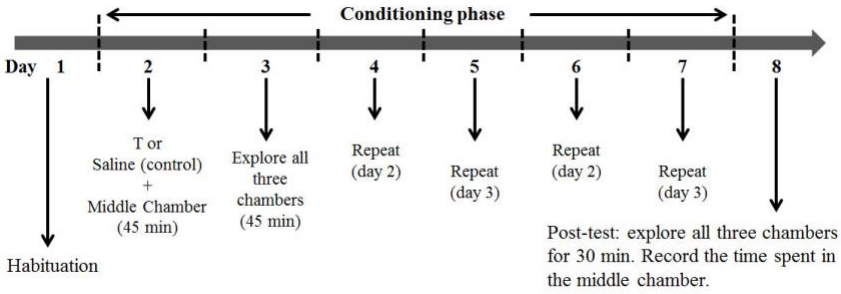

Figure 1. Experimental Timeline. The CPP procedure consists of three phases: habituation (day 1), conditioning (day 2-7) and post-test (day 8). During the conditioning phase, the focal males received either T or saline (control), which was conditioned with either home or unfamiliar middle chambers for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

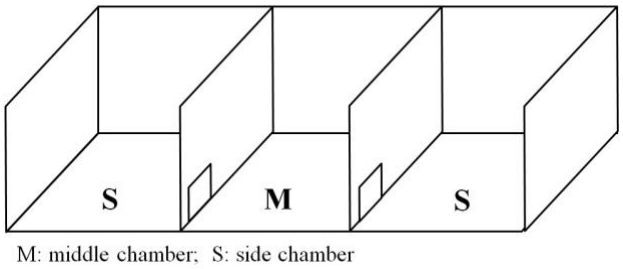

Figure 2. CPP apparatus is divided into three equal chambers. The two side chambers were connected to the middle chamber by manually controlled, sliding guillotine doors.

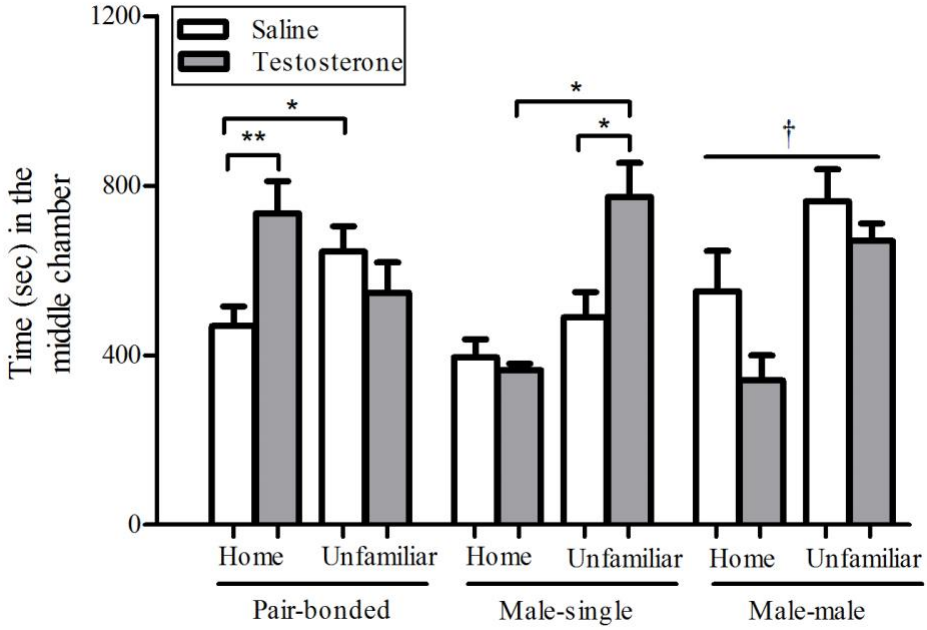

Figure. 3 Mean time (± SEM) spent in the middle chamber (home vs. unfamiliar) of the conditioned place preference apparatus for pair-bonded, sexually naive single males (male-single) and sexually naive males with another male cage mate (male-male). * $P < 0.05$ and ** $P < 0.01$ independent t test. † $P<0.05$ main effects for two-way (environment × treatment) ANOVA. N=8-10 in each group.

Chapter 3

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

Abstract

Despite the wealth of empirical studies on the territorial behavior, little is known about the processes by which animals establish territories. We speculate that the formation of conditioned place preferences (CPP), an increased time allocation to the environment where the 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 that the T-induced CPPs developed in singly-housed, but not group-housed males. This parallels the natural transition of dispersal from the natal area (group-housed), to becoming motivated to explore novel environments alone (singly-housed) and establish territories. In this study, we further examined the functions of the T-induced CPP in modulating aggressive motivation and the neural changes that may contribute to territoriality establishment during the transition period. Our results revealed that T pulses interact with the single housing experience and appears to enhance approach towards an intruder male. T pulses also increased aggression although independently of location. On a neural level, the single housing experience upregulates expression of androgen receptors in the preoptic area, which may facilitate the T-induced CPP. This is supported by a positive association between androgen receptors in the preoptic region and conditioned location preferences. Our results suggest that preparation for responsiveness to T changes after dispersal may reflect increased androgen receptors in brain regions such as the preoptic area, which may mediate the formation of CPP and enhance aggressive motivation towards a novel male intruder. Also, synaptic plasticity in the nucleus accumbens (NAc), ventral hippocampus (vHIP) and dorsal hippocampus (dHIP) is increased by single housing. These neural changes are likely associated with dispersal, territorial establishment and/or preparation for reproductive opportunities in male California mice. Our findings suggest that a series of behavioral and neural changes occur when males disperse,

including the upregulation of androgen receptors in the preoptic area, that may be involved in the life history transition from residing in the natal territory to establishment of their own territories.

Introduction

Territoriality is one of the most widespread behaviors among animal species (Burt 1943). It is usually defined as the defense or maintenance of an area to the exclusion of others and the ability to establish and defend such an area can be crucial for an individual's fitness (Maher and Lott 1995). To date, territoriality has been extensively studied from based on the behaviors displayed in an already settled territory, such as self-advertisement and direct, interactive aggressive behaviors (Fuxjager et al. 2017). However, as Maynard Smith (1982) stated in his seminal work *Evolution and the Theory of Games*, ''Although much is known about how animals behave once territories are established, we know little about how those territories are established in the first place''. For territorial species, the territory would be acquired through either inheriting the natal territory, or dispersing and searching for new territories. How dispersal facilitate the territoriality establishment in an originally unfamiliar space and how does the territoriality establishment enhance the defense behavior have always fascinated biologists but the underlying proximate mechanism remains largely unclear.

To explain how learning from the social experience contribute to the process of territoriality establishment, Stamps and Krishnan (1999) proposed a hypothesis that the territory is gradually formed via animals returning to the areas in which they previously had rewarding experience. This notion is supported by empirical evidence showing that aggression-related rewarding experience resulted in an enhanced preference for the site of previous aggression in green anole lizards (*Anolis carolinensis*) (Farrell and Wilczynski 2006). It also suggested that the formation of a conditioned place preference (CPP) is likely to be an important mechanism underlying the establishment of a territory. CPP refers to the development of a preference for the location in which an animal was exposed to a stimulus that activates the internal reward systems (Tzschentke 1998). From an ethological perspective, CPPs may allow animals to associate a rewarding experience (e.g., winning a fight or mating) with the specific

environment and motivates animals to allocate more time to that environment, which commonly occurs in the beginning of the territory establishment (Tirpak 2007; White and Harris 1994).

Testosterone (T) can elicit a CPP (Alexander et al. 1994; Packard et al. 1997) and plays an important role in the underlying hormonal mechanisms of territory establishment as it is important for the modulation of many territorial behaviors (Chandler et al. 1994; Enstrom et al. 1997; Lynn et al. 2000; Saino and Møller 1995; Silverin 1980). Working with California mice (*Peromyscus californicus*), a territorial and strictly monogamous species, we previously demonstrated that T injections designed to mimic natural T pulses found in intact California mice after winning male-male aggressive encounter (Marler et al. 2005a; Oyegbile and Marler 2005) and male-female encounters (Zhao & Marler, unpublished data), can produce CPPs in this species (Zhao and Marler 2014). Furthermore, we revealed that the development of T-induced place preferences shows plasticity: a CPP to a novel environment was only produced when males were singly housed, but not when males were housed with a sibling or paired with a female (Zhao and Marler 2016). This enhanced CPP development in males after being singly housed parallels the increased motivation of territory establishment after dispersing from the natal area. We hypothesized that social experience such as singly housing may thus facilitate territoriality by affecting the T-induced CPP in the appropriate environment. Yet, it is unclear if a T-induced CPP in singly housed males only increases time allocation to an area or, since aggression towards an intruder usually increases with territory establishment (Fuxjager et al. 2010a), if it also increases aggressive motivation towards an intruder in the preferred place.

Despite what is known about hormonal mechanisms, there is a dearth of research on the neural substrates for territoriality. For territorial and monogamous species (e.g., California mice and prairie voles, *Microtus ochrogaster*), the transition from living with parents and/or siblings in the natal area to living with a pair-bonded partner and defending a territory may require the experience of dispersing to a novel area and exploring that environment alone, in the absence of their natal group (Getz et al. 2003; Getz et al. 1994; Ribble 1992). Here we explore some of the neural changes that might occur during the above transition. We speculate that these changes may facilitate the development of a T-induced CPP

and/or the enhanced aggressive motivation in the preferred place ("territory"). Previous studies have shown that microinjections of T into the preoptic area (POA) and the nucleus accumbens (NAc) are sufficient to induce a CPP (Frye et al. 2002; King et al. 1999; Packard et al. 1997), indicating a potential role for these two areas in mediating T's reinforcing effects. The hippocampus has been implicated in facilitating the CPP formation (Ferbinteanu and McDonald 2001) and administration of T into hippocampus influences spatial learning and memory abilities (Babanejad et al. 2012; Hodgson et al. 2008). T can regulate the expression of target genes through binding to the intracellular androgen receptor (AR) (Rahman and Christian 2007) and may be the mechanism through which housing singly enhanced T's ability to induce the formation of a CPP. The regulation of AR expression has been implicated in mediating the experience-dependent behavioral plasticity (Trainor et al., 2004). For example, in California mice, winning male-male encounters (and experiencing natural T pulses) in the home cage increases AR expression in brain areas associated with reward (Fuxjager et al., 2010).

To further explore the functions of the T-induced CPP in territory establishment and its underlying neural mechanisms, we replicate a previous CPP paradigm in which the T-induced CPP was produced in singly-housed male California mice and group-housed mice that did not form a CPP act as a comparison. To test the effect of the CPP on aggressive motivation, we measured the latency to approach and the time interacting with a male intruder. We predicted that in the environment where the CPP was formed, the focal male would approach the novel intruder faster and spend more time interacting with the intruder behind a wire mesh, which were reported to be positively correlated with aggressive motivation to an novel male intruder in territorial species (Kudryavtseva 2003; Smagin et al. 2015). On the neural level, we measured the expression of AR in the areas implicated in processing reward, memory and social behaviors. These areas include the POA, the NAc, the bed nucleus of the stria terminalis (BNST), the amygdala (AMY), and the dorsal and ventral hippocampus. Also, the levels of synapsin and phosphorylated synapsin (phos-synapsin) were measured in the above regions. Synapsin and its activated form, phos-synapsin play important roles in synaptic plasticity and neurotransmission (Cesca et al. 2010), making them a useful protein marker of synaptogenesis and the activity of neurotransmitter release.

Methods

Subjects

We used 60 male *P. californicus* aged 6-12 months. They were reared in a laboratory colony at the University of Wisconsin (Madison, WI). After weaning, subjects were housed with two same-sex siblings or non-siblings within approximately one week of each other in small cages for one month and then moved to standard cages (48×27×16 cm L x W x H) under a 10L: 14D light cycle with lights off at 01:30 pm. Purina 5001 mouse chow and water were given ad libitum. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and were in adherence with the University of Wisconsin-Madison Research Animal Resource Committee (RARC) and Institutional Animal Care and Use Committee (IACUC; L0021-0-03-10). Males were randomly assigned to either group housed (n = 24), or singly housed (n = 24). For the group housed animals, focal males were housed with two other males since weaning to mimic the natal environment. For the singly housed animals, a randomly selected sexually naive male was separated from its cage mate and was singly-housed for three days before the start of the CPP pre-test. A separate group of singly housed males (n=12) were used as stimulus "intruders" to assess whether the enhanced aggressive motivation is dependent on the location of drug administration.

Testosterone treatment

We used 36 μg/kg T-injections (T-cyclodextrin inclusion complex) because in a previous study this injection 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 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 (Alexander et al. 1994; De Beun et al. 1992), it mimics natural changes in T found in intact male California mice after winning a male-male aggressive encounter (Oyegbile and Marler 2005) and male-female sexual encounters (Zhao and Marler, unpublished data). 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 animals were randomly selected to receive T-injections during the conditioning phase (T-group). As T-cyclodextrin was dissolved in saline, the controls received injections of saline (saline group).

CPP apparatus

Conditioning took place in large polycarbonate testing cages (90 x 46 x 43 cm, L x W x H) divided into three equal chambers (30 x 46 x 43 cm, L x W x H). The two side chambers were connected to the middle chamber by manually controlled, sliding guillotine doors. The outer walls of each side chamber was decorated by horizontal or vertical black-and-white stripes. Within each side chamber, a mesh wall (30 x 43 cm) extending from the floor to the ceiling formed a small enclosure (30 x 13 x 43 cm, L x W x H) behind which a stimulus male could be placed thereby limiting physical interaction with the subject but presumably not inhibiting auditory or olfactory communication.

CPP procedure

The CPP procedure was conducted over eight days (see (Zhao and Marler, 2014)). On day 1, each male's initial chamber preference was tested (pre-conditioning test). Following a 5-min habituation period in the middle chamber, we raised the doors 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. From days 2-7, males received a series of 45 min conditioning sessions, one session per day on six consecutive days. On days 2, 4 and 6, each male was removed from his home cage and placed into the chamber that he initially preferred (CS- chamber) during the pre-conditioning test, where the male was given a subcutaneous saline injection and allowed to explore the chamber alone for 45 min. On days 3, 5, and 7, males in the T-group were removed from their home cages, placed in the initially non-preferred compartments (CS+ chamber) and administered subcutaneous T-injections while controls received subcutaneous saline injections. Twenty-four hours after the last conditioning session, males were tested for their place preferences in a post-conditioning test using the same procedure as in the pre-

conditioning test. During all conditioning sessions and tests, the male cage mates of the focal male was moved out of the testing room. After the 30-min test concluded, the entrance to the two side chambers were closed and the activity of the focal male was restricted to the middle chamber. A novel conspecific stimulus male was then placed in the small enclosure of the CS+ chamber (30 x 13 x 43 cm, L x W x H) behind a wire mesh. A 5-min interaction test was started by lifting the gate to the side chamber. The latency to approach the intruder (starting from lifting the gate to CS+ chamber to the first time the focal male contact the intruder), the time in the side chamber and the time spent interacting with the intruder (nose to nose and nose to anogenital sniffing) were recorded.

To assess if the increased aggressive motivation is relying on the place of preference (CS- vs. CS+ chamber), a separate group of 12 singly housed males underwent the CPP conditioning with T injections (the same procedure as above). After the CPP post-conditioning test, half (n=6) of the animals were randomly selected and engaged in a 5-min interaction test in the CS+ chamber, while the remaining six males engaged in a 5-min interaction test in the CS- chamber.

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 defrosted to −11 °C and cut into 300-µm coronal sections using a cryostat. For each brain, micropunches of the NAc, POA and amygdala were collected using gauge#11 sample corer (Fine Science Tools, item No. 18035-02; diameter = 2 mm; 2 pellets for each region), and micropunches of BNST, dorsal hippocampus and ventral hippocampus were collected using gauge#11 sample corer (Fine Science Tools, item No. 18035-01, diameter = 1 mm; 4 pellets for each region). All samples were immediately stored at -80 °C.

Tissue was then homogenized after adding 300 µl of ice-cold lysis buffer consisting of 50 mM Tris-HCl, 1% Na-deoxycholate, 0.25% 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 concentration was determined by 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), phos-synapsin (1:1000 dilution; catalog#2311S; cell signaling technology, Danvers, MA, USA). 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).

Data analysis

We used a preference score to assess whether the T-injection induced a CPP. The preference score has been widely used in CPP studies (Bell et al. 2010; Martínez and Paredes 2001; Meerts and Clark 2007; Tenk et al. 2009) and was computed as the time spent in the CS+ chamber divided by the sum of the time in the CS+ and CS- chambers. The formation of a CPP was defined as a significant increase in the preference score by an independent-sample t test. For testing the aggressive motivation, we used the latency to approach the intruder and time spent interacting with the intruder. Two-way ANOVAs (2×2)

were conducted to analyze the interaction between treatment (T vs. saline) and housing condition (singly housed vs. group housed). After a significant two-way interaction, we used independent t-tests to compare the two treatments (T vs. saline) in either the group housed or singly housed males. Pearson test was used to analyze the correlation between neural changes (AR, synapsin and phos-synapsin expression) and behavioral responses (preference score, latency to approach the intruder and the duration of interacting with the intruder).

Results

Behavioral test

Consistent with our previous findings, in singly-housed male California mice, preference scores during pre-conditioning tests and post-conditioning tests were not significantly different for the control group ($p > 0.05$), but increased significantly for the T-group after conditioning ($t_{(11)} = 2.758$, $p = 0.02$). In contrast, group-housed males did not show a CPP for the side chamber associated with the T-injections (CS+ chamber) ($p > 0.05$) (Fig. 3a). For the latency to approach the intruder during the 5-min interaction test, a two-way ANOVA revealed a significant interaction ($F_{(1,38)} = 4.28$, $p = 0.47$). Further independent t-tests revealed that the singly-housed males that received T, which is also the only group forming CPP, displayed significantly shorter latencies to approach a novel male intruder ($t_{(16)} = 2.331$, $p = 0.03$), while singly-housed males that received saline showed no difference ($p > 0.05$) (Fig. 3b). For the duration of interaction with the intruder during the 5-min interaction test, a two-way ANOVA revealed a significant effect of interaction between drug treatment and housing condition ($F_{(1,39)} = 7.703$, $p < 0.01$). Further independent t-tests revealed that the singly-housed males that received T, which is also the only group forming CPP, interacted with the intruder for longer periods of time ($t_{(10)} = 2.757$, $p = 0.01$), compared to the singly-housed males that received saline (Fig. 3c). The shorter latency to approach the intruder and longer duration of interacting with the intruder indicated a stronger aggressive motivation towards an unfamiliar male conspecific (Kudryavtseva 2003; Smagin et al. 2015).

In a separate group of 12 singly housed males that received T, the independent t-test did not reveal a significant difference between the behavioral responses in CS+ versus CS- chamber in terms of latency to approach (47.8 ± 24.04, n=5 vs. 34.6 ± 12.58, n=5) and the duration of interacting with the intruder (131.2 ± 34.71, n=5 vs. 126.8 ± 43.04, n=5) ($p > 0.05$). This suggests that the enhanced aggressive motivation is not dependent on the location of preference.

Neural changes

For the expression of AR, the two-way ANOVA did not reveal a significant interaction ($p > 0.05$) or main effect of treatment ($p > 0.05$) in the POA, but did reveal a significant main effect of housing condition, with the singly housed males expressing significantly higher AR expression than the group housed males ($F_{(1,22)}=10.953$, $p<0.01$) (Fig. 4a). Neither a significant interaction nor main effect was detected by ANOVA in NAc, AMY, BNST, dHIP or vHIP (all $p > 0.05$). In the singly housed males that received T (the group that formed a CPP), but not the other three groups, AR expression was positively correlated with the preference score for the location of drug administration ($R^2=0.578$, $p=0.028$) (Fig. 4b). Neither the latency to approach the intruder nor the duration of interaction with the intruder were correlated with the POA AR expression in the singly housed males that received T. When the data from all groups were combined, the AR expression was positively correlated with the duration of interaction with the intruder ($R^2=0.349$, $p<0.01$) (Fig. 4c). There were no significant effects of drug treatment, housing condition or interaction on AR expression in all other analyzed areas ($p>0.05$).

We also examined synapsin and phos-synapsin in the brain regions listed above. In the NAc, two-way ANOVA did not reveal a significant interaction ($p > 0.05$) or main effect of treatment for either the expression of synapsin or phos-synapsin ($p > 0.05$). A significant main effect of housing condition, however, was found, with the singly housed males exhibiting significantly higher levels of both synapsin ($F_{(1,19)} = 18.959$, $p <0.01$) (Fig. 5a) and phos-synapsin ($F_{(1,20)} = 5.466$, $p = 0.03$) (Fig. 5b) than the group housed animals. In the dHIP, two-way ANOVA did not reveal a significant interaction or main effect of treatment for the expression of phos-synapsin ($p > 0.05$), but a significant main effect of housing

condition was found, with the singly housed males significantly higher than the group housed males ($F_{(1,22)} = 6.819$, $p = 0.02$) (Fig. 5c). In the vHIP, two-way ANOVA revealed a significant interaction ($F_{(1,20)} = 4.662$, $p = 0.04$), but not any significant effects ($p > 0.05$). Further simple t-test showed that the singly-housed males that received T, again the group that formed the CPP, expressed a significantly higher level of phos-synapsin compared to the saline control in the vHIP (t (10) = 2.262, $p = 0.04$) (Fig. 5d). This difference was not significant in the group-housed males ($p > 0.05$), Neither a significant interaction nor main effect was detected by the ANOVA for the synapsin expression in either the dHIP or vHIP ($p > 0.05$). No significant effects were detected in all other brain regions including POA, AMY or BNST ($p > 0.05$).

Our analysis also revealed a positive correlation between the BNST synapsin level and the duration of interaction ($R^2 = 0.406$, $p < 0.01$), and a positive correlation between the AMY synapsin level and the duration of interaction ($R^2 = 0.784$, $p < 0.01$) (Fig. 6).

Discussion

The current study confirms that single housing facilitates T-induced CPP to an unfamiliar environment in sexually naïve males. Additionally, we reveal how single housing, by acting alone or by interacting with T pulses, changes the expression of ARs and neural markers associated with synaptic plasticity in regions of the social decision-making network. In a laboratory setting, short-term isolation may mimic the natural experience of exploring a novel environment after dispersal and reflect the transition to territorial defense of a defendable area with resources. Our results suggest that the experience of dispersal must interact with T's effects to promote the expression of CPP and aggressive motivation. The requirement of single housing and T pulses parallels the development of the winner effect which requires both the winning experience and associated T pulses. It is becoming clear that the effects of T pulses are highly dependent on social context. For territorial species, like California mice, we speculate that some of the neural changes in singly housed males may alter the response to T and upon the

activation of T pulses, the behavioral output will be manifested (e.g. establishing their own territory and acquiring a mate).

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 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 appears to be critical for the reproductive fitness of dispersed sexually naïve males. In nature, endogenous pulses of T could be elicited by either winning male–male antagonistic encounters or engaging in male-female sexual encounters (review see Gleason et al., 2009). Both aggressive and sexual encounters can induce CPPs (Bell et al., 2010; Martínez et al., 1995). We speculate that these rewarding experiences of social encounters may facilitate the territory establishment by reinforcing the monopolization of novel resources; and the CPP induced by the T-pulses following these social encounters 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 an environment where he is more likely to encounter a female or drive off a persistently intruding male. In comparison, the dampened CPP in group housed males may reflect an inhibited motivation of establishing 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).

In singly housed males, the effects of T pulses on aggressive motivation may be independent of the formation of a CPP. In the current study, we did not directly measure the physical aggressive behaviors between the focal male and the novel male intruder, but we assume based on our unpublished data showing that the resident males receiving a transient T increase will initiate attack once the mesh barrier between the two males has been removed (Zhao and Marler unpublished data; Chapter 5 of the dissertation). This also harmonizes with the notion of the "partition test" that shows that the time spent close to the partition with an intruder positively correlates with the measurements of aggressive motivation (Kudryavtseva 2003). Our results showed that only the singly housed males that received T,

but not saline or group housed, showed decreased latency to approach the intruder and increased duration of interaction with the intruder, indicating an enhanced aggressive motivation. However, when we further assessed if the enhanced aggressive motivation relies on the place of preference (CS- vs. CS+), our results demonstrated that the enhanced aggressive motivation may not rely on the environment where the place preference was formed. This suggests the formation of a CPP may not directly enhance the aggressive motivation, but mainly influences the time allocation to the place of drug administration.

Androgen signaling in the POA may play an important role in mediating the enhanced sensitivity to exogenous T injections. The POA is an area in the diencephalon that responds to sex steroids for the regulation of reproductive and aggressive behaviors (Hutchison, 1976; Panzica et al., 1996; Ball and Balthazart, 2002). The projections from the medial POA to the ventral tegmental area have been implicated in processing the reward and can be modulated by estrogen (McHenry et al. 2017). One potential mechanism through which the T pulses can enhance the rewarding processing is through enhancement of aromatase activity, which activates the projections from the POA to the ventral tegmental area via facilitation of the production of estrogen (Roselli and Resko 1984). This is supported by our result showing that in the singly housed males that received T, the individuals with higher level of AR expression in the POA have higher location preference scores. It would be interesting to examine the level of the aromatase activity in the POA in future studies. Across all four groups, the AR expression in the POA was also positively correlated with duration of interaction with the intruder. It is unlikely that the interaction with the intruder upregulated the AR expression because the brain tissue was collected immediately after the 5-min interaction test. We were not able to test the direct relationship between the POA AR and the aggressive behavior, but our results indicates a potential role for POA androgen signaling in mediating aggressive motivation, which seems to 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). Beyond that, the POA has been implicated in male-male aggression (Albert et al. 1986) and maternal care (Lee and Brown 2007). In male European starlings (*Sturnus vulgaris*), both preproenkephalin (PENK) and mu opioid receptor mRNA expression in the

medial POA correlated positively with both individual reward state (as reflected in CPP) and undirected singing behavior (Riters et al. 2014). Adding to these, our findings further revealed the potential role of the POA in mediating the establishment of a territory.

It is well established that NAc plays an important role in processing sensorimotor information that facilitates a favorable behavioral output of either approach or avoidance of a stimulus (O'Connell and Hofmann 2011). In the current study, we did not find any significant change in the levels of NAc AR, which initially seems to conflict with a previous study showing that winning male-male encounters (and experiencing natural T pulses) increases expression of AR in the NAc when the encounters occurred in a subject's home cage and not in an unfamiliar cage (Fuxjager et al., 2010). 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 males that we focus on here were sexually naïve, whereas the winner effect study used pair-bonded males (Fuxjager et al. 2010a). 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 home environment (Zhao and Marler 2014). It would be interesting to test the effects of T-induced CPP (to home) on the regulation of NAc AR in pair-bonded males. Taken together, our findings did not support the role of NAc AR in facilitating the production of CPP in singly housed males.

Our data also revealed the change in the synapsin system as a function of the T-induced CPP or singly housing experience. Synapsin is one of the most extensively characterized synaptic terminal proteins, and is known to play a major role in synaptic plasticity (Greengard, Valtorta et al. 1993). During an action potential, synapsins are phosphorylated by PKA (cAMP dependent protein kinase), releasing the synaptic vesicles and allowing them to move to the membrane and release their neurotransmitter (Cesca et al. 2010). Thus, the phosphorylation state of synapsin, rather than total amount of phos-synapsin and unphosphorylated synapsin was used as an indicator of neurotransmitter release. In the singly housed

males that received T, the increased phos-synapsin in the vHIP suggests that the activity of neurotransmitters in this region may be involved in the responses specific to the T-induced CPP. However, our correlation analysis did not reveal any significant relationship between the phos-synapsin level in the vHIP and the behavioral measurements, indicating that this region may be involved in functions other than directly influencing the formation of CPP or aggressive motivation. In the dHIP, the level of phos-synapsin, but not synapsin, increased after being singly housed. This effect is independent of the treatment. 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 rodents, the BNST and AMY play important roles in chemoinvestigatory behavior (Powers et al., 1987) and there are extensive connections between the two areas (Coolen and Wood, 1998; Price and Amaral, 1981). The synapsin levels in both the BNST and AMY were positively correlated with the duration of interaction, suggesting the synaptic plasticity in these two regions may contribute to the aggressive motivation in response to a novel intruder, as might be expected from other studies linking these brain areas to aggression (Karli et al. 1977; Shaikh et al. 1986; Sheehan et al. 2001).

In conclusion, our 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. This may act as a potential mechanism for the establishment of a territory. Besides social encounters, other factors such as exploring a novel resource rich environment may also influence T pulses and remains an explored research direction.

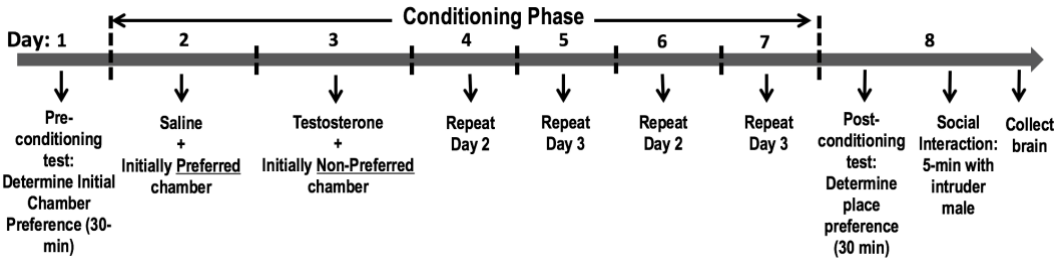

Figure 1. Diagram depicts time course and flow of experimental procedures

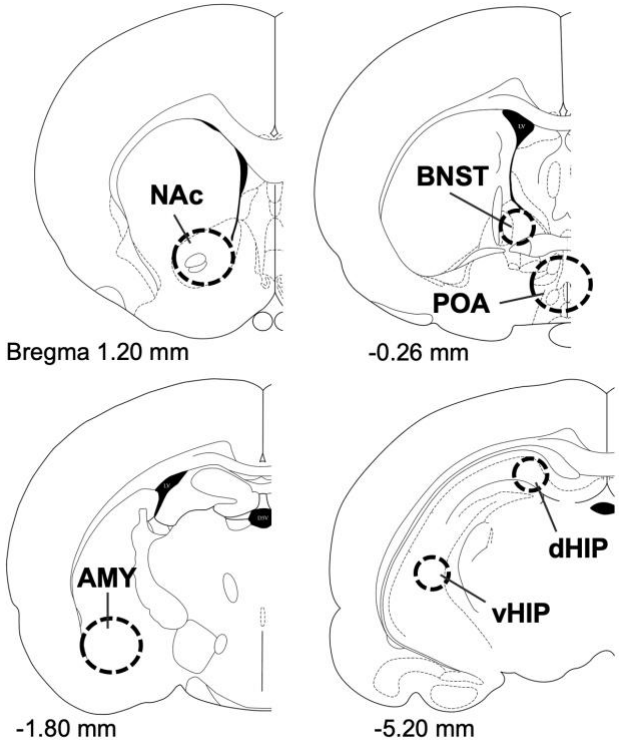

Figure 2. Coronal brain sections from which nucleus accumbens (NAc), bed nuclues of the stria terminalis (BNST), preoptic area (POA), amygdala (AMY), dorsal and ventral hippocampus (dHIP and vHIP) were collected. Stereotaxic coordinate of each section (bregma) is provided below each illustration. Circles depict areas of punches.

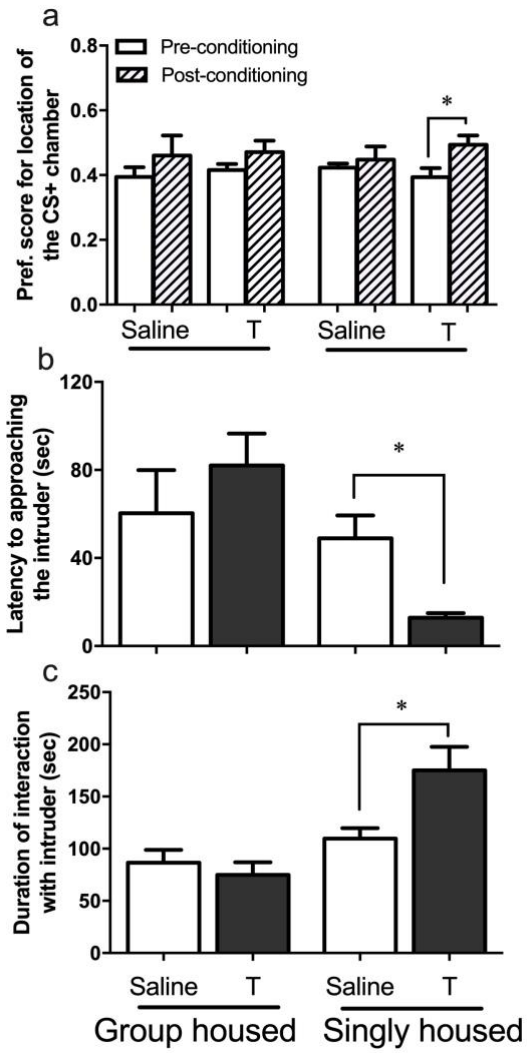

Figure 3. (a) Preference score during pre-conditioning tests (open bars) and post-conditioning tests (shaded bars), (b) the latency to approach the intruder and (c) the duration of interacting with the intruder for saline and testosterone (T) groups of group-housed (male-male) and singly-housed (male-single) male California mice. Data are mean ± SEM; * indicates significant difference at $P < 0.05$.

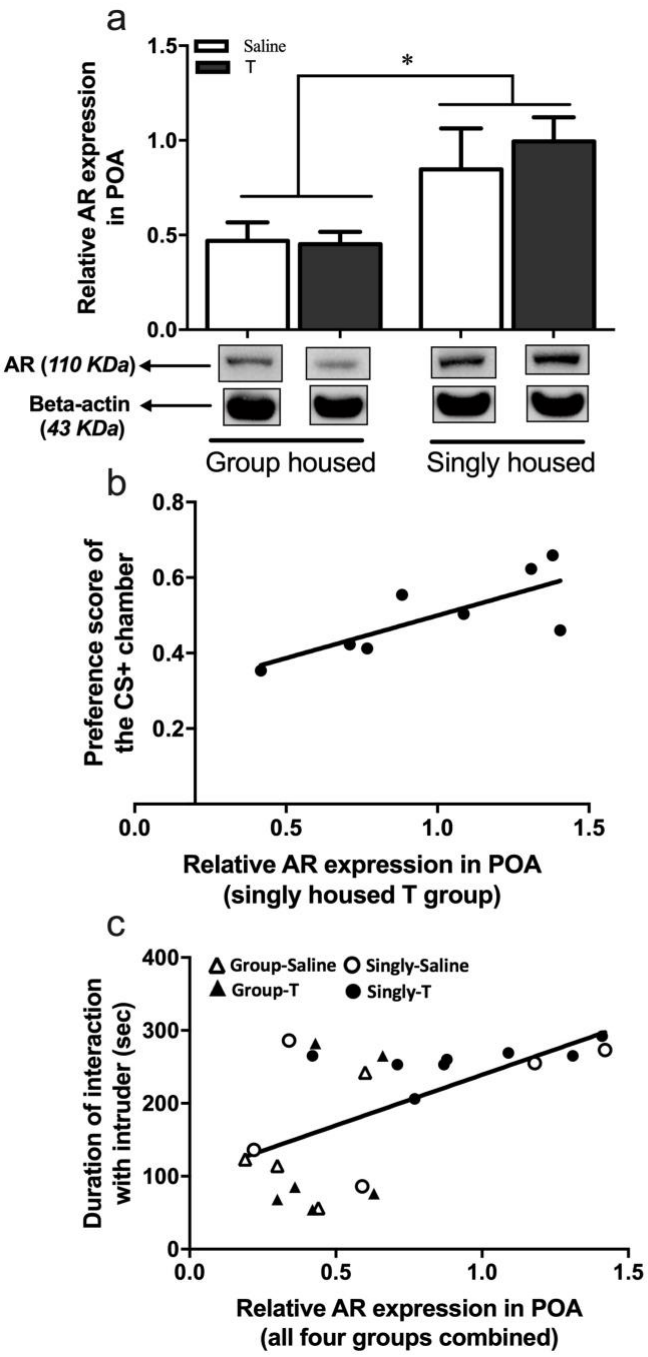

Figure 4. (a) Relative AR expression (AR/beta-actin) in the POA. (b) Correlation of the relative AR expression and the preference score of the CS+ chamber in singly housed males that received T. (c) Correlation of the relative AR expression and the duration of interaction with intruder when data from all four groups combined. Data are mean ± SEM; * indicates significant difference at $P < 0.05$.

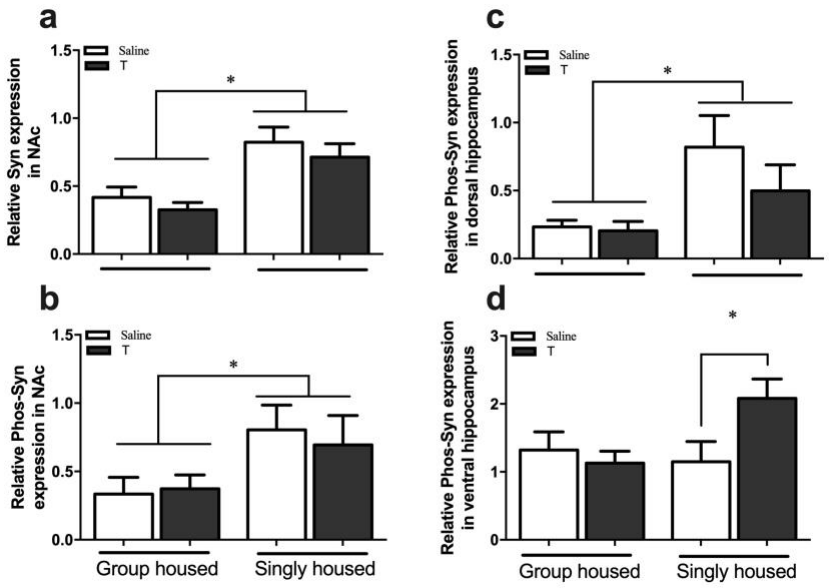

Figure 5. The expression of synapsin (Syn) in the NAc and the expression of phosphorylated synapsin (Phos-Syn) in the NAc, dHIP and vHIP. Data are mean ± SEM; * indicates significant difference at $P < 0.05$.

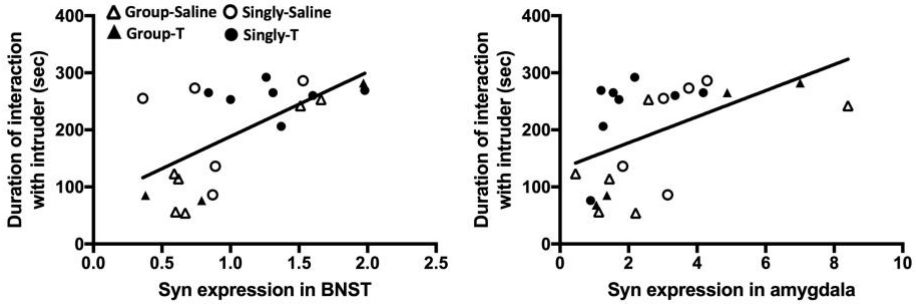

Figure 6. Correlation of the duration of interaction with intruder and the synapsin expression in BNST (left) and AMY (right).

Chapter 4

Rapid effects of testosterone on social decision-making in a monogamous rodent

Abstract

In social species, individuals must cope with challenges and opportunities by adjusting how they react to a salient stimulus. Although the mechanisms that underlie such decision making have garnered significant attention, much remains unclear. Here we address this issue by studying (*i*) rapid effects of androgen pulses on social decision making, and (*ii*) whether social experience shapes how such effects are manifested. In Experiment 1, we examined whether testosterone (T) pulses have rapid effects on a male's decisions to approach a novel male (challenge) versus a receptive female (opportunity) in both sexually naïve and pair-bonded California mice (*Peromyscus californicus*). Sexually naïve males administered saline injections preferentially approached unfamiliar females over unfamiliar males, in contrast, 10 minutes after receiving a single T-injection, males expressed a preference for approaching unfamiliar males. This suggests that T pulses may rapidly change the social-decision making by facilitating the approach to the potential competition as opposed to approaching a potential mate. Such an effect of T was specific to sexually naïve males; pair-bonded males typically approached males regardless of whether they received a saline or T injection, suggesting that the rapid effects of T on approach behavior may rely on the pair-bonding experiences. Experiment 2 investigated social decision-making across three repeated exposures to the challenge/opportunity situations. Only the initial decision, approach to the challenge, predicted future aggressive behaviors, and such an effect relies on the rapid actions of T. We also found that experience with the controlled challenge situation (the male intruder was restrained behind a wire mesh) dampened the approach to the male side (potential threat) when later exposed to the same conditions. We speculate that these results are consistent with the "dear enemy" hypothesis proposing that a resident's motivation of defending against a threatening individual will be decreased as the threat posed by the "neighbors" is reduced. Overall rapid T effects revealed decision making that suggests that the

transient T pulses that occur in response to behavioral interactions across species may also alter consequent behavioral decisions.

Introduction

Social animals need to deal with varied forms of social interaction that occur in different contexts and phases of the lifespan. Appropriate decision-making in coping with these different interactions is critical for an animal's individual fitness. For instance, male rats increased their approach behavior to an estrus female compared to a non-estrus female or a male (López et al. 1999) and male mice that experienced defeat showed fewer approaches to an intruder than the undefeated mice (Lumley et al. 2000). Based on the functional context and outcome of the behaviors, O'Connell and Hofmann (2011) classified social interactions into two simplified categories: challenge, such as territorial defense, requires animals to approach and expel the intruder; and opportunity, such as finding or guarding a mate, requires animals to approach the mating resources for reproductive purpose. In nature, animals may find themselves in situations in which the acquisition of opportunity (e.g. mating resource) entails dealing with challenges (e.g. competitor) simultaneously. When such conflicting stimuli are presented simultaneously, the decision-making is likely the net result of multiple, and sometimes conflicting, sets of motivations and is speculated to be subject to the hormonal state and social experiences (Hau and Goymann 2015; Koolhaas et al. 2010).

Testosterone (T) release can be elicited following male-male agonistic encounters as well as male-female sexually encounters and lies at the core of social interactions by facilitating social approach and dominance-seeking behaviors across species (Gleason et al. 2009). According to the challenge hypothesis, T levels are elevated in response to a challenging encounter in which social status might be threatened, thereby initiating approach, motivation and simultaneously reducing fear (Archer, 2006, Bos et al., 2012). Rapid effects of the T pulses are hypothesized to help animals cope with the immediate situation that stimulate the T-pulse (Nyby, 2008). For example, in humans, exogenous T rapidly enhanced responsiveness to social threat (Hermans et al. 2008) and increased aggression in dominant and impulsive

man (Carré et al. 2017). In male California mice (*Peromyscus californicus*) that have pair-bonded with a female, T-pulses rapidly decreased ultrasonic vocalizations toward a novel female, whereas it induced rapid increases in hypothesized courtship vocalizations in unpaired males (Pultorak et al. 2015), suggesting pair-bonding can influence an animal's responses to the rapid effects of T. In male California mice, pair-bonding also influenced reinforcing effects of T (Zhao and Marler 2014, 2016), that can act upon the neural circuits controlling social decision-making (Ikemoto and Panksepp 1999; O'Connell and Hofmann 2011). We therefore hypothesized that T can rapidly influence social decision-making in the challenge/opportunity situations and that such an effect will be subject to the pair-bonding status.

Adding a layer of complexity to this idea is the fact that prior social experience may influence social decision making. For example, male Thomas langur (*Presbytis thomasi*) reduce loud calls and chases when they are familiar with the potential threat (Wich and Sterck 2007) and male collared lizards (*Crotaphytus collaris*) decrease aggression as the perceived threat of the opponents diminishes (Husak 2004). These findings suggest that previous experience of dealing with the challenge/opportunity may affect the future decision-making when exposed to the same situation. Therefore, we also explored animal's social decision-making and T's effects on the decision-making with repeated exposure to the challenge/opportunity situations. We hypothesized that either the preference would be repeatable or that the preferences would change once the males assessed the controlled challenge situation, such as a decreased approach to the male restrained by the wire mesh barrier. We had no *a priori* predictions regarding which preference test(s) would predict the future levels of social interaction with an intruding male.

In the current study, Experiment 1 investigates both the effects of transient T pulses and pair-bonding experience on social decision-making (approach behavior) when in the presence of both a male intruder and a receptive female, which reflects a challenge/opportunity context. Based on Experiment 1 results, T only has a significant effect on social decision-making in sexually naïve males. We therefore only used sexually naïve males in Experiment 2 and allowed males to establish residency for the sake of assessing the relationship between the decision-making and future aggression. We also examined the

change of social decision-making along repeated exposures to the challenge/opportunity situations. We used two choice tests which have been employed as an indirect measure of social decision-making in different animal taxa (Henley et al. 2010; Hetta and Meyerson 1978; Merkx 1983; Meyerson and Lindström 1973; Portillo and Paredes 2003); time spent in proximity to a stimulus is used as a measure of preference for that stimulus. In the context of opportunity, sexually naive and experienced male rats spend more time near sexually receptive versus non-receptive females or males (Ågmo 2003; Edwards and Einhorn 1986). In the context of challenge, animals often show sex-specific aggression in defending a territory or a mate, with males targeting other males and females targeting other females (Adkins-Regan and Robinson 1993).

Method

Overview of experiments

Two experiments were conducted in this study. In Experiment 1, sexually naive and pair-bonded males were injected with either T or saline, and then subjected to a two-choice test assay (see below) to assess their approach to either a novel male (conflict) or a receptive female (mating). Because we only found a significant T-induced difference in approach behavior of sexually naïve males in Experiment 1, we only used sexually naïve males in Experiment 2 and allowed them to establish residency (characteristic of territoriality) in the middle, neutral chamber of the two-choice test apparatus, before subjecting these individuals to the same two-choice test between a male and a female once per day over three consecutive days. In this final experiment, on the day after the last preference test, we examined each focal animal's aggressive behavior to a male using a resident-intruder paradigm and assessed the relationship between the decision-making through approach behavior and the aggression levels.

Experiment 1

Animals

Animals were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. We used 52 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. Based on the pair-bonding experience, all mice were divided into two groups: a pair-bonded group consisting of 26 randomly selected males, each of which was paired with a female 1-week before the experiment; a sexually naive group consisting of 26 randomly selected males that were sexually naive and housed with two other male cage mates (housed together after weaning).

Testing Apparatus

Preference testing took place in a large polycarbonate apparatus (91 cm long x 46 cm wide x 43 cm high) that was divided equally into three chambers (Zhao and Marler 2014). Access to each side chamber was controlled through sliding doors. The rear third of each side chamber was separated from the front two thirds by a metal wire mesh. During the preference test, stimulus animals were placed in the area behind the wire mesh, which prevents physical interaction between the focal and stimulus animals, while visual, auditory as well as olfactory cues were accessible.

Testosterone dose

We used 36 μg/kg T-injections (T-cyclodextrin inclusion complex) because in a previous study this injection 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). This dose mimics natural changes in T found in intact California mice after winning an aggressive encounter (Oyegbile and Marler 2005) and in male-female encounters (Zhao and Marler, unpublished data); in keeping with this, the same dosage enhances aggression and future winning ability (Fuxjager et al. 2011a; Fuxjager et al. 2011b; Trainor et al. 2004), induces conditioned place preferences (Zhao and Marler 2014, 2016), and can rapidly influences ultrasonic vocalization (Pultorak et al. 2015) . In the current study, half of the animals 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 animals constituted the controls and received injections of saline (saline group).

Procedure

The whole procedure consisted of three phases: habituation (30 min), injection (10 min) and preference test (15 min). Immediately after selection, each focal male was placed into the middle chamber of a testing apparatus. At this time, the sliding doors were opened, permitting access to the side chambers. The focal males were then given 30 min to habituate to their new environment, including the side chambers. After this period, the focal animal was gently guided into the central chamber and the sliding doors were sealed. The focal males then received either T or saline. In each group, half of the randomly selected animals received a weight-adjusted 36µg/kg (weight range 39-45 g) subcutaneous injection of T, while the other half received an injection of saline as a control. We then allowed 10 min before testing for the injection to take effect. After this period, stimulus males and females were placed behind the wire mesh partition in each apparatus such that the focal male and the stimulus males could see, hear, smell, and maintain proximity, but could not engage in physical interactions such as agonistic fighting or mating. To avoid pre-existing side preferences, we counterbalanced the side of the apparatus in which the stimulus male was placed; in each treatment (T or saline) group, half of the male (or female) stimulus were placed in right side and the other half were placed in the left side.

All preference tests were conducted within 2 hrs after the lights off and recorded using Panasonic HD cameras on the low-light setting. Each test was run for 15 min after the sliding guillotine doors were opened. At the conclusion of each test, the focal male was gently guided back into the central chamber and the doors were closed.

Experiment 2

Animals

We used 34 sexually naïve male P. *californicus* aged 6 -12 months. Animals were randomly assigned to receive T or a saline vehicle as a control. Prior to testing, each animal was group-housed with two other males (cage dimensions: 48 x 27 x 16 cm) under a 10 L: 14 D light cycle. Twenty-four hours before the two-choice test, the randomly selected focal males were moved to the middle chamber of the testing apparatus until the end of the aggression test on the fourth day. In the middle chamber, they were housed with one of their two former cage-mates to avoid isolation-induced stress. Shelter (a plastic tube), food (Purina 5001 mouse chow) and water were also provided. We have shown that > 24 hrs is sufficient to establish residency status in California mice (Bester-Meredith and Marler 2001; Fuxjager et al. 2010a; Oyegbile and Marler 2005).

We selected stimulus females and males (including intruders) that were unrelated to the focal males (separated by at least two generations). Prior to testing each day, the estrus cycle stage was assessed using vaginal lavage for sexually naïve, group-housed candidate females housed separately from the breeding colony (Byers et al. 2012; Davis and Marler 2003), and only females in the estrus state were used. Each intruder male was shaved prior to testing so that they could be distinguished from the focal male. Stimulus males and females were used up to three times, but never more than once in the same treatment group.

Procedure

We first habituated the focal males to the testing apparatus for 30 min. After this period, the focal animal was gently guided into the central chamber and the sliding doors were sealed. The cage-mate was then added to the central chamber. The animals were left undisturbed for 24 hrs. The focal animal and his cage-mate were housed in the central chamber with a red plastic tube toy, and food and water ad libitum.

The procedure of injection (10 min) and two-choice test (15 min) were the same as in Experiment 1 except that we repeated the same procedure three times (once per day) on three consecutive days. On each testing day, the cage-mate was removed from the testing apparatus and placed in a small cage. All animals received the same treatment and the same dosage on each test. We then waited 10 min before

testing for rapid effects of T on behavior. The side placement of stimulus males and females was counterbalanced across groups.

At the conclusion of each test, the focal male was gently guided back into the central chamber and the doors were closed. We then returned the cage-mate to the central chamber, and removed the stimulus animals. We tested the animals only once per day, and tested each focal male in this way a total of three times.

Aggression tests.

On the fourth day of testing, the focal males were subjected to a resident-intruder test as a proxy for territorial aggression. This test used only the central chamber of the testing apparatus and the side chamber in which stimulus males had been presented to each focal male. The cage-mates were removed from the cage and the testing room before each trial as in the male-female two-choice tests described above. We allowed the focal male to freely explore the side chamber for two minutes. We then shut the door, and transferred a weight-matched intruder (± 3 g) (one per focal male) into the side chamber, and gave it two min to habituate to the side chamber. We then opened the sliding doors and recorded the next five min of interaction between the two males with Panasonic HD cameras. After five min had elapsed, the two animals were separated. After the end of aggressive tests, all males were returned to their original cages. The winner was defined as the individual who initiated at least three consecutive attacks that elicited eliciting avoidance or freezing behavior (Oyegbile and Marler 2005).

Data Analysis

All videos were coded by an observer blind to both the treatment group of the animal (T or S) and the male/female arrangement of the side chambers in each test. In each male/female choice test, the amount of time the focal male spent in each chamber was recorded. The choice of approaching either the male or female stimulus was measured by a difference score (time spent in the male side minus time in the female side), a measure of approach that has been used in two-choice test (Cummings et al. 2008;

Henley et al. 2010). A positive difference score means more time was spent with the stimulus male, whereas a negative difference score indicates more time was spent with the stimulus female. The normality of the data was determined by the Shapiro-Wilk test. For Experiment 1, two-way ANOVA was used to analyze the interaction between treatment (saline vs. T) and pair-bonding status (sexually-naive vs. pair-bonded). If ANOVA revealed a significant interaction, an independent t-test was used to compare the two treatments (T vs. saline). For Experiment 2, the effect of interaction between trial and treatment was analyzed using two-way repeated measures ANOVA followed by pairwise comparisons. For aggression tests, the effect of treatment on the probability of winning a contest was analyzed using a modified Fisher's exact test adjusted for data arranged in a 2×4 table (Freeman and Halton 1951). The data of attack latency was not normally distributed according to Shapiro-Wilk test and therefore Spearman tests were used to analyze the correlation between attack latency and difference score. Three males in Experiment 1 and four males in Experiment 2 were excluded from analysis due to a complete absence of exploratory behavior in the two-choice test.

Results

Experiment 1

In Experiment 1, the two-way ANOVA did not reveal any significant interaction ($F_{(1,45)}$=1.395, p=0.244) or main effects of either pair-bonding status ($F_{(1,45)}$<0.0001, p=0.989) or drug treatment ($F_{(1,45)}$=1.380, p=0.246) for the time spent in the chamber containing the male stimulus (Fig. 1a). For the time spent in the female chamber, the two-way ANOVA yielded a significant interaction ($F_{(1,45)}$=6.134, p=0.017), but no significant main effects of pair-bonding status ($F_{(1,45)}$=0.802, p=0.375) or drug treatment ($F_{(1,45)}$=1.987, p=0.166) (Fig. 1b). Independent t-tests revealed that, compared to saline-treated pair-bonded males, the saline-treated sexually-naive males spent significantly more time in the female side (t_{21}=2.235, p=0.036); no such significant effect of experience was found in the T-treated groups (t_{21}=1.197, p=0.243) (Fig. 1b). Also, compared to saline-treated sexually-naive males, the T-treated sexually-naive males spent significantly less time in the female side (t_{21}=2.30, p=0.03); no such

significant effects of treatment occurred in the paired groups (t_{21}=0.934, p=0.359) (Fig. 1b). The above results suggest that sexually-naive males show an overall tendency to approach females in the absence of a T pulse; however, a T pulse appears to motivate sexually-naive males to allocate more of their time to the novel males, which is a typical behavioral pattern observed in all pair-bonded males. For the time spent in the middle chamber, the two-way ANOVA did not reveal any significant interaction ($F_{(1,45)}$=1.556, p=0.219) and main effects of pair-bonding status ($F_{(1,45)}$=0.833, p=0.366) and drug treatment ($F_{(1,45)}$=0.022, p=0.882) (Fig. 1c). For the difference score, the two-way ANOVA yielded a significant interaction ($F_{(1,45)}$=4.152, p=0.047), but no significant main effects of pair-bonding status ($F_{(1,45)}$=0.244, p=0.624) and drug treatment ($F_{(1,45)}$=2.110, p=0.153) (Fig. 1d). Independent t-tests revealed that, compared to saline-treated sexually-naive males, the T-treated sexually-naive males spend significantly less time in the female side (t_{21}=2.179, p=0.041); no such significant effects of treatment occurred in the paired groups (t_{21}=0.472, p=0.641) (Fig. 1d).

Experiment 2

Experiment 2 examined the consistency of the preference of choice and the correlation between the preference of choice and the aggressive behavior.

Two-choice Test

Two-way repeated measures ANOVA revealed a significant overall effect of trials (Trial 1 vs. 2 vs. 3) ($F_{(2,56)}$=4.489, p=0.016), with no significant effect of interaction ($F_{(2,56)}$=4.489, p=0.016) and treatment ($F_{(2,56)}$=0.583, p=0.561). This suggests that the time allocation to the male and female chambers is not consistent across trials. Pairwise comparisons showed that the difference score of Trial 1 is significantly higher than that of Trial 2 (p=0.012) and 3 (p=0.029), suggesting animals decreased the approach to the male side across the trials; no significant difference between Trial 2 and 3 was found (p=0.791). Also, in the sexually naïve males that had established residency, T did not influence the approach towards males in each trial (Fig. 2).

Aggression Tests

According to Fisher's Exact Test, the probability that sexually naïve males won their aggressive encounter did not differ between saline and T groups (p=0.272). However, based on the treatment (saline or T) and preference of choice (spending more time in male or female side) in Trial 1, animals were further sorted into four groups: saline+female (S+F), saline+male (S+M), T+female (T+F), T+male (T+M). We found that the percentage of mice that won the test encounter was significantly different among these four groups (χ^2=9.579, df=3, P=0.021, Fig. 3), with the T+M group obtaining significantly higher proportion of wins than the other three groups.

For males that received T (combined T+M and T+F), we found that attack latency during the aggressive trial was negatively correlated with the difference scores in Trial 1 (r = -0.79, P = 0.001, n = 15, Fig. 4), but not with difference scores in Trials 2 or 3 (p>0.05). Also, we found no correlation between attack latency and difference scores in the saline group (p>0.05). The results suggest that the persistency of approaching the male stimulus in Trial 1 may predict the motivation of aggression of sexually naïve mice that have become residents, but is not revealed without exposure to T.

Discussion

To our knowledge, our study shows for the first time that T pulses can rapidly mediate social decision-making processes, but in a way that hinges on prior social experience. We find, for example, that sexually naïve male California mice normally prefer to associate with receptive females. However, a single pulse of T, similar to ones that occur in response to social stimuli, completely reverses this effect, causing males to instead associate with potential competitors. We also find that social decision making under the challenge/opportunity choice tests relies on an interaction between the T pulse and pair-bonding status. Compared to the effects in sexually naïve males, T injections do not further enhance the approach to the male side in pair-bonded males. We find that when a resident male is repeatedly exposed to challenge/opportunity situations, the decision-making in Trial 1 (and not Trials 2 and 3) reflects the

internal motivation of a male and predicts how he will later cope with a challenge, but such motivation is only revealed in the presence of a T pulse (individuals that preferred males and also received T); this is manifested in the later aggressive behavior towards the male intruder. Furthermore, multiple exposures to the challenge/opportunity situation appear to cause the adjustment of decision-making when exposed to the same situation. We speculate that the resident male is able to assess that the threat posed by the male "competitor" is decreased by the physical separation of the wire mesh and thereby dampens the approach to the challenge situation. This echoes the "dear enemy" hypothesis that proposes that a resident's motivation of defending against a threatening individual will be decreased as the threat posed by the "neighbors" is reduced (Getty 1987; Temeles 1994).

The rapid effects of T on the decision making may be a hormonal strategy that enables animals to cope with immediate threats (Nyby, 2008). The approach to receptive females in sexually-naive males may reflect the tendency of seeking reproductive opportunities, while T rapidly changes the goal and redirects decision-making to motivate males to focus on the potential threat of the same-sex conspecifics within 25 min. We speculate that the rapid effects of a T pulse may support the endogenous mechanisms that underlie sexually-naive males' drive to begin establishing a territory. We previously demonstrated the rewarding effects of T pulses by showing that sexually-naive males can form conditioned place preferences to the environment wherein they received T injections (Zhao and Marler 2014, 2016). Such preferences to a specific environment may contribute to the formation of a territory, and the transient T pulse may initiate this process particularly when in the presence of a same-sex intruder. The cellular mechanism underlying T's rapid effects on social decision-making is still unclear, but through conversion to estrogen or 3α-androstanediol, T can rapidly modulate reproductive behaviors in birds (Cornil et al. 2006) and rodents (Frye 2001). We argue similar mechanism of converting T to estrogen or 3α-androstanediol may also underlie its effects on social decision-making in the challenge/opportunity situations. Alternatively, the winner effect appears to involve experienced induced changes in aggression that seem to function through androgen and not estrogen receptors (Trainor et al. 2004)

Variation in the social decision-making between the sexually naïve and pair-bonded males may reflect different life history stages. In the wild, sexually naïve males that dispersed from the natal area are motivated to explore novel environments and establish their own territories (Ribble 1992), and conditioned place preferences induced by T pulses have the potential of facilitating territorial establishment (Zhao and Marler 2016). In the current study, T pulses may further contribute to behavioral changes by rapidly altering the social decision-making of the sexually naïve males to approach males may be a mechanism during the initiation of territoriality. Compared to sexually-naive males, pair-bonded males decrease time spent on the unfamiliar female's side, which may be because the pair-bonded males already have long term mates. In a study by Ribble (1991) conducted in the field, no extra-pair mating were detected in California mice. In the laboratory, male sexual fidelity in male California mice appears to be more self-imposed by the male mice who did not copulate with the estrous female when given the opportunity regardless of whether their partner is present or not. (Gubernick and Nordby 1993). Mechanisms for sexual fidelity in the male is further supported by the inhibitory effect of a T pulse on rapid vocal responses of a paired male to an unfamiliar male (Pultorak et al. 2015).

Although the effect of residency is not directly examined in the current study, some evidence hints that the effects of T pulses on decision making differed between the Experiment 1 and Trial 1 of Experiment 2 might be accounted for by the residency status of the sexually naïve males; The significant rapid effect of T is only displayed in sexually naïve males from Experiment 1, which is conducted in an environment in which residency has not been established. As discussed above, we speculate that the rapid effect of T in sexually naïve males may mainly function to initiate territory establishment but not to maintain the territory. In such a scenario, the T pulse would not further enhance the approach to males in Experiment 2. We previously demonstrated that in sexually naïve males, the T-induced CPP was only produced in unfamiliar environments, and not in conditions where males had established residency (thereby mimicking territoriality); while in pair-bonded males, the T-induced CPP was only produced at home but not in an unfamiliar environment (Zhao and Marler 2014, 2016). Thus, compared to unfamiliar environments, we speculate that home is a more salient environment to pair-bonded males and if we

allowed pair-bonded males to become residents, the T pulse may have a more significant effect on the approach to male intruder, which may function to enhance territorial defense.

Neither the approach to the male nor T's rapid effects alone predict the level of future aggression towards the novel male intruder. When the animals were repeatedly exposed to the challenge/opportunity situations, the decision-making in Trial 1, but not Trial 2 and 3 predicts the aggressive motivation but this effect relies on the action of T. We speculate that the approach to the male stimulus in Trial 1 may reflect the variability in aggressive motivation which is related to the way individual males react to environmental challenges (Koolhaas et al. 1999). Such variation in aggression may be achieved, at least partially, through hormonal mechanisms. For example, previous studies showed that the prenatally circulating T levels (Compaan et al. 1994) and T secreting capacity of the testes (De Ruiter et al. 1993) are greater in aggressive (short attack latency) than in non-aggressive (long attack latency) males. Expanding upon these previous findings, our results reveal that the rapid effects of T may facilitate the manifestation of the individual variation in the aggressive propensity. For the males that received T injections but preferred the female side, their aggressive motivation might be lower and therefore the time spent on the male side does not predict later aggression. The direct evidence for the mechanisms underlying the variation of the approach tendency is currently lacking. Previous studies in birds and mice demonstrated that the expression of androgen and estrogen alpha-receptors (Rosvall et al. 2012; Sperry et al. 2010; Trainor et al. 2006) and the aromatase activity (Compaan et al. 1994) in distinct brain areas may explain the variation in aggression. The similar plasticity in the levels of enzyme and receptor may also account for the difference in the preference to the male stimulus; the more time spent in the male side might indicate the higher levels of enzymes and receptors for T to act on and therefore behave more aggressively after repeated T injections. Also, the repeated T injections may also allow animals to form a preference to the chamber where they preferred. Later in the same chamber (male side) animals behave more aggressively.

The effect of T on decision-making is not only influenced by males' mating status, but also previous experience of exposing the challenge/opportunity situations. We can only speculate, but such plasticity in

the social decision-making may be consistent with the "dear enemy" hypothesis, which assumes that the decreased threat will reduce the response to the potential territorial intruder (Temeles 1994). In Experiment 2, animals were housed in the middle chamber of the apparatus for 24 hrs before Trial 1. During the 30-min exploration in Trial 1, the focal males may have assessed that the threat of the male intruder was limited because of being restricted to part of a side chamber via a wire mesh. This may have induced males to decrease the time allocation to the male side chamber in Trials 2 and 3. Such plasticity in the decision-making may be an evolved mechanism that could help territorial animals minimize the energy expended on aggression towards the secured neighboring territories (Ydenberg et al. 1988). According to the "dear enemy" hypothesis, a resident should invest more in defending against a more threatening individual that is capable of inflicting greater losses on the resident (Getty 1987) while the energy spent dealing with the challenge will be reduced as the threat posed by the "neighbors" is low (Temeles 1994). Such a scenario could be tested by comparing resident's behavioral responses to an activity-restricted versus non-restricted intruder.

Altogether, the current study reveals that the T pulses, which naturally occur following male-male agonistic encounters or male-female sexual encounters (Gleason and Marler 2010), can rapidly affect animal's social decision-making in response to challenge/opportunity situations. Moreover, we find that this response varies depending on whether males are sexually naïve or pair bonded. We also have evidence that indirectly suggests that residency status may influence T's rapid effects on social decision-making, at least in sexually naïve males. Finally, plasticity in the social decision-making process may also occur when males are repeatedly exposed to the same challenge/opportunity situation, possibly by assessing the limited threat of an intruder introduced behind a wire mesh barrier. All these findings depict a complex interplay between hormone, environment and prior social experience, which may act as a mechanism for animals to cope with the immediate situation following the agonistic or sexual encounters in nature.

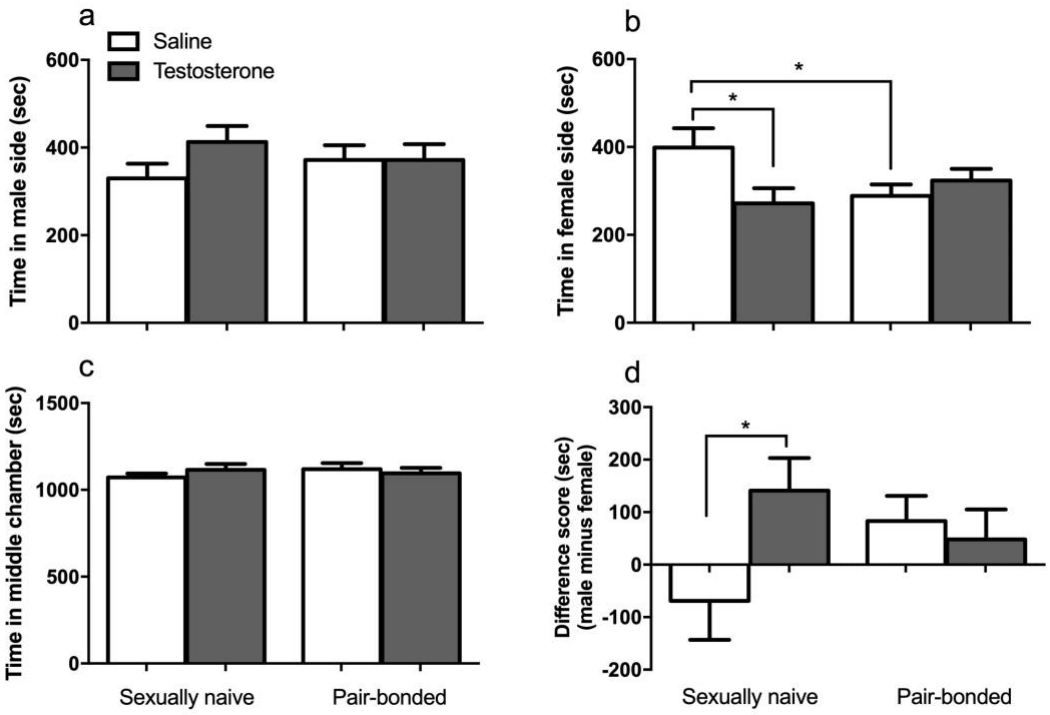

Figure. 1 Time in the (a) male, (b) female and (c) middle chamber for sexually naive and pair-bonded males in the two-choice test. (d) The difference score (time in male side – time in female side) for sexually naive and pair-bonded males in the two-choice test. * $P < 0.05$ independent t test. N=11-13 in each group. Data are presented as Mean ± SEM.

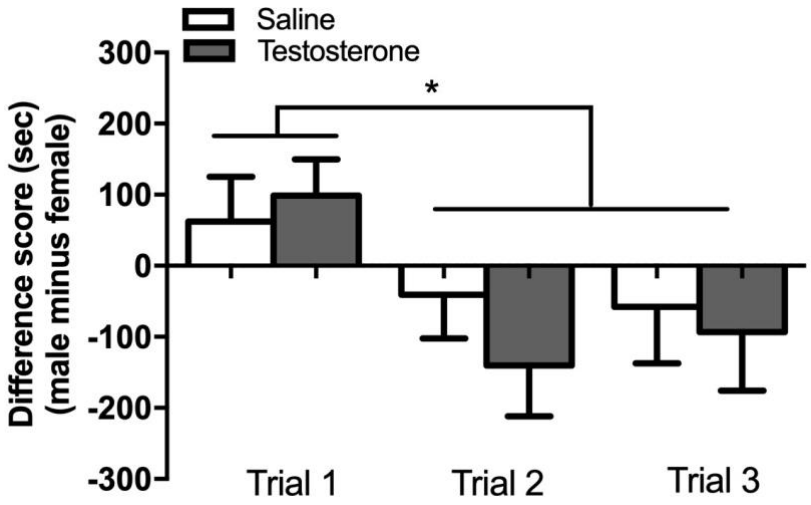

Figure. 2 The difference score (horizontal axis represents animals spent equal time in the male and female side; positive represent male-biased and negative represents female-biased) for sexually naive males. * $P < 0.05$ significant effects of pairwise comparison. Data are presented as Mean ± SEM.

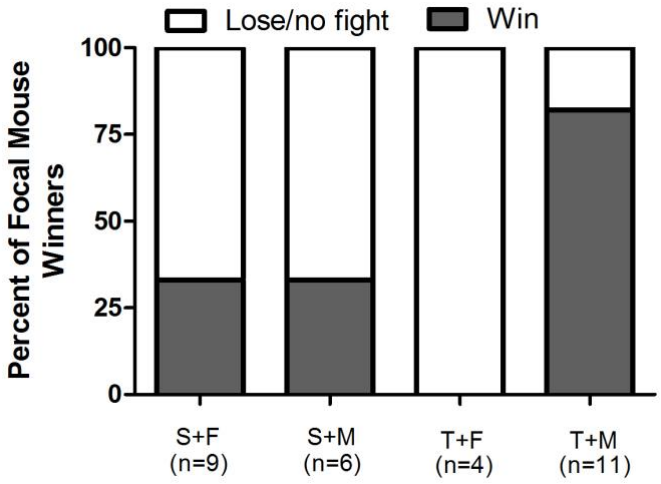

Figure. 3 The percent of focal mouse winners for saline group that preferred female (S+F) and preferred male (S+M) in Trial 1, and T group that preferred female (T+F) and preferred male (T+M) in Trial 1.

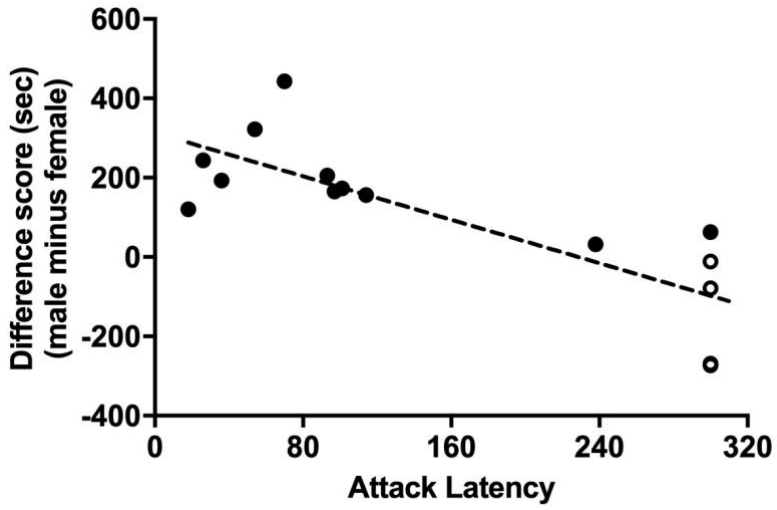

Figure. 4. Correlation of attack latency during the resident-intruder test and the difference score (sec) in two-choice test Trial 1 for the males that received T. Black dots represent individuals that preferred males in Trial 1 and circles represent individuals that prefer females in Trial 1.

Chapter 5

Summary of the Main Findings

In Chapter 1, I created a behavioral paradigm showing that the CPP can be developed through T injections (36 μg /kg) that mimic natural changes in T found in intact California mice after winning an aggressive encounter. I also found that the reinforcing effects of T rely on the pair bonding status; T created CPPs for a previously neutral environment in SN males, but not PB males. To our knowledge, this is the first study demonstrating variation in T-induced CPPs based on social experience.

In Chapter 2, I examined the plasticity in the T-induced CPP. I found that the CPP to a novel environment was only produced when the male mouse was singly housed, but not in a male housed with a sibling or paired with a female; the pair-bonded male can form a CPP to an environment where the residency has been established. This plasticity in the T-induced CPP and potential aggressive motivation parallels the variation in natural territorial behaviors, such as the diminished territoriality in the natal area (group housed with siblings), increased motivation of territory establishment after dispersing from the natal area (singly housed) and enhanced territory defense after bonding with a female partner.

In Chapter 3, I confirmed that singly housing males facilitated T-induced CPP to an unfamiliar environment in sexually naïve males. Beyond that, I also reveal that, by interacting with the T pulses, the experience of singly housed (mimic dispersing) enhanced the aggressive motivation towards a novel male intruder. On a neural level, I found that upregulation of androgen receptors in the preoptic area (POA) occurred after animals were singly housed, suggesting that preparation for responsiveness to T pulses changes after dispersal may be reflected by increased POA AR. Single housing also increased the synapsin/phosphorylated synapsin in the nucleus accumbens, ventral and dorsal hippocampus, suggesting that the synaptic plasticity in these regions may prepare males for reproduction, likely including the establishment of a territory. Altogether, these results suggest that the territoriality establishment/enhancement may be achieved by developing T-induced CPPs in appropriate contexts, and such a process may require neural changes that are associated with the social experience.

In Chapter 4, I found that sexually naïve male California mice normally preferred to associate with receptive females. However, a single pulse of T, similar to ones that occur in response to social stimuli, completely reversed this effect, causing males to instead associate with potential competitors. I also found that compared to the effects in sexually naïve males, T injections did not further enhance the approach to the male side in pair-bonded males. Additionally, when a resident male was repeatedly exposed to challenge/opportunity situations, the decision-making in the initial exposure (and not the subsequent exposures) reflected the internal motivation of a male and predicted how he will later cope with a challenge, but such motivation was only revealed in the presence of a T pulse (individuals that preferred males and also received T); this was manifested in the later aggressive behavior towards the male intruder. Furthermore, multiple exposures to the challenge/opportunity situation appeared to cause the adjustment of decision-making when exposed to the same situation. To my knowledge, this is the first time a study showed that T pulses can rapidly mediate social decision-making processes, but in a way that hinges on prior social experience.

In the next sections, the results from all chapters will be considered together, and hypotheses to explain these results collectively with previous research will be presented. Furthermore, limitations as well as future directions will be discussed.

As a paradigm to measure the rewarding and incentive motivational effects of drugs, CPP has become increasingly used in preclinical research. However, what is surprisingly understudied is the natural functions of developing a CPP. The place conditioning is based on the principles of classical conditioning that environmental cues can gain the capacity of evoking approach behaviors after being repeatedly conditioned with a rewarding stimulus. It would be reasonable to assume that such an evolved ability may help animals to adapt to varied environments and benefit individual's fitness. Several studies have shown that social stimuli or interactions can induce a CPP. For example, engaging in an aggressive interaction can be rewarding and that aggressive experience can result in an enhanced preference for the

site of previous aggression in territorial species such as male mice and green anoles (Farrell and Wilczynski 2006; Martínez et al. 1995). CPP to the environment previously paired with sexual interaction can be developed in male rats and mice (Paredes 2009), and Syrian hamsters (Bell et al. 2010). Also, interacting with pups can induce CPP in rat dams (Fleming et al. 1994). An additional intriguing finding is that the degree of the CPP decreases as the pups growing older (Seip and Morrell 2007), indicating plasticity in the rewarding value of stimuli. The plasticity in the rewarding stimuli has been a critical finding across my studies and indicates that there are likely a number of mechanisms that are turning reward on and off. Using the perspective of territoriality, the combined findings implicate the adaptive values of forming a CPP in nature such as securing territory, defending mating resources and protecting pups, which will eventually increase the reproductive fitness. Engaging in agonistic or sexual encounters can produce a transient T pulse. Findings in this dissertation, such as the plasticity in the T-induced CPP and the subsequent change in the aggressive motivation suggest the T-induced CPP may serve as a hormonal mechanism to mediate the rewarding properties of social interactions. It would be interesting to test if blocking the post-encounter T pulses would inhibit the CPP induced by social interactions. Moreover, it would be advantageous to further examine the neural mechanisms involved in such plasticity.

The focus in this study is on pulses of T, which differ from the baseline T levels in terms of the manner of release – transient surge and decline compared to slow but stable changes, often associated with seasonal breeding. Long-term changes in the baseline levels of T has been shown to influence spatial behaviors. For instance, male dark-eyed juncos (*Junco hyemalis*) implanted with T tend to increase the allocation of time to locomotion and foraging, and decrease the time allocated to sleeping and preening (Lynn et al. 2000). In spiny lizards (*Sceloporus jarrovi*), T-implanted males spend more time in territorial defense during the day (Marler and Moore 1989) and increased frequencies of male-female interactions following a territorial encounter with an introduced male (Marler and Moore 1991). As a complement to previous studies using T implants, our result indicates that a few T pulses may also impact time allocation to different environments and social decision-making. In territorial lizards, administration of T in the non-

breeding season causes individuals to become territorial and compete with each other as though the breeding season has begun (Marler & Moore, 1988; Moore & Marler, 1987). This follows other work that shows that males increase T during periods of social instability, when fighting and rivalry are more rampant (Goymann et al., 2007; Hirschenhauser & Oliveira, 2006). Indeed, some of this research has elegantly pinpointed the causes of an increase in T to aggressive interactions among conspecifics, as opposed to sexual opportunities that arise concurrently (Muller & Wrangham, 2004). An adaptive framework for the function of T pulses has been elegantly encompassed by the "Challenge Hypothesis," which was proposed by Wingfield (1984) hypothesizing that androgen levels above breeding baseline function to increase the frequency and intensity of aggression in males, especially when hierarchies are being established or when dominance relationships are challenged.

While the T pulses in response to salient social stimuli have been demonstrated in many species, few experiments have manipulated these endocrine events and determined their rapid effects. In the preceding studies, we focused on T's rewarding effects that occurred repeatedly 45 min after the injection and its effect on social decision-making that occurred within 25 min after a single injection. A previous study in adult male white-footed mice found that a single pulse of T rapidly (within 20 min) altered the urinary marking behavior (Fuxjager et al. 2015). These results suggest that the rapid effects of T may be part of a mechanism by which animals can quickly adjust their behavior in accordance to highly dynamic social environments. These findings are also consistent with past work in fish, which similarly showed rapid effects of androgens on behavior (Remage-Healey and Bass 2005, 2006) and physiology (Mangiamele and Thompson 2012). However, it should be noted that I cannot rule out the possibility that the long-term effect of T across the three injections also contribute to the formation of CPP and the enhancement of aggressive behavior, which are tested after the animal received all three T injections. For the development of a full winner effects, three T pulses are necessary (Fuxjager et al. 2011b; Oyegbile and Marler 2005) and this effect lasts at least 7 days. This implies that the expression of certain behaviors may require a physiological 'summation' of androgenic pulses.

Social experience and context are needed to be taken into account when assessing the effects of T pulses on social behaviors. This is reasonable because the optimal reproductive strategies could be varied in different life stages and environments. Findings from Chapter 1, 2 and 4 demonstrated that pair-bonding is an important factor that could influences animals' responses to T injection(s). The pair bond 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 (Aragona et al. 2005; Insel et al. 1995). Compared to sexually naïve California mice, pair-bonded males dampen their scent-marking responses towards novel females (Becker et al. 2012) and decreased ultrasonic vocalizations toward a novel female after receiving a single T injection (Pultorak et al. 2015). As I show here, pair bonding also alters whether or not T-induced CPPs occur and this further interacts with the location, such as whether it is a familiar residency or a novel area. While some neural changes (i.e. dopamine system) associated with pair bond formation have been uncovered (Aragona et al. 2003; Gingrich et al. 2000), less is known about how the pair bonding experiences affects the reinforcing properties of hormones or neurochemicals. Formation of a pair bond in male prairie voles (*Microtus ochrogaster*) decreases the effect of the drug amphetamine on the formation of a CPP (Liu et al. 2011).

Despite the large number of studies that ascribe the behavioral and endocrine changes obtained in isolated animals to the effects of "the isolation-induced stress syndrome", there are controversies on whether social isolation/individual housing of male rodents should be considered highly stressful (Benton and Brain 1979; Brain 1975; Valzelli 1973). Although the markers of stress (e.g. adrenocortical reactivity) were not assessed in Chapter 2 and 3, the results suggest that a short-term isolation per se may trigger some characteristics of territoriality in species that would naturally disperse from the natal area and explore unfamiliar environment alone for a period of time. California mouse males display low levels of stress and corticosterone levels do not change in response to a social challenge from a male (Marler et al. 2005a). After dispersal in the wild, male California mice are known to remain in an area without a mate for up to 8 months (Ribble and Salvioni 1990). I would speculate that during this stage, the behavioral tactics used to cope with challenges (e.g. competition) or opportunities (e.g. mating) are

different from the tactics used in the natal area and a mechanism should be evolved along with the dispersal (short-term isolation). Chapter 3 also revealed that housing males individually induced an upregulation of AR in the POA and increased the synaptic plasticity in the NAc and the activity of the neurotransmitter release in the dorsal hippocampus. These neural changes may be a part of the neural mechanisms that prepare animals to adapt to environments after dispersing.

The role of context in modulating behavioral and hormonal responses has been well demonstrated. For example, the enhanced aggression by adding residency has been demonstrated in a wide variety of taxa, including invertebrates (Takeuchi and Honda 2009), amphibians (Baugh and Forester 1994), reptiles (Olsson and Shine 2000), birds (Snell-Rood and Cristol 2005), and mammals (Corlatti et al. 2013). Compared to unfamiliar environments, the home environment in Chapter 2 also consisted of familiar odors from the focal animals and/or from their female partners or cage mates, which may add salience to the home. Why do unfamiliar environments become more salient in the presence of T's effects and in sexually naïve males that are singly housed? One explanation, as I hypothesized, is that in sexually naive males that are individually housed, T pulses contribute to the establishment of a new territory but do not function for maintenance of an already established territory. This makes sense because for sexually naïve animals that dispersed to a novel environment, T may function to establish a new territory and monopolize more resources critical for reproductive fitness. Therefore, the effects of T pulses in singly housed sexually naïve males are more significant in an unfamiliar environment, but not at home. This is supported by Chapter 2 showing that the development of a CPP was inhibited in sexually naïve males in an unfamiliar environment. Although I did not directly assess the effect of residency in Chapter 4, some evidence hints that the effects of T pulses on decision making was also dampened in the place where the residency was established.

One limitation of Chapter 3 is that the measurements of aggressive motivation were limited by using only the latency to approach and duration of interacting with an intruder and not the physical aggressive behaviors between the focal male and the novel male intruder. In studies using rats to assess individual's motivation of social contact, the longer duration of interaction was used as a stronger drive of

seeking out nonaggressive social approach (Kentner et al. 2018). Rats, however, live in colonies, where they may share the burrow and raise their young together. In comparison, for territorial and monogamous species such as California mice, individuals do not share the burrow or territory unless it is the pair-bonded male and female and their offspring. We speculate that for California mice, the demand of seeking out positive social interactions with the same-sex conspecifics may not as strong as that in rats. In fact, male California mice are highly aggressive (Bester-Meredith and Marler 2001; Fuxjager et al. 2010a; Oyegbile and Marler 2005; Trainor et al. 2004). Within my research in Chapter 4, resident males that received T injections (but not saline) and spent more time interacting with the novel males initiated attack more quickly and are more likely to win in a later resident-intruder test. This approach towards an unfamiliar male is highly likely to be an aggressive approach in male California mice. I also speculate that the hormonal state (e.g. adding T pulses) may also influence the valence of predicting aggressive motivation by using the duration of interaction.

The CPP apparatus and procedures that we used in the preceding studies are smaller than the natural conditions under which male California mice form territories. In nature, the California mice could disperse up to 80 meters and the size of a territory is in average around 1161 m^2 (Ribble and Salvioni 1990). However, it is impossible to exactly mimic such a natural environment in laboratory settings. Future research under more natural conditions will be required to examine further the contribution of T pulses and associative learning to territory formation. Actually, some unpublished preliminary data from a field study has shown that receiving T injections near pair's nest will increase the pair-bonded male's time allocation to the nest, suggesting they may form a preference to the nest (Petric, Kalcounis-Ruepell and Marler, unpublishe data).

It is unknown if the differences in the behavioral responses observed across groups are accounted for by the variation in the baseline T levels, which were not measured in the preceding studies. I speculate it is unlikely at least for the difference between sexually naïve and pair-bonded males because previous study showed that baseline levels of T are not significantly different between sexually naïve and pair-bonded male California mice that have not had pups (Gubernick and Nelson 1989). Also, in California

mice, the T levels reach high levels during the first 24 hours after a pair is introduced and fall back to baseline by three weeks (Gleason and Marler 2010).

General conclusion

The studies presented in the preceding chapters addressed the effect of T pulses on behavior and specifically location preferences under the umbrella of territoriality. Results support the hypotheses that the T pulses may contribute to the territoriality establishment/enhancement through forming a conditioned place preference. Naturally, the social experience such as dispersal (or forming a pair-bond) may result in a series of neural and behavioral changes that would prepare animals for the life history transition from residing in the natal territory to establishment of their own territories (or defending territory and producing offspring with partners). My findings in the dissertation revealed potential roles of T in mediating those adaptive neural and behavioral changes by studying plasticity in T's effects on CPP formation and social decision-making. My findings also further supported the challenge hypothesis and provides a framework for thinking about why and how circulating levels of T vary throughout lifespan or in different environments.

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