THE ORGANIZATIONAL EFFECTS OF PUBERTAL TESTOSTERONE ON THE MATURATION OF ADULT SOCIAL COGNITION AND SOCIAL PROFICIENCY

By

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ABSTRACT

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Maturation of social cognition and a gain in social proficiency are universal aspects of adolescent development that prepare an individual for adulthood. Social cognition involves the perception and interpretation of social cues, followed by the generation of a behavioral response. Social proficiency is acquired through the ability to make behavioral adaptations as one learns from social experience. The pubertal rise in testosterone secretion results in both activation and organization of the neural circuits underlying adult male social behaviors, including sexual behavior, which may contribute to the developmental changes in social cognition and gain in sexual proficiency. To assess the contribution of pubertal testosterone to the maturation of adult social behavior, we utilize the NoT@P/T@P experimental animal model. In this model, male Syrian hamsters are deprived of testosterone during puberty (defined as NoT@P) or for an equivalent amount of time during adulthood (defined as T@P) and then given testosterone replacement in adulthood for two weeks before behavior testing. If NoT@P and T@P males differ in behavior, we infer that the difference is due to organizational effects of pubertal testosterone. If NoT@P and T@P males show similar behavior, then we infer that the adolescent maturation of that behavior is due to hormone-independent or activational actions.

We first tested the hypothesis that the adolescent acquisition of social reward does not depend on organizational effects of pubertal testosterone. We found that both

NoT@P and T@P males show a CPP for vaginal secretions and a receptive female, indicating that the adolescent maturation of social reward is independent of pubertal testosterone. Second, we tested the hypothesis the adolescent gain in sexual proficiency does depend on organizational effects of testosterone. When sexual experience is equated for both NoT@P and T@P males, NoT@P males do not inhibit maladaptive behaviors with sexual experience, whereas T@P males do. These data suggest that testosterone during puberty programs the ability to adapt behaviors (via inhibition) in a social context-dependent manner. Third, we tested whether pubertal testosterone organizes the adolescent gain in sexual proficiency through the regulation of the transcription factor ΔFosB in the infralimbic medial prefrontal cortex (IL). We found that ΔFosB was induced in the IL after sexual experience in T@P, but not NoT@P, males. Furthermore, over-expression of ΔFosB in the IL of NoT@P males prior to sexual behavior testing was sufficient to reverse the deficits in behavioral inhibition found in NoT@P males. Taken together, these data provide evidence that social reward develops independently of pubertal testosterone, whereas the ability to inhibit inappropriate behavior is organized by pubertal testosterone through the regulation of ΔFosB in the IL.

I dedicate this dissertation to:
my parents, Shar and Larry, for encouraging me to be myself no matter what and putting up with me during adolescence;
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Chapter 1: Introduction

Adolescent development and relevance to human mental health

Adolescence involves a decrease in parental dependence and an increase in peer interaction, known as social reorientation (Nelson, Leibenluft, McClure, & Pine, 2005). As social spheres change during adolescence, so do perceptions of social stimuli and behavioral responses in specific social contexts. Perceiving and interpreting social cues, and then responding appropriately (i.e., behavioral enactment) are important aspects of social cognition that ensure successful social interactions within a species (Adolphs, 2001). Through the ability to learn from social experience and adapt behavior accordingly, one gains social proficiency. Successful adolescent acquisition of social cognition and social proficiency plays a key role in ensuring the ability of an individual to become independent and appropriately interact with her or his peers. Many of the behavioral changes related to social cognition that take place during adolescence have been attributed to puberty, which coincides with adolescence and is characterized by an increase in sex hormone secretion as reproductive maturation begins. Therefore, the adolescent maturation of social cognition and gain in social proficiency arises as the result of complex interactions between hormones, experience, and the developing adolescent brain.

Adolescent maturation of social cognition can go awry. Indeed, several psychological disorders characterized by inappropriate social cognition emerge during adolescence, including depression, eating disorders, schizophrenia, conduct disorder, and anxiety disorders (Hayward & Sanborn, 2002). These psychological disorders are also sexually dimorphic, with depression, eating disorders, and anxiety disorders being

typically female-biased, and schizophrenia and conduct disorder being typically male-biased (Hayward & Sanborn, 2002). There is a positive association between pubertal onset, gender/sex, and the development of certain psychological disorders. For example, late pubertal onset has been linked to schizophrenia in girls and boys, and depression and antisocial behavior only in boys. In contrast, early pubertal onset has been linked with certain conduct disorder symptoms in boys and girls, and eating disorders and depression only in girls (Conley & Rudolph, 2009; Franko & Striegel-Moore, 2002; Gruzelier & Kaiser, 1996; Kaiser & Gruzelier, 1996, 1999; Susman et al.; Zehr, Culbert, Sisk, & Klump, 2007). These data suggest that gonadal hormones may contribute to the neural and behavioral changes seen during puberty, as the brain is a major target of gonadal hormones.

Investigating the contribution of hormones to the appropriate expression of social behavior is important to further understand normal and abnormal human development. However, conclusions based on human studies are limited by the necessarily correlational data derived from developmental neuroimaging research on human subjects. Animal studies can provide greater insight into the hormonal mechanisms affecting the morphology of the brain and behavior, as we can experimentally manipulate their hormonal status and environment to test our hypotheses.

Neurobiology of adolescent development

The maturation of social cognition and gain in social proficiency may occur as a result of the numerous neural changes that occur during adolescence. For example, when adults and adolescents are asked to perform a goal-directed task (i.e. assessing

nose width) while viewing fearful facial expressions, adults show greater activation than adolescents in brain regions that are involved in attentional demands, whereas adolescents show greater activation than adults in brain regions related to processing emotional content (Monk et al., 2003). These data suggest that the transition from adolescence into adulthood involves the maturation of relevant brain areas to maintain goal-directed attention in social situations, even when an emotional stimulus is presented (Monk et al., 2003). Adults and adolescents also differ in regional brain activation in response to negative and positive outcomes, with adolescents showing greater activation of the nucleus accumbens (NAc) and weaker activation of the amygdala compared to adults (Ernst et al., 2005). Interestingly, in another study, adolescents and adults had similar behavioral responses to a task measuring risk perception, but adolescents show greater activation of the medial prefrontal cortex (mPfC) than adults, suggesting that adolescents require more neural recruitment to refrain from risk-taking (Barkley-Levenson, Van Leijenhorst, & Galvan, 2013). Adults and adolescents also process social emotions differently. Unlike basic emotions, social emotions such as embarrassment and guilt require people to consider the mental state of others. When asked to imagine a scenario involving social emotions, adolescents activated relevant brain regions for mental state attribution, whereas adults activated relevant brain regions for social semantic knowledge (i.e., knowledge about social concepts and rules) (Burnett, Bird, Moll, Frith, & Blakemore, 2009). This may be due to adults having more social experience than adolescents, and thus, relying less on mentalizing and more on social knowledge.

These functional neural changes during adolescence are accompanied by structural changes. There is a gradual, linear increase in cortical white matter volume throughout adolescent development, reflecting increased myelination (Giedd, 2004; Giedd et al., 1999; Sowell, Trauner, Gamst, & Jernigan, 2002). Additionally, grey matter in the frontal and parietal lobes develops in an inverted U-shaped pattern with a peak of grey matter volume at pubertal onset, followed by a plateau, and then a steady decrease throughout adolescence (Giedd, 2004; Giedd et al., 1999; Sowell et al., 2002). The peak of grey matter occurs earlier in girls than in boys, which corresponds to sex differences in pubertal onset, further implicating gonadal hormones as a possible mechanism of this change (Giedd, 2004; Giedd et al., 1999). Both the prefrontal cortex (PfC) and NAc go through significant reorganization during adolescence. Similar to grey matter volume, synaptic proliferation peaks at puberty, followed by a plateau, and then synaptic and dendritic pruning in the PfC (Blakemore & Choudhury, 2006). In the NAc, late adolescents have a larger regional volume compared to young adults suggesting a decrease in regional volume from late teens to early 20s, possibly due to synaptic pruning (Urosevic, Collins, Muetzel, Lim, & Luciana, 2012). This reorganization of synapses in the PfC and NAc is thought to fine-tune the adolescent brain to enhance the capacity to regulate appropriate responses to social stimuli.

Neural circuitry of social cognition and social proficiency

Both the NAc and mPfC are part of the neural circuit regulating reward-based behavior. The NAc is a nodal relay in neural reward circuitry and consists of two distinct subregions: core, which contributes to cue-conditioned and goal-directed behaviors, and

shell, which is more associated with food and drug reward (Sesack & Grace, 2010). The NAc is interconnected with the ventral tegmental area (VTA), amygdala, and mPfC, and serves as the interface between arousal and motivational systems and motor output directing an animal's behavior to approach or avoid unconditioned and conditioned stimuli (Sesack & Grace, 2010). Lesions of the NAc inhibit sexual arousal and sexual behavior in male rats (Kippin, Sotiropoulos, Badih, & Pfaus, 2004; Liu, Sachs, & Salamone, 1998). Thus, the NAc is critical for behaviors relating to social cognition such as processing social information appropriately and generating a behavioral response.

The mPfC regulates decision making, moderating appropriate social behavior, reward-based learning, and behavioral flexibility, and thus, is a central region for gaining social proficiency (Euston, Gruber, & McNaughton, 2012; Vertes, 2006). The mPfC is comprised of three subregions that differ both functionally and structurally: infralimbic (IL), prelimbic (PrL), and anterior cingulate (Cg1) (Hoover & Vertes, 2007; Vertes, 2006). Our lab previously found that pubertal testosterone organizes the pattern of neural activation (via c-fos) of the male hamster Cg1 to promote behavioral flexibility of dominant males in order to maintain their dominant status; in contrast, the IL is involved in the adolescent gain in sexual reward (M. R. Bell, De Lorme, Figueira, Kashy, & Sisk, 2013; De Lorme & Sisk, 2013). The IL and PrL are more implicated in sexual behavior than Cg1, and both send projections to multiple other brain regions involved in motivation and sexual behavior including the NAc, medial preoptic area, bed nucleus of the stria terminalis, amygdala, and VTA (Balfour, Brown, Yu, & Coolen, 2006). Additionally, unlike intact male rats, males with IL and PrL lesions will continue to show

sex behavior even after it has been paired with the aversive stimulus lithium chloride (Davis et al., 2010). Therefore, these mPFC subregions are not necessary to *express* sexual behavior, but instead are thought to play a key role in mediating behavioral adaptations via inhibition when a once rewarding stimulus has aversive or maladaptive consequences.

Syrian hamster animal model to study adolescence and social cognition

Adolescent maturation of sexual behavior in the male Syrian hamster is an ideal model system for studying hormonal contributions to changes in social cognition and social proficiency. Sexual behavior is only one facet of social behavior, but it is well characterized in the male Syrian hamster and serves as a great model for studying an individual during social interactions. Specifically, unlike female rats, female hamsters remain still in the lordosis position during mating and do not display proceptive behaviors (i.e., hopping, darting, and ear wiggling). Therefore, a male hamster's sexual behavior is more independent of the female than that of a male rat and allows us to study the male's behavior in isolation from the female's behavior. However, it should be considered that this also limits the social aspect of the sexual interaction. Although not all aspects of human social cognition can be studied using animal models (e.g., perspective taking), like humans and other mammals, the Syrian hamster goes through physiological and behavioral changes during adolescence that affect how they perceive, interpret, and respond to social stimuli in an appropriate manner. Before going through this developmental transition, prepubertal male hamsters respond differently than adults to social stimuli. For example, during sexual behavior tests, testosterone-treated

prepubertal males do not mate with the females, but instead display submissive and fearful behaviors (Meek, Romeo, Novak, & Sisk, 1997; Schulz et al., 2004; Schulz, Zehr, Salas-Ramirez, & Sisk, 2009). The response of prepubertal males to the receptive female is likely the result of immature social information processing of female pheromones, which is necessary for expression of sexual behavior in this species. We infer this from experiments showing that adult, but not prepubertal, male hamsters respond to female pheromones with an increase in both preoptic area dopamine and circulating testosterone (Romeo, Parfitt, Richardson, & Sisk, 1998; Schulz et al., 2003). Furthermore, as shown by conditioned place preference experiments, adult male hamsters perceive female hamster pheromones as a natural reward, whereas prepubertal males do not show interest or perceive them as rewarding (M. R. Bell et al., 2013; M. R. Bell, Meerts, & Sisk, 2010; Johnston & Coplin, 1979). The expression of sexual behavior gradually emerges during adolescence, and sexual proficiency increases as a function of both hormone secretion and experience, marking a significant change in neural and social development (Miller, Whitsett, Vandenbergh, & Colby, 1977). In adulthood, male Syrian hamsters must appropriately display sexual behaviors to increase chances of reproduction, the marker of evolutionary success.

Organizational and activational effects of testosterone

The pubertal rise in testosterone secretion results in both activation and organization of the neural circuits underlying adult male social behaviors. Activational effects of testosterone facilitate the expression of appropriate behaviors in specific social contexts, and require the continued presence of hormone to be observed. In

contrast, organizational effects of testosterone create permanent alterations in brain and behavior at particular points during development that persist even in the absence of hormone later in life. Organizational effects of testosterone often program activational responses, usually by increasing or decreasing sensitivity or responsiveness to the activating effects of hormones in adulthood (Wallen, 2009). To assess the organizational effects of pubertal testosterone on the maturation of social cognition and social proficiency, male hamsters are deprived of testosterone either during puberty (designated NoT@P) or for an equivalent amount of time in adulthood (designated T@P), and then tested in a variety of behavioral paradigms after testosterone replacement (Figure 1). If the behavior of NoT@P and T@P males is similar, then expression of the behavior is not dependent on organizational effects of pubertal testosterone. However, if the behavior of NoT@P and T@P males differs, then adult-typical expression of the behavior depends on organizational effects of pubertal testosterone.

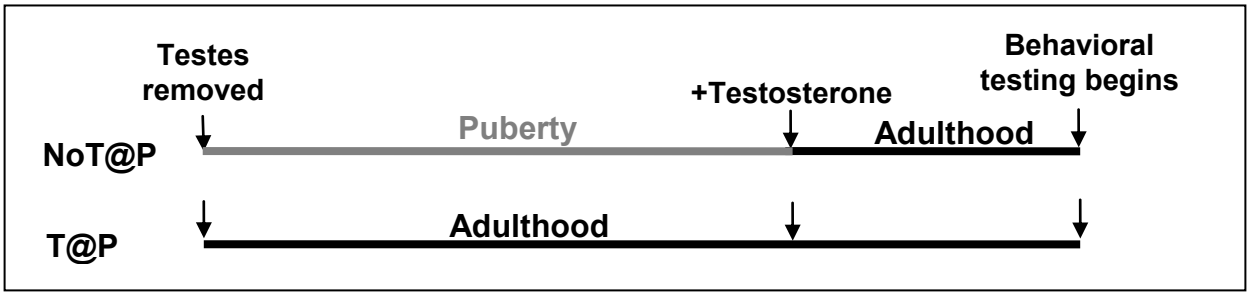

Figure 1. NoT@P/T@P Experimental Model. Prepubertal (NoT@P) and adult (T@P) male hamsters are castrated such that animals go through adolescent development either without or with endogenous testosterone, respectively. Four weeks later in adulthood, all NoT@P and T@P males receive testosterone-filled capsules two weeks before behavior testing begins.

Conditioned place preference to assess social reward

Social reward can be evaluated using the conditioned place preference (CPP) paradigm. CPP is a well-established paradigm that uses classical conditioning to assess the rewarding properties of a variety of stimuli (Schechter & Calcagnetti, 1993; Tzschentke, 1998, 2007). This is done through the repeated pairing of a rewarding stimulus such as an addictive drug, food, or social stimulus (unconditioned stimulus; UCS) with an initially non-preferred environment to induce a preference for that same environment (conditioned stimulus; CS). The *primary* motivational properties of the UCS can elicit *secondary* motivational properties attached to the CS such that the once non-preferred environment now attracts the animal based on the association between the UCS and CS (Tzschentke, 1998).

CPP has been used to determine rewarding properties of sexual behavior in many adult rodents, including male hamsters. For example, female hamsters and rats and male rats all show a CPP for access to a sexual partner (Meerts & Clark, 2007, 2009b; Mehrara & Baum, 1990; Meisel & Joppa, 1994; Meisel, Joppa, & Rowe, 1996; Oldenburger, Everitt, & de Jonge, 1992; Paredes & Alonso, 1997). In addition, male rats will also show a CPP for ejaculations and female rats will show a CPP to vaginocervical and clitoral stimulation (Agmo & Berenfeld, 1990; Agmo & Gomez, 1993; Meerts & Clark, 2009a; Parada, Chamas, Censi, Coria-Avila, & Pfaus, 2010; Tenk, Wilson, Zhang, Pitchers, & Coolen, 2009). Our lab has specifically shown that gonad-intact sexually naïve adult male hamsters find both female pheromones and interaction with a receptive female intrinsically rewarding, as they formed a CPP for each stimulus (M. R. Bell et al., 2010). However, gonad-intact sexually naïve prepubertal male hamsters do not show a CPP to female pheromones, suggesting that the rewarding properties of

these stimuli develop across puberty (M. R. Bell et al., 2013). Chapter 2 sought to investigate whether the adolescent maturation of social reward (i.e. female sexual stimuli) is due to organizational effects of pubertal testosterone by applying the CPP paradigm to our NoT@P model.

Sexual experience to assess behavioral enactment and social proficiency

Once a social stimulus has been perceived, evaluation of an appropriate response and enactment of the chosen response occurs. Social proficiency is then acquired through the ability to learn and adapt one's behavior with social experience in a social context. One specific social context is male-female sexual interaction. Sexual experience is highly rewarding and has a profound effect on subsequent sexual behavior in male rodents. Sexually experienced males require fewer intromissions to achieve ejaculations and take less time to mount, intromit, and ejaculate over time (Bialy, Rydz, & Kaczmarek, 2000; Dewsbury, 1969; Larsson, Fuxe, Everitt, Holmgren, & Sodersten, 1978; Miller et al., 1977). In addition, the number of ectopic (mis-directed) mounts decreases over time, thus increasing the number of intromissions and ejaculations (Sato, Schulz, Sisk, & Wood, 2008; Schulz et al., 2009). Therefore, the facilitation and motivation of sexual behavior increases with sexual experience as a result of gaining social proficiency.

We hypothesized that pubertal testosterone programs appropriate behavioral enactment and social proficiency in adulthood. In timed tests, NoT@P males display fewer mounts, intromissions, and ejaculations compared to T@P males (Schulz et al., 2004; Schulz & Sisk, 2006). Additionally, both gonad-intact adult and T@P males

show a significant decrease in ectopic mounts with sexual experience, whereas NoT@P males continue to show high numbers of ectopic mounts *even after sexual experience* (Schulz & Sisk, 2006). To further investigate our hypothesis, NoT@P males were given repeated sexual tests, equating their sexual experience with that of T@P males. The males' sexual behavior was quantified to detect any deficits in appropriate behavioral enactment and/or social proficiency.

ΔFosB and behavioral learning

Experience can have long-lasting effects on both behavior and neural mechanisms. The ability for the brain to adapt and change in response to repeated exposure to stimuli is of great interest. One mechanism by which the brain makes stable, long-lasting adaptations is through the regulation of gene expression. Transcription factors have been a primary research focus for neural and behavioral plasticity, as they bind to specific sequences of DNA and increase or decrease the rates at which certain genes are transcribed. One transcription factor involved in stable, long-term behavioral and neural adaptations is ΔFosB, which is a member of the Fos-Jun family and a truncated splice variant of FosB (Morgan & Curran, 1995; Nakabeppu & Nathans, 1991). ΔFosB is unique due to its stability and persistence in the brain for extended periods of time from weeks to months after chronic exposure to drugs, stress, and natural rewards, in contrast to the other Fos and Jun family members that are transiently expressed (as reviewed in McClung et al., 2004; Nestler, Kelz, & Chen, 1999). Therefore, ΔFosB plays an important role in mediating long-term adaptations involving a 'molecular switch' which contributes to social proficiency by converting acute

responses to relatively stable shifts in behavior, presumably through long term alterations in gene expression and increases in dendritic spines (McClung et al., 2004; Meisel & Mullins, 2006; Nestler et al., 1999).

This molecular switch can be found in response to a variety of rewarding stimuli. Most research on ΔFosB has focused on drug addiction, with ΔFosB induction occurring after chronic administration of cocaine, amphetamines, opiates, and nicotine (McClung et al., 2004; Nestler et al., 1999). However, it has been recently discovered that ΔFosB also mediates natural rewards such as sucrose intake, voluntary wheel-running, and sexual behavior in male and female rodents (Hedges, Chakravarty, Nestler, & Meisel, 2009; Pitchers et al., 2010; Wallace et al., 2008; Werme et al., 2002).

Sexual experience increases the rewarding aspects of sexual behavior in male rodents, which in turn enhances the expression of sexual behavior over time (Balfour, Yu, & Coolen, 2004; M. R. Bell et al., 2010; Pitchers et al., 2010; Tenk et al., 2009). Not only does sexual experience induce an accumulation of ΔFosB in mPfC and NAc in male rats and female hamsters, but over-expression of ΔFosB in NAc enhances the facilitation of sexual behavior with less experience needed compared to controls (Hedges et al., 2009; Meisel & Mullins, 2006; Pitchers et al., 2010; Wallace et al., 2008). Interestingly, the increase in ΔFosB expression is specific to sexual experience. Male rats that are exposed to females multiple times without being able to interact with them do not have increased ΔFosB expression as seen in males that have actual sexual experience (Wallace et al., 2008). Furthermore, blocking ΔFosB-mediated transcription decreases facilitation of sexual behavior and sexual motivation, and increases latencies of sexual behavior even after sexual experience in male rats (Pitchers et al., 2010). Taken

together, the results suggest that ΔFosB is an essential mediator of behavioral learning and plasticity in regards to sexual behavior and experience.

Interestingly, even after sufficient sexual experience, NoT@P male hamsters do not respond appropriately to social cues or adjust their behavior accordingly (Schulz et al., 2004; Schulz et al., 2009). Instead, they continue to show low levels of sexual behavior and high numbers of ectopic mounts relative to T@P males. The neural mechanism behind these deficits is unclear. Considering the important role of ΔFosB in long-term behavioral adaptations specific to sexual experience, it is a likely candidate for exploration.

Summary of Dissertation Experiments

The overall goal of this dissertation was to test organizational effects of pubertal gonadal hormones on the maturation of adult-typical sexual behavior, as an easily quantified social behavior that involves both social cognition and gain in social proficiency with experience. I predicted that rewarding properties of sex behavior, such as social and chemosensory information from both a female hamster and/or vaginal secretions (VS), are not dependent on the organizational effects of pubertal gonadal hormones. In contrast, I predicted that appropriate behavioral enactment and social proficiency are dependent on organizational effects of pubertal gonadal hormones, possibly through the regulation of ΔFosB in NAc and/or mPfC, which has been implicated in reward-based behavioral learning.

Chapter 2: Test the hypothesis that adolescent maturation of social reward does not depend on organizational effects of pubertal hormones. Social reward was assessed using the well-established paradigm conditioned place preference (CPP) to evaluate the rewarding properties of both vaginal secretions (VS) and interaction with a receptive female. To tease apart whether pubertal testosterone plays a role in the adolescent acquisition of rewarding properties of these natural stimuli, prepubertal (P28) and adult (P60) male hamsters were gonadectomized (GDX; NoT@P and T@P, respectively). Four weeks later (P56 and P88) the hamsters in each group received subcutaneous capsules of testosterone. After 2 weeks of testosterone replacement, at P70 and P102, the CPP paradigm was used to assess whether the presence or absence of pubertal testosterone affects the ability of males to form a CPP to VS or a receptive female.

Chapter 3: Test the hypothesis that maturation of social proficiency is dependent on organizational effects of pubertal testosterone. NoT@P and T@P males (as described above) were exposed to the same sexual experience, equating for 5 intromissions over 4 sex behavior tests each 48 hours apart. Mounts, intromissions, and ectopic (misdirected) mounts were quantified and analyzed to evaluate whether NoT@P males differed in sexual behavior compared to T@P males across all four tests.

Chapter 4: Test the hypothesis that maturation of behavioral response and social proficiency is dependent on organizational effects of pubertal testosterone through the regulation of ΔFosB induction in prefrontal cortex and/or nucleus

accumbens. For Experiment 1, there were 4 groups of males: NoT@P and T@P, as previously described, plus sham-NoT@P and sham-T@P males to control for age at time of surgery and age at time of sex testing. Half of the males in each group remained sexually naïve, while the other half was exposed to the same sexual experience once a week over five consecutive weeks. Mounts, intromissions, ejaculation, and ectopic mounts were quantified and analyzed for test 1 (sexually naïve) and test 5 (sexually experienced) to evaluate whether NoT@P males are as proficient in sexual behavior as T@P males. ΔFosB expression in prefrontal cortex (PfC) and nucleus accumbens (NAc) was also quantified using immunohistochemistry in sexually naïve males and sexually experienced males to assess if ΔFosB in PfC and/or NAc is dysregulated in NoT@P males as predicted. We found that T@P males show an increase in ΔFosB in the infralimbic cortex (IL) of the PfC with sexual experience, whereas NoT@P males do not. Therefore, for Experiment 2, ΔFosB was over-expressed in IL of NoT@P males before exposing them to sexual behavior testing as previously described in Experiment 1. Sexual behavior and ectopic mounts of ΔFosB over-expressed NoT@P males, unmodified NoT@P males, and unmodified T@P males was quantified and compared.

Chapter 2: Maturation of social reward in adult male Syrian hamsters does not depend on organizational effects of pubertal testosterone

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Introduction.

Maturation of social information processing is a universal feature of mammalian adolescence that maximizes the probability of appropriate and successful social interactions in adulthood (Dodge, 1993; Nelson et al., 2005). This phenomenon is exemplified in male Syrian hamsters by the adolescent maturation of behavioral responses to vaginal secretions, which contain female pheromones required for successful mating (M. R. Murphy & Schneider, 1970). For example, sexually naïve adult male hamsters show an unconditioned attraction to vaginal secretions that is not seen in prepubertal males (Johnston & Coplin, 1979). In addition, sexually naïve adult male hamsters show a conditioned place preference (CPP) to both vaginal secretions alone and sexual interactions with a female, whereas prepubertal males do not show a CPP to vaginal secretions, indicating that this chemosensory stimulus is unconditionally rewarding to adult, but not prepubertal, males (M. R. Bell et al., 2013; M. R. Bell et al., 2010). Thus, neural processing of vaginal secretions changes over the course of pubertal development such that they acquire positive valence, even without sexual experience. The mechanisms underlying this change in social reward are unknown.

One possible mediator is testosterone, which is produced by adult but not prepubertal males. Indeed, vaginal secretions induce a surge of testosterone within 30-60 minutes of exposure in sexually naïve adult male hamsters, but this neuroendocrine response does not occur in prepubertal males (Macrides, Bartke, Fernandez, & D'Angelo, 1974; Pfeiffer & Johnston, 1992; Romeo et al., 1998). Testosterone is intrinsically rewarding to adult male hamsters and rats (Alexander, Packard, & Hines, 1994; Packard, Cornell, & Alexander, 1997; Wood, 2004; Wood, Johnson, Chu, Schad, & Self, 2004), and one intriguing possibility is that the rewarding value of female chemosensory stimuli is mediated by the endogenous rise of testosterone elicited by them. If so, then the absence of this surge in prepubertal males could explain their inability to form a CPP to this social stimulus.

Alternatively, elevated testosterone during puberty may organize the neural circuitry responsible for evaluating the social relevance of vaginal secretions and sexual interactions with a receptive female. Using an experimental model that can distinguish organizational from activational effects of pubertal testosterone, we have shown that during puberty, testosterone organizes neural circuits underlying male sexual behavior (Schulz et al., 2004; Schulz & Sisk, 2006). In this model, male hamsters are deprived of testicular hormones, either during the normal time of puberty (via castration before 28 days of age; NoT@P), or for an equivalent amount of time in adulthood (via castration after 56 days of age; T@P), and testosterone is replaced in adulthood. Although adult testosterone treatment is sufficient to activate sexual behavior in NoT@P males, these animals still show aberrant patterns in the expression of sexual behavior, such as high levels of ectopic mounts even after sexual experience and low levels of intromissions,

mounts, and ejaculations relative to T@P males (Schulz et al., 2004; Schulz & Sisk, 2006). In addition, testosterone replacement restricted to the normal period of puberty is sufficient to normalize adult sexual behavior in males gonadectomized at postnatal day 10 (Schulz et al., 2009). Thus, pubertal testosterone (or a metabolite) organizes the adolescent brain to enhance behavioral enactment in adulthood. Because NoT@P males do mate with females, although not proficiently, we infer that the social information contained in vaginal secretions is appropriately processed, at least to the extent that male sexual behavior is activated. However, the behavioral deficits observed in NoT@P males could be related to deficits in sexual reward.

The present studies were therefore designed to investigate two potential roles of endogenous testosterone in mediating social reward in adulthood. Experiment 1 determined whether the acute testosterone surge induced by vaginal secretions is necessary for adult males to show a CPP, and Experiment 2 determined whether the ability of adult males to show a CPP to vaginal secretions or a receptive female is the result of organizational effects of pubertal testosterone.

Methods.

Animals: Sexually naïve male Syrian hamsters were ordered from Harlan Sprague-Dawley Laboratories (Madison, WI) and individually housed upon arrival in clear polycarbonate cages (30.5 x 10.2 x 20.3 cm) with *ad libitum* access to food and water in a 14:10 light/dark cycle (lights out at 1300 h). Sixty ovariectomized (OVX) adult female Syrian hamsters were used as stimulus animals. All animals were treated in accordance

with the NIH Guide for the Care and Use of Laboratory Animals and protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

Animal treatments for Experiment 1: Is the vaginal secretion-induced testosterone surge necessary for adult males to show a CPP?

Thirty-one adult male hamsters (56-70 days old; P56-70) were used in this experiment. Ten males were gonadectomized (GDX) and given two subcutaneous testosterone-filled capsules (13 mm and 5 mm of testosterone with 4 mm of sealing glue on both ends; inner diameter 1.98 mm; outer diameter 3.18 mm). These males all received pairings of the stimulus (vaginal secretions; stimulus-paired) with a specific chamber in a CPP paradigm (described below). The remaining 21 males were left gonad intact and were either stimulus-paired or controls (no stimulus pairings) in the CPP paradigm. For all hamsters, the CPP procedure began between P64-P78, one week after GDX and testosterone treatment in one group.

Animal treatments for Experiment 2: Is pubertal testosterone necessary for adult males to show a CPP for vaginal secretions or a receptive female?

Experiments 2a and 2b tested two different stimuli (vaginal secretions or receptive female, described below), but were of identical design (Figure 2). Specifically, 21 prepubertal (P28; NoT@P) and 21 adult (P58-65; T@P) male hamsters were GDX, while 22 young adult (P47-62) males remained gonad intact. Four weeks after GDX, the NoT@P and T@P males received two subcutaneous testosterone-filled capsules as described in Expt 1. After two weeks of testosterone replacement, or continued

development in gonad-intact animals, the CPP procedure began. NoT@P and T@P males served as stimulus-paired subjects, while intact males served as no-stimulus-paired controls.

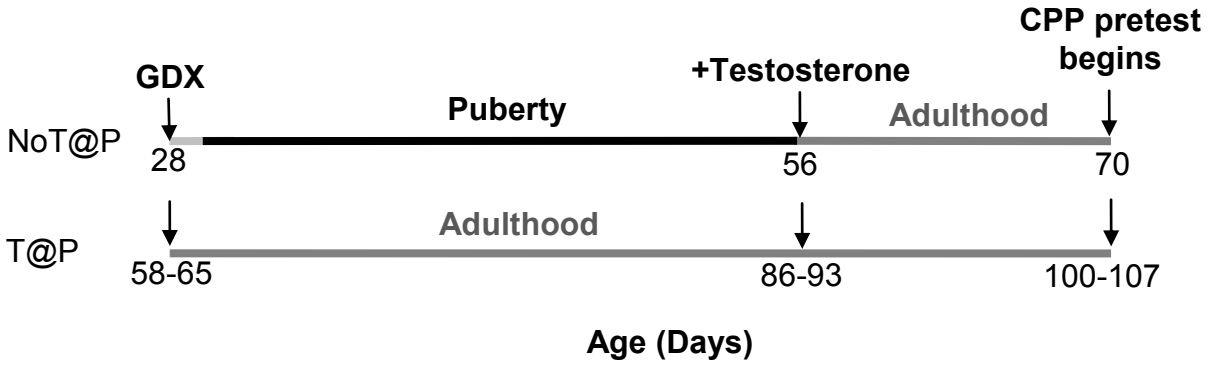

Figure 2. Experimental Design. Prepubertal (P28; NoT@P) and adult (P58-63; T@P) male hamsters were gonadectomized (GDX) such that animals experienced adolescent development either without or with endogenous testosterone, respectively. Four weeks later in adulthood, all NoT@P and T@P males received testosterone-filled capsules two weeks before CPP testing began.

Stimulus Preparation: Behavioral receptivity was induced in 60 OVX females by an injection of estradiol benzoate (10 µg in 0.05 mL sesame oil, subcutaneous) and progesterone (500 µg in 0.1 mL sesame oil, subcutaneous) 52 hours and 4-5 hours, respectively, prior to use either for collection of vaginal secretions or as a stimulus female. For Experiment 1 and 2a, an hour before conditioning sessions began, vaginal secretions were collected from 30 hormonally primed females by vaginal palpation and mixed together to total approximately 500µl. For Experiment 2b, each female was tested for receptivity 30 minutes before conditioning sessions began by placing a non-experimental, sexually experienced male from our colony into her home-cage until she displayed lordosis, at which time the male was immediately removed. Only females who showed behavioral receptivity were used in conditioning sessions.

Conditioned Place Preference (CPP) Apparatus: CPP testing occurred in an apparatus with three distinct compartments (Med Associates, St. Albans, VT). The middle compartment (12×21×21 cm) was gray with a smooth Plexiglas floor and was connected to the two outer compartments (28×21×21 cm) by manually controlled sliding doors. One outer compartment was white, with metal grid flooring. Fresh pine pellets were placed in the waste pan beneath the floor before each conditioning session. The other outer compartment was black, with black scalloped solid Plexiglas flooring, and scented with a 2% glacial acetic acid solution swabbed along the top of the walls and ceiling before each conditioning session. Time spent in each compartment was recorded using MED-PC software connected to infrared photobeams spaced at 5-cm intervals along the bottom of the apparatus. Prior work with Syrian hamsters has demonstrated that CPP is successful both in the light and dark phases of the daily light/dark cycle, as long as training and testing occur at the same time across days (Ralph et al., 2002). We have found this to be the case in our laboratory as well [unpublished observations]. Therefore, in order to complete each experiment with just one cohort of animals, control animals underwent conditioning and testing under normal white light during the late phase of their light phase. All conditioning and tests with stimulus-paired animals were conducted under dim red light 1 hour into the dark phase. The Pretest (described below) for control hamsters occurred between 1000 and 1200 h and the Pretest for stimulus-paired hamsters occurred between 1400 and 1800 h. Testing and conditioning sessions were arranged so that for a single animal, sessions occurred at the same time each day +/- 40 minutes.

Pretest: An initial place preference test, here called the Pretest, was used to determine each hamster's initial compartment preference. Following a 5-minute habituation period in the middle gray compartment, the doors were raised and the hamster was able to move freely throughout the apparatus for 15 minutes. The outer compartment in which the hamster spent the most time was defined as the initially preferred compartment. If a male did not enter both compartments at least 5 times during the Pretest, then he was excluded from the experiment.

Conditioning (adapted from M. R. Bell et al., 2010): Following the Pretest, males received a series of conditioning sessions, with one session per day across consecutive days. No Stimulus (NoS) or Stimulus (S; vaginal secretions or receptive female) conditioning sessions took place on alternating days, beginning with the NoS conditioning session. NoS conditioning sessions were in the initially preferred compartment and S conditioning sessions were in the initially non-preferred compartment. Control animals were never exposed to the stimulus, and were placed in either the initially preferred or non-preferred compartment on alternating days. The control animals were used to confirm that preference scores do not change over the period of exposure to the two different compartments in the absence of conditioning. This biased assignment approach to CPP has been used previously for both sexual and drug rewards (M. R. Bell et al., 2010; Camacho, Portillo, Quintero-Enriquez, & Paredes, 2009; Dominguez-Salazar, Camacho, & Paredes, 2008; Meerts & Clark, 2009b; Paredes & Alonso, 1997; Pierman, Tirelli, Douhard, Baum, & Bakker, 2006). The CPP

apparatus was cleaned thoroughly with 25% ethanol following each Test and conditioning session between animals, and 75% ethanol after each S day.

Experiment 1 and 2a conditioning: CPP for vaginal secretions

Conditioning sessions were each 30 minutes long and held on 10 consecutive days. Of the 500 µl of vaginal secretions, approximately 15 µl were applied to water-moistened cotton gauze packed into a 2-ml Eppendorf tube, one tube for each male, and the remaining ~200 µl were mixed with 1.5 ml of mineral oil. Stimulus-paired males were removed from their home cages and a metal spatula was used to apply approximately 50 µl of either blank (NoS) or vaginal secretion-containing (S) mineral oil directly onto their noses immediately before they were placed into the initially preferred or non-preferred compartment, respectively. Either a clean or vaginal secretion-containing Eppendorf tube was taped to the top of the back wall of each respective compartment, out of reach of the male. The purpose of the two modes of vaginal secretions delivery was to ensure exposure to both volatile and non-volatile components, as both are important and potentially have different roles in male sexual behavior (as discussed in M. R. Bell et al., 2010). Control animals received blank oil and empty tubes on all sessions.

Experiment 2b conditioning: CPP for receptive female

Conditioning sessions were each 20 minutes long and held on 6 consecutive days. Stimulus-paired males were placed alone (NoS) or with a receptive female (S) into the initially preferred or non-preferred compartment, respectively. Males were paired with a

different stimulus female for each S conditioning session. Behavior was observed to ensure that all males mated with the females throughout the S conditioning sessions, but was not quantified due to visibility limitations inherent in CPP apparatus design. Control animals were alone on all sessions.

Tests for CPP: Twenty-four hours after the last conditioning session, males were tested for their place preference following the same procedure used for the Pretest (Test 1). Two weeks later, without further training, all males were tested again (Test 2). Test 2 was used to determine if the males would maintain the stimulus-induced CPP for up to 2 weeks (Experiment 1 and 2), and if pubertal testosterone played a role in the maintenance (Experiment 2).

Plasma testosterone concentration: Twenty-four hours after Test 1, the males were put under isoflurane anesthesia and blood was collected via survival cardiac puncture. Twenty-four hours after Test 2, the males were given an overdose of sodium pentobarbital (130 mg/kg) and blood was collected via terminal cardiac puncture. Plasma testosterone concentrations were determined by radioimmunoassay. Duplicate 50-µl samples were analyzed within a single assay per experiment using the Coat-A-Count Total testosterone Kit (Diagnostic Products, Los Angeles, CA). For Experiment 1, the minimum detectable concentration for the assay was 0.1 ng/ml of testosterone and the intra-assay coefficient of variance was 4.1%. For Experiment 2, the minimum detectable concentration for the assay was 0.1 ng/ml of testosterone and the intra-assay coefficient of variance was 7.6%.

Statistical Analyses (adapted from M. R. Bell et al., 2010): To assess whether the stimuli induced a CPP, data from the Pretests and Tests were used to calculate a preference score, defined as time in the stimulus-paired compartment/(time in stimulus-paired compartment + time in no-stimulus compartment), and a difference score, defined as the time in the no-stimulus compartment−time in the stimulus-paired compartment. A repeated-measures ANOVA using a Geisser-Greenhouse correction was used to determine if there was a significant change in preference and difference scores between Pretest, Test 1, and Test 2 within each group of males with the alpha level set at $p < 0.05$. If a significant difference was revealed by the ANOVA within a specific group, post-hoc paired t-tests were used to evaluate the change in preference and difference scores between the Pretest and Tests within that group with the alpha level set at $p < 0.05$. The group sample sizes varied between and within experiments due to animals either not meeting the Pretest criteria (n = 3), loss of testosterone capsule (n = 3), or death from unknown causes between Test 1 and 2 (n = 2). Thus, there were 7-11 males/group for Experiment 1, 9-12 males/group for Experiment 2a, and 10 males/group for Experiment 2b.

Results.

Testosterone concentrations

All males used in Experiments 1 and 2 had circulating testosterone concentrations within normal adult male physiological range at both Test 1 and Test 2 (Table 1).

Plasma Testosterone (ng/ml)

	Experiment 1			*Experiment 2a*			*Experiment 2b*		
	Control	Intact	GDX+T	Control	T@P	NoT@P	Control	T@P	NoT@P
Test 1	2.37 ± 0.87	2.3 ± 0.75	2.77 ± 1.18	3.17 ± 0.43	2.26 ± 0.51	2.46 ± 0.65	2.52 ± 0.75	3.24 ± 1.38	2.86 ± 0.94
Test 2	2.91 ± 0.92	1.85 ± 0.78	2.11 ± 0.75	2.29 ± 0.80	2.03 ± 0.53	2.47 ± 0.37	3.16 ± 1.50	2.26 ± 0.49	2.01 ± 0.49

Table 1. Concentrations of circulating plasma testosterone (ng/ml) per group of each experiment taken 24 hours after Test 1 and Test 2.

Experiment 1: The vaginal secretion-induced testosterone surge is not necessary for adult males to show a CPP

As shown in Figure 3, a repeated measures ANOVA revealed a significant change in preference and difference scores between tests for both intact [$F(1, 9) = 5.11$, $p = 0.042$; $F(1, 10) = 7.43$, $p = 0.014$, respectively] and GDX+T [$F(1, 10) = 14.85$, $p = 0.002$; $F(1, 10) = 22.01$, $p = 0.001$, respectively] males, but not controls [$F(2, 19) = 0.83$, $p = 0.452$; $F(2, 19) = 1.27$, $p = 0.302$, respectively]. Follow-up paired t-tests for intact and GDX+T males revealed a significant increase in preference score and decrease in difference score between Pretest and Test 1 for both intact [$t(9) = -3.374$, $p = .008$; $t(9) = 3.987$, $p = .003$, respectively] and GDX+T [$t(8) = -6.872$, $p = .000$; $t(8) = 8.411$, $p = .000$, respectively] males. Similarly, paired t-tests revealed a significant increase in preference score and decrease in difference score between Pretest and Test 2 for both intact [$t(7) = -2.782$, $p = .027$; $t(7) = 3.168$, $p = .016$, respectively] and GDX+T [$t(7) = -5.040$, $p = .001$; $t(7) = 6.123$, $p = .000$, respectively] males. There was no significant difference between Test 1 and Test 2 preference or difference scores within either group.

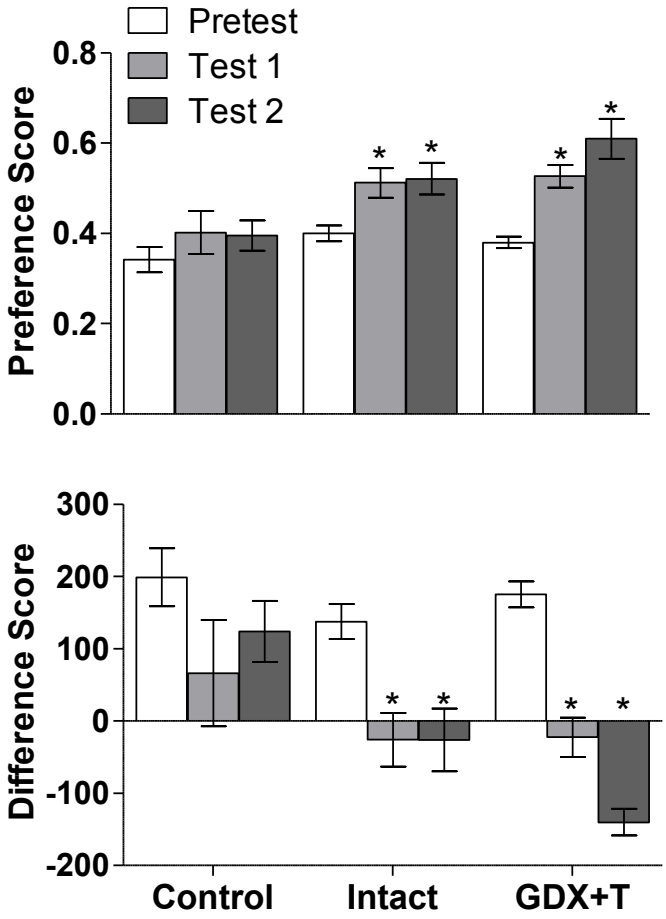

Figure 3. Mean (±SEM) preference score and difference score on Pretest, Test 1, and Test 2 demonstrate that both Intact (n = 10 for Pretest and Test 1, n = 8 for Test 2) and GDX+T (n = 9 for Pretest and Test 1, n = 8 for Test 2) males showed a CPP for vaginal secretions. There was no difference in either score between Pretest, Test 1, and Test 2 for the intact, no-stimulus-paired controls (n = 11). *indicates a significant change in preference and difference score between the Pretest and Tests within a group, $p < 0.05$.

Experiment 2a: The presence of pubertal testosterone is not necessary for adult males to show a CPP for vaginal secretions

As shown in Figure 4, a repeated measures ANOVA revealed a significant change in preference and difference scores between tests for both T@P [$F(2, 14) = 14.61$, $p = 0.001$; $F(2, 13) = 19.10$, $p = 0.000$, respectively] and NoT@P [$F(2, 18) = 9.30$, $p = 0.002$; $F(1, 15) = 7.83$, $p = 0.008$, respectively] males, but not controls [$F(2, 17) = 2.45$,

p = 0.124; $F(2, 18) = 2.63$, p = 0.108, respectively]. Follow-up paired t-tests for T@P and NoT@P males revealed a significant increase in preference score and decrease in difference score between Pretest and Test 1 for both T@P [$t(8) = -4.653$, p = .002; $t(8) = 5.053$, p = .001, respectively] and NoT@P [$t(10) = -3.468$, p = .006; $t(10) = 3.598$, p = .005, respectively] males. Similarly, paired t-tests revealed a significant increase in preference score and decrease in difference score between Pretest and Test 2 for both T@P [$t(8) = -4.159$, p = .001; $t(8) = 4.623$, p = .002, respectively] and NoT@P [$t(10) = -3.674$, p = .004; $t(10) = 3.121$, p = .011, respectively] males. There was no significant difference between Test 1 and Test 2 preference or difference scores within either group.

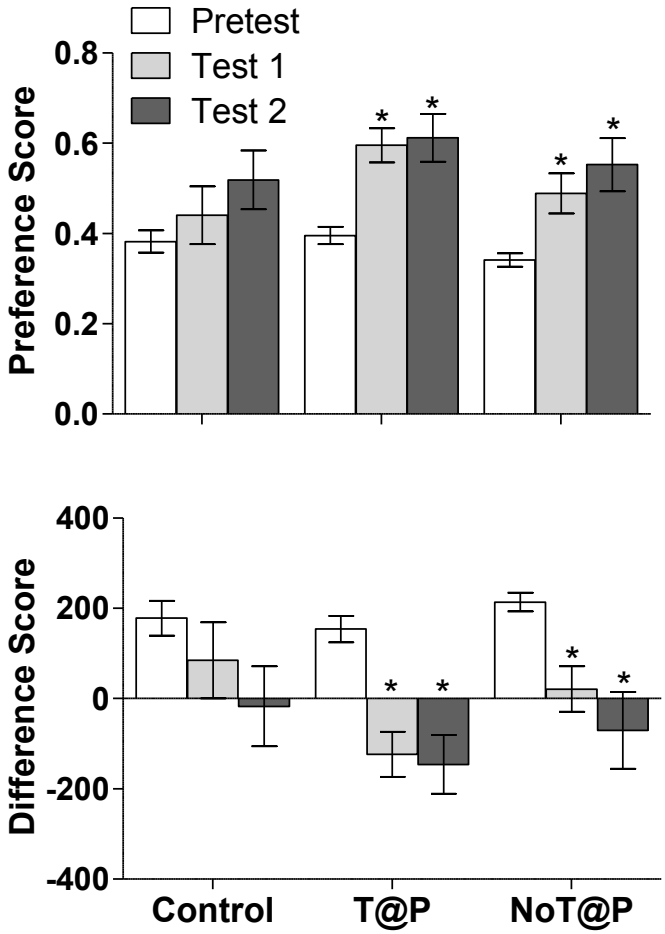

Figure 4. Mean (±SEM) preference score and difference score on Pretest, Test 1, and Test 2 demonstrate that both T@P (n = 9) and NoT@P (n = 11) males showed a CPP for vaginal secretions. There was no difference in either score between Pretest, Test 1, and Test 2 for the intact, no-stimulus-paired controls (n = 12). *indicates a significant change in preference and difference score between the Pretest and Tests within a group, $p < 0.05$.

Experiment 2b: The presence of pubertal testosterone is not necessary for adult males to show a CPP for a receptive female

As shown in Figure 5, a repeated measures ANOVA revealed a significant change in preference and difference scores between tests for both T@P [$F(1, 12) = 54.22$, $p = 0.000$; $F(1, 12) = 48.45$, $p = 0.000$, respectively] and NoT@P [$F(2, 17) = 17.20$, $p = 0.000$; $F(2, 17) = 17.44$, $p = 0.000$, respectively] males, but not controls [$F(2, 15) = 1.32$,

p = 0.290; $F(2, 15) = 1.34$, p = 0.287, respectively]. Follow-up paired t-tests for T@P and NoT@P males revealed a significant increase in preference score and decrease in difference score between Pretest and Test 1 for both T@P [$t(9) = -7.287$, p = .000; $t(9) = 6.880$, p = .000, respectively] and NoT@P [$t(9) = -5.562$, p = .000; $t(9) = 5.496$, p = .000, respectively] males, but not controls. Similarly, paired t-tests revealed a significant increase in preference score and decrease in difference score between Pretest and Test 2 for both T@P [$t(9) = -11.747$, p = .000; $t(9) = 10.644$, p = .000, respectively] and NoT@P males [$t(9) = -4.011$, p = .003; $t(9) = 3.937$, p = .003, respectively]. There was no significant difference between Test 1 and Test 2 preference or difference scores within either group.

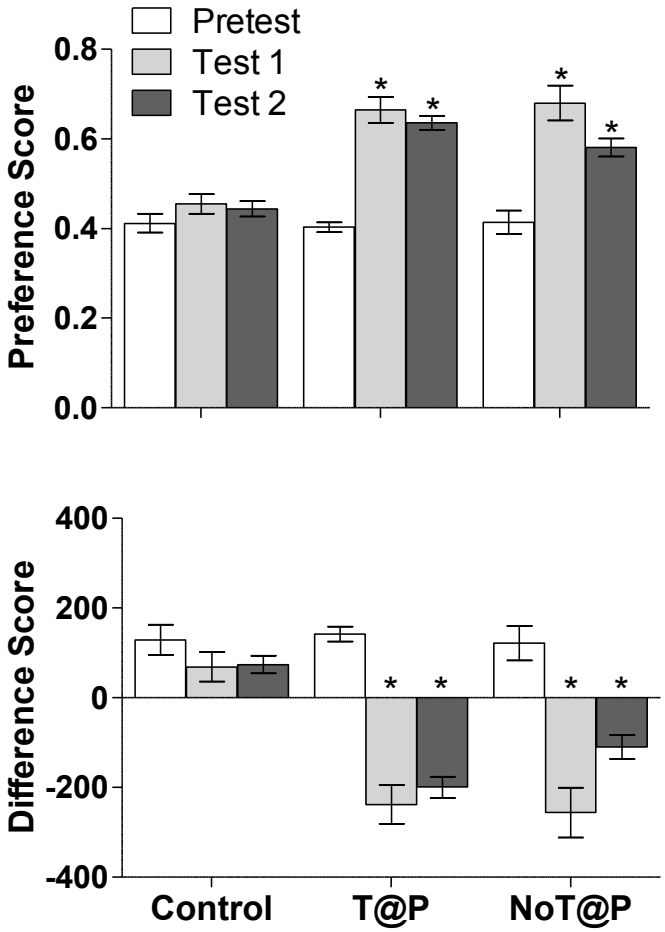

Figure 5. Mean (±SEM) preference score and difference score on Pretest, Test 1, and Test 2 demonstrate that both T@P (n = 10) and NoT@P (n = 10) males showed a CPP for a receptive female. There was no difference in either score between Pretest, Test 1, and Test 2 for the intact, no-stimulus-paired controls (n = 10). *indicates a significant change in preference and difference score between the Pretest and Tests within a group, $p < 0.05$.

Discussion.

These experiments demonstrate that social reward derived from female sexual stimuli does not depend on either an endogenous surge of testosterone in response to the social sensory experience, or on organizational effects of pubertal testosterone. In Experiment 1, testosterone-treated gonadectomized adult male hamsters formed a CPP

to vaginal secretions, even though they could not have elicited a testosterone surge in these males. Similarly, in Experiment 2, both NoT@P and T@P males formed a CPP to vaginal secretions and sexual interactions with a female, indicating that the presence of endogenous testosterone during puberty is not a requirement for the evaluation of vaginal secretions or sexual behavior as rewarding. Thus, it appears that adult social reward is not mediated by rewarding properties of testosterone per se, nor is adolescent maturation of social reward organized by testosterone.

Because testosterone is inherently rewarding (Wood, 2004; Wood et al., 2004), it seemed plausible that the testosterone surge induced by vaginal secretions could mediate the rewarding value associated with them, but this was not found to be the case. The stimulus-induced increase in testosterone is observed 30-60 minutes after the introduction of vaginal secretions (Macrides et al., 1974; Pfeiffer & Johnston, 1992; Romeo et al., 1998). Therefore, the time lapse between the chemosensory experience and the surge in testosterone may preclude a psychological association of vaginal secretions with testosterone reward. Male hamsters are dependent on neural processing of vaginal secretions in order to mate, and therefore it may be disadvantageous for the rewarding value of vaginal secretions to be tightly coupled with a physiological response (i.e. testosterone surge) that does not occur relatively soon after the chemosensory experience.

It is noteworthy that pubertal testosterone does not organize the acquisition of positive valence of female sexual stimuli, considering the importance of pubertal testosterone to the expression of adult-typical male sexual behavior (Schulz et al., 2004; Schulz & Sisk, 2006). NoT@P males show low levels of mounts, intromissions, and

ejaculations, as well as consistently high numbers of ectopic mounts after sexual experience, compared to T@P males. These data, together with those from the present experiments, suggest that pubertal testosterone organizes the neural circuitry involving sexual proficiency, but not motivation to mate. Indeed, sexual motivation and sexual performance are regulated by different neural circuitries (Becker, 2009), and may be differentially influenced by pubertal hormones. The dichotomy between sexual motivation and sexual proficiency is paralleled in social information processing theory, which distinguishes the appropriate *perception and interpretation* of social stimuli from the appropriate *enactment* of a response through behavior (Dodge, 1993). Our results suggest that organizational effects of pubertal testosterone are necessary for proficient enactment of behavioral responses to vaginal secretions and a receptive female (i.e., sexual behavior), but not for the perception of vaginal secretions or sexual behavior as rewarding. The independence from organization by pubertal hormones of early stage enactment of sexual behavior may be beneficial in preventing potential hormonal disturbances during adolescence from completely abolishing reproductive success in adulthood.

To further investigate the involvement of testosterone in the perception of social reward, we tested the persistency of the positive valence assigned to female sexual stimuli. In both of the current experiments, all stimulus-paired males maintained a CPP for vaginal secretions and a receptive female after two weeks even with no further conditioning. These data indicate that associative learning about vaginal secretions or a receptive female persists for some time regardless of whether a testosterone surge or pubertal testosterone is present. Thus, while deficits in learning to modify copulatory

behavior exist in NoT@P males, their atypical sexual behavior compared to T@P and intact males is not the result of insufficient long-term associations between sexual behavior and reward. The persistent and unconditioned reinforcing properties of vaginal secretions in sexually-naïve males demonstrate the strong saliency of these specific natural rewards, which are necessary for the expression of sexual behavior in male hamsters. Although maintenance of drug-induced CPPs have been demonstrated to last up to 12 weeks in rats (Mueller, Perdikaris, & Stewart, 2002; Mueller & Stewart, 2000), this is the first known report of sexual stimuli-induced CPPs persisting for at least two weeks.

In conclusion, the current study sought to investigate the role of endogenous testosterone in the differential behavioral responses to vaginal secretions and a receptive female between prepubertal and adult male hamsters. The data revealed that the positive valence associated with vaginal secretions in adults is independent of the chemosensory-evoked surge of testosterone in adulthood. Additionally, the ability of adult males to show a CPP to vaginal secretions and a receptive female does not depend on organizational effects of pubertal testosterone. These data, in conjunction with previous reports (Schulz, Menard, Smith, Albers, & Sisk, 2006; Schulz et al., 2004), demonstrate that adolescent maturation of social cognition involves both pubertal hormone-dependent and hormone–independent mechanisms. This dichotomy provides insight into the role of pubertal hormones in the adolescent remodeling of neural circuitry underlying social behaviors.

Chapter 3: The organizational effects of pubertal testosterone on sexual proficiency in adult male Syrian hamsters

Introduction.

During adolescence, the valence of certain social cues shifts, resulting in modifications to social behavior. An important social behavior that develops during adolescence is sexual behavior, which is induced by reproductive maturation and the secretion of gonadal hormones during puberty. Competent sexual behavior is essential for reproductive fitness, and sexual experience generally increases sexual proficiency through social learning and behavioral adaptation.

Research on adolescent maturation of sexual behavior in the male Syrian hamster has identified multiple roles for testosterone in the development of sexual competence and proficiency. First, the pubertal rise in testosterone increases the salience and rewarding properties of female pheromones, which are a necessary sensory stimulus for expression of sexual behavior (M. R. Bell et al., 2013; M. R. Bell et al., 2010; Wood, 1998). This effect of testosterone is activational, that is, it can be induced by treatment of prepubertal and adult males with testosterone, and also in males that are gonadectomized prior to puberty and treated with testosterone in adulthood (M. R. Bell et al., 2013; De Lorme, Bell, & Sisk, 2012). Second, testosterone programs sexual performance or competence via long-term, organizational influences that must occur prior to attainment of adulthood. In this instance, sexual competency is compromised if male hamsters are deprived of testosterone during adolescence, even if testosterone is replaced in adulthood. For example, in timed trials with a receptive female, male

hamsters that do not experience testosterone during puberty (i.e., gonadectomized prior to puberty and receiving testosterone replacement several weeks later, in adulthood; defined as NoT@P) display fewer mounts, intromissions, and ejaculations compared with males that do experience testosterone during puberty (i.e., gonadectomized in adulthood and receiving testosterone replacement several weeks later; defined as T@P) (Schulz et al., 2004; Schulz & Sisk, 2006). Third, testosterone appears to program the ability to become sexually proficient, that is, making behavioral adaptations with sexual experience that increase behavioral efficiency. For example, both gonad-intact adult and T@P male hamsters show a significant decrease in ectopic (mis-directed) mounts with increasing sexual experience, whereas NoT@P males continue to show high numbers of ectopic mounts *even after sexual experience* (Schulz & Sisk, 2006).

The failure of NoT@P males to adapt their behavior with experience may be because sensitivity to rewarding sensory feedback during intromissions is diminished by the absence of organizational effects during puberty. Alternatively, sensory feedback may be uncompromised, but because NoT@P males achieve fewer intromissions than T@P males in timed trials, they experience less reward. In the current study, we asked whether NoT@P and T@P males would both show a decrease in ectopic mounts if given equivalent sexual experience.

Methods.

Animals: Sexually naïve male Syrian hamsters were ordered from Harlan Laboratories (Madison, WI) and individually housed upon arrival in clear polycarbonate cages (30.5 x 10.2 x 20.3 cm) with *ad libitum* access to food and water in a 14:10 light/dark cycle

(lights out at 1400 h). The male hamsters were used as experimental animals and 48 ovariectomized female Syrian hamsters from our colony (also from Harlan Laboratories) were used as stimulus animals. All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals and protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

Animals and testosterone treatments for Experiment 1: Fifteen prepubertal (26 days old; P26) and 16 adult (P56-63) males were used for this experiment. These prepubertal and adult hamsters were all gonadectomized two days after arrival, P28 and P58-65, respectively. Thus, the prepubertal males did not experience testosterone during the normal time of puberty and adolescence (NoT@P), whereas the adult hamsters had undergone normal pubertal development before being gonadectomized (T@P). Four weeks after gonadectomy, all NoT@P (P56) and T@P (P86-93) males received two subcutaneously implanted testosterone capsules (one 21 mm and one 13 mm; inner diameter 1.98 mm; outer diameter 3.18 mm). Two weeks following testosterone replacement, sexual behavior testing began (P70 for NoT@P males and P100-107 for T@P males).

Twenty-four hours after behavior testing, blood samples were collected to ensure the effectiveness of the testosterone capsules. Blood was collected either by cardiac puncture or immediately following rapid decapitation. All males had adult physiological concentrations of circulating testosterone, with T@P males having an average concentration of 5.23 ± 2.12 ng/ml and NoT@P males having an average concentration of 6.78 ± 2.33 ng/ml.

Animals for Experiment 2: Due to unexpected behavioral results found in Experiment 1 (i.e. a drop-off in rates of mounting and intromitting in the 4th trial), Experiment 2 was performed to observe sexual behavior in unmanipulated, gonad-intact male hamsters. Ten adult males arrived in the laboratory on P56-70, and two weeks later, sexual behavior testing began when they were P70-84.

Stimulus Females: Behavioral receptivity was induced in ovariectomized female hamsters by treatment with estradiol benzoate (10 µg in 0.05 mL sesame oil, sc injection) and progesterone (500 µg in 0.1 mL sesame oil, sc injection) 52 hours and 4-5 hours, respectively, prior to use in sexual behavior tests with males. Each receptive female was used only once per sexual behavior test and was never paired with the same male more than once throughout the experiments.

Sexual behavior tests: Testing began 1 hour into the dark-cycle under dim red light in clean large glass aquaria. Four tests were conducted at 48 hour intervals. Following a 2 minute acclimation period to the aquaria, each male was allowed to interact with a receptive female until the male achieved 5 intromissions or up to 30 minutes if 5 intromissions did not occur. Behavior was video recorded for later quantification.

Sexual behavior analysis: The three behaviors investigated for both experiments were ectopic mounting (male grips female tightly and displays fast thrusting, but the mount is not vaginally oriented), mounting (male orients himself up on the female's hind flanks, grips her tightly with his forepaws, and displays fast thrusts), and intromissions (male is

vaginally-oriented and makes a long-lasting thrust resulting in vaginal penetration). Ejaculations did not occur during any of the trials. Because males reached the 5 intromission criterion within varying times, the frequency of the behaviors displayed was divided by the total test time per male per trial. If a male displayed no mounts of any kind within 30 minutes, then his ectopic mounting data were not included in the statistical analysis (i.e., zeroes were not recorded), because inclusion would skew the data to reflect more efficient behavior than actually displayed.

Statistical Analysis: Multilevel modeling (MLM) was used to provide an integrated assessment of pubertal testosterone and/or sexual experience (trial 1-4) on three separate measures of behavior (ectopic mounts, mounts, and intromissions). The model treated the animal as the upper-level sampling unit and trial as the lower-level sampling unit. For Experiment 1, pubertal testosterone (NoT@P, T@P, between subject variable) and sexual experience (trials 1-4, within subject variable) were independent variables. For Experiment 2, sexual experience (trials 1-4) was the sole independent variable. The error structure was modeled to impose the traditional homoscedasticity assumption used in analysis of variance (ANOVA). MLM provides a more powerful analysis than a traditional repeated measures ANOVA because it integrates non-independence between samples from the same subject in the model and allows unequal sample sizes within the repeated measures. The nature of the main effect of sexual experience was determined using a Bonferroni correction for both experiments, and interactions for Experiment 1 were followed up by MLMs using a

Bonferroni correction within a subset of animals, as appropriate. $p \leq 0.05$ was considered significant.

Results.

Experiment 1: Effects of pubertal testosterone and sexual experience on ectopic mounting, mounting, and intromissions

Rate of ectopic mounts is dependent on pubertal testosterone

MLM revealed a significant main effect of pubertal testosterone on ectopic mounts per minute [$F(1, 29) = 16.03$, $p < 0.001$; Figure 6A] with T@P males displaying less ectopic mounts per minute than NoT@P males across all 4 trials.

Rate of mounts and intromissions is dependent on pubertal testosterone and sexual experience

MLM revealed a pubertal testosterone x trial interaction on mounts per minute [$F(3, 87) = 3.75$, $p = 0.014$; Figure 6B]. A follow-up MLM found a significant main effect of trial on mounts per minute within T@P males [$F(3, 45) = 4.66$, $p = 0.006$] with T@P males mounting more per minute on trial 3 compared to trial 4. In contrast, trial did not significantly affect mounts per minute within NoT@P males [$F(3, 42) = 0.78$, $p = 0.510$].

MLM revealed a pubertal testosterone x trial interaction on intromissions per minute [$F(3, 87) = 3.94$, $p = 0.011$; Figure 6C]. A follow-up MLM found a significant main effect of trial on intromissions per minute within T@P males [$F(3, 45) = 4.22$, $p = 0.010$] with T@P males mounting more per minute on trial 3 compared to trials 1 and 4. In

contrast, trial did not significantly affect intromissions per minute within NoT@P males [$F(3, 42) = 1.22$, $p = 0.314$].

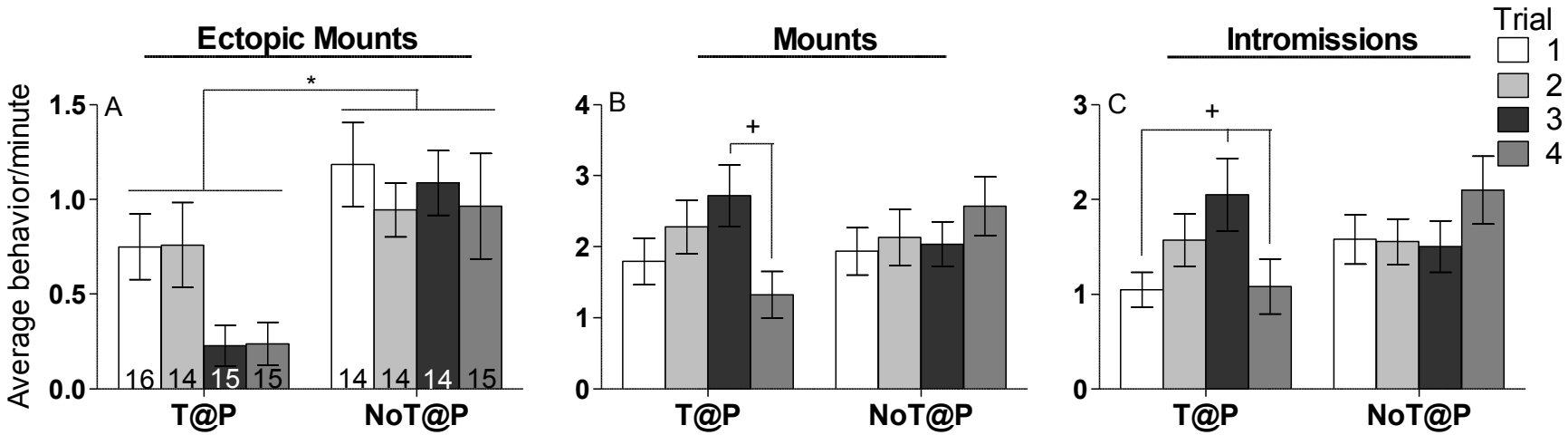

Figure 6. Effect of pubertal testosterone and sexual experience on mean (±SEM) number of ectopic mounts (A), mounts (B), and intromissions (C) per minute across 4 sexual behavior trials. A: There was a main effect of pubertal testosterone with T@P males showing significantly lower rates of ectopic mounting compared to NoT@P males. Sample sizes per trial for each group represented in bars. B: There was an interaction between pubertal testosterone and trial with T@P males (n = 16) showing a decrease in rate of mounting from trial 3 to trial 4. This was not found in NoT@P males (n = 15). C. There was an interaction between pubertal testosterone and trial with T@P males (n = 16) showing an increase in rate of intromitting from trial 1 to trial 3, and then a decrease in rate of intromitting from trial 3 to trial 4. This was not found in NoT@P males (n = 15). * indicates a significant difference between NoT@P and T@P males with $p \leq 0.05$. +indicates an interaction between pubertal testosterone and trial with $p \leq 0.05$.

Experiment 2: Effects of sexual experience on ectopic mounting, mounting, and intromissions in intact males

Rate of ectopic mounting and sexual experience

MLM did not reveal a significant main effect of sexual experience on ectopic mounts per minute [$F(3, 22) = 2.90$, $p = 0.058$; Figure 7A]. However, the *p* value indicates a trend approaching significance with intact males displaying fewer ectopic mounts per minute in trials 3 and 4 compared to trials 1 and 2.

Rate of mounting is not dependent on sexual experience

MLM did not reveal a significant main effect of sexual experience on mounts per minute [$F(3, 27) = 2.46$, $p = 0.085$; Figure 7B].

Rate of intromissions is dependent on sexual experience

MLM revealed a significant main effect of sexual experience on intromissions per minute [$F(3, 27) = 4.06$, $p = 0.017$; Figure 7C] with males displaying more intromissions per minute on trial 3 compared to trials 1 and 2.

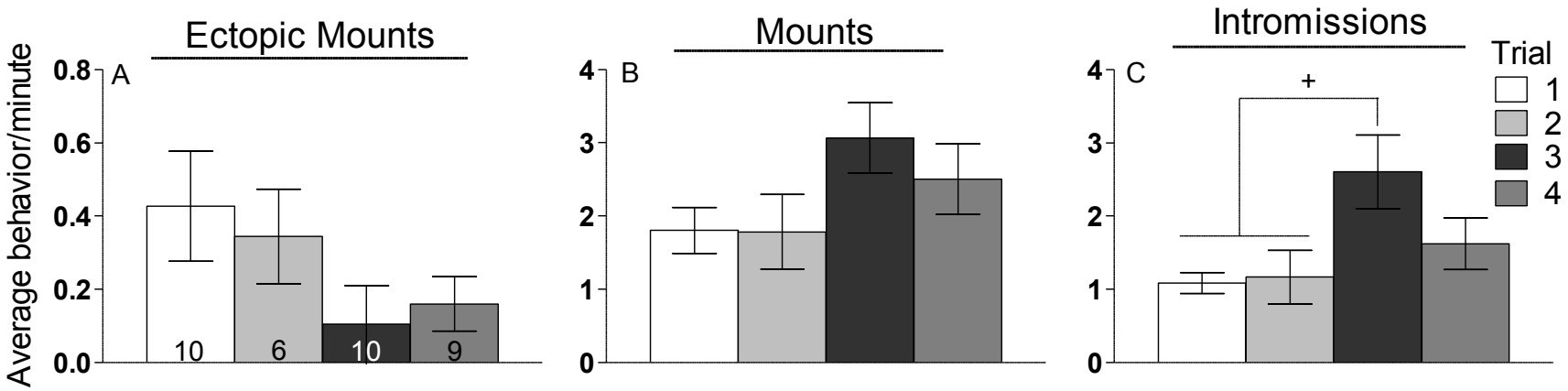

Figure 7. Effect of sexual experience on mean (±SEM) number of ectopic mounts (A), mounts (B), and intromissions (C) per minute across 4 sexual behavior trials in gonad-intact males. A: There was a trend towards a main effect of trial (p = 0.058) with intact males showing a decrease in rates of ectopic mounting across the 4 trials. Sample sizes per trial represented in bars. B: There was no effect of trial on the rate of mounting (n = 10). C. There was a main effect of trial with intact males (n = 10) showing an increase in rate of intromitting from trials 1and 2 to trial 3. * indicates a significant difference between trials with $p \leq 0.05$.

Discussion.

The current study investigated whether males deprived of pubertal testosterone would make behavioral adaptations similar to those of males that experienced pubertal testosterone if given equivalent sexual experience. We found that when limited to only five intromissions per test, both T@P and gonad-intact males adjusted their behavioral responses to a receptive female after repeated interactions, e.g. decreasing ectopic mounts per minute and increasing intromissions per minute, whereas NoT@P males did not. These results provide evidence that testosterone, acting during puberty and adolescence, programs sexual proficiency by promoting behavioral adaptations that increase sexual efficiency.

Ectopic mounting is commonly displayed by sexually naïve males during their first sexual encounter. Although ectopic mounting is not necessarily inappropriate in inexperienced males, it becomes maladaptive if it is maintained with sexual experience because it decreases behavioral efficiency. Therefore, ectopic mounting should decrease over time as a male becomes sexually proficient. NoT@P males had consistently high rates of ectopic mounting across all four trials, whereas T@P males had overall lower rates compared to NoT@P males, and also decreased their rate of ectopic mounting with sexual experience. Intact males also showed the expected decreased rates of ectopic mounting with sexual experience. Thus, even when NoT@P males have similar sexual experience as T@P males, they still continue to maintain ectopic mounting, which is no longer context-appropriate after multiple sexual encounters.

This inability to adjust behavior with experience found in NoT@P males was not limited to ectopic mounting. Although NoT@P males had similar initial rates of mounts and intromission compared to T@P males, T@P males eventually decreased their rates of these behaviors by the fourth trial, whereas NoT@P males had consistent rates across all 4 trials. These data were unexpected as we predicted T@P males would show steady increases in rates of mounts and intromissions across all 4 trials. However, intact males had similar patterns of behavior as T@P males showing an initial increase in mounts and intromissions and then a decrease by the fourth trial. These data indicate that just as ectopic mounting becomes inefficient with sexual experience, displaying high rates of mounts and intromissions without ejaculations may also be inefficient. Therefore, the consistent rates of mounts and intromissions shown by NoT@P may be maladaptive as the behavioral paradigm used in the current study did not allow for ejaculations to occur, and thus, continuing to mate with the female in that context is not an efficient reproductive strategy.

Testosterone during puberty may organize neural circuitry underlying adaptability and proficiency with experience. One possible region of interest is the medial prefrontal cortex as it is implicated in decision making, behavioral flexibility, reward-based learning, and moderating appropriate social behavior (Euston et al., 2012; Vertes, 2006). The prefrontal cortex comprises three subregions that differ both functionally and structurally, infralimbic (IL), prelimbic (PrL), and anterior cingulate (Cg1) (Hoover & Vertes, 2007; Vertes, 2006). We previously reported that along with deficits in behavioral adaptations during repeated male-male interactions, neural activation of Cg1 after following an interaction with another male was less in NoT@P males than in T@P

males (De Lorme & Sisk, 2013). Additionally, male rats with PrL and IL lesions will continue to mate with a receptive female, even after being paired with the aversive stimulus lithium chloride, whereas males with intact PrL and IL do not continue mating (Davis et al., 2010). These data imply that the PrL and IL are important for inhibiting behavior that was once rewarding, but that has become maladaptive or aversive after a particular experience. While T@P males showed a decrease in rates of sexual behavior after being paired with a female without the rewarding stimuli of achieving an ejaculation, NoT@P males continued to show high rates of ectopic mounts, mounts, and intromissions. Therefore, testosterone during puberty may organize the PrL and IL to regulate behavioral inhibition when a once rewarding stimulus develops maladaptive consequences.

In conclusion, testosterone during puberty exerts organizational effects on the ability to adapt behavior with social experience. Ectopic mounting is not optimal for successful reproduction, and thus, should decrease with sexual experience as males learn to exhibit appropriate sexual behavior. Sexually naïve NoT@P males showed high rates of ectopic mounting compared to sexually naïve T@P males, which is not necessarily maladaptive initially, but NoT@P males continued to show these high rates of ectopic mounting with sexual experience while T@P and gonad-intact males declined in rate of ectopic mounting after sexual experience. Interestingly, when sexual experience was equated for T@P and NoT@P males, NoT@P males did not seem to have deficits in enacting a behavioral response to a receptive female as they displayed similar *overall* rates of mounts and intromissions to those of T@P males. However, the pattern of sexual behavior across the 4 trials was quite different between the groups.

NoT@P males had consistent rates of mounts and intromission across the 4 trials, whereas T@P males showed an inverted U-shape pattern with an increase in rates of sexual behavior followed by a drop-off by the 4th trial. This inverted U-shape pattern was also observed in gonad-intact males. A possible explanation for the drop-off is that our behavioral paradigm did not allow ejaculations to occur, and therefore, mounts and intromissions were no longer rewarding to T@P and gonad-intact males. Because NoT@P males continued to show consistent rates of mounts and intromission, we conclude that testosterone during puberty programs the ability to adapt behaviors (via inhibition) in a social context-dependent manner possibly through organization of the PrL and IL of the medial prefrontal cortex.

Chapter 4: Pubertal testosterone regulates the induction of ΔFosB in the infralimbic cortex to program sexual proficiency in male Syrian hamsters

Introduction.

Sexual behavior emerges during adolescence as a result of the pubertal rise in gonadal hormones. In males, testosterone exerts both organizational and activational actions to promote adult-typical sexual behavior. Organizational effects of testosterone refer to permanent changes in brain structure and function that persist beyond the time of exposure to testosterone, whereas activational effects are transient and depend on both the continued presence of testosterone and prior organization of the underlying neural circuitry. Research initially focused on the organizational effects of gonadal hormones during perinatal development; however, gonadal hormones also exert organizational effects on the brain and behavior during puberty. For example, male hamsters deprived of testosterone during puberty via prepubertal gonadectomy (NoT@P) have a reduced sexual behavioral response to testosterone in adulthood, displaying fewer mounts, intromissions, and ejaculations compared to males that experienced a typical puberty but then were gonadectomized in adulthood (T@P) (Schulz et al., 2004; Schulz and Sisk, 2006). Thus, testosterone during puberty is necessary for the organization of neural circuitry underlying adult-typical testosterone-activated sexual behavior in males.

In order to increase reproductive success, animals must also learn to adapt their behavior with sexual experience. The ability to make appropriate behavioral adaptations with sexual experience leads to gaining sexual proficiency. Often, making behavioral

adaptations requires the inhibition of certain behaviors that are no longer rewarding or advantageous. Ectopic (mis-directed) mounting is one such behavior that is displayed by sexually naïve male hamsters, but with sexual experience, it decreases and remains low to enhance sexual proficiency and reproductive success. However, NoT@P males show high numbers of ectopic mounts compared to T@P males even after sexual experience, whether either time with the female or absolute amount of sexual experience is equated (see Chapter 3; Schulz and Sisk, 2006). In addition, NoT@P males also continue to display consistent rates of mounts and intromissions during sexual encounters that do not result in ejaculations, whereas T@P males show a decrease in the rates of these behaviors, presumably because it is no longer adaptive to display mounts and intromissions without being able to achieve ejaculation (see Chapter 3). These data suggest that testosterone during puberty may play a role in organizing neural circuitry involved in behavioral inhibition to promote increased social proficiency.

The medial prefrontal cortex (mPfC) and nucleus accumbens (NAc) are both part of the neural circuitry that regulates motivated behaviors such as sexual behavior. The mPfC is involved in behavioral flexibility and decision-making, whereas the NAc is critical for processing social information, evaluating the incentive salience (motivational strength) of social stimuli, and generating a behavioral response (Euston et al., 2012; Sesack & Grace, 2010). Long-term induction of the transcription factor ΔFosB within both the mPfC and NAc occurs after sexual experience in both male and female rodents (Hedges et al., 2009; Pitchers et al., 2010; Wallace et al., 2008). In addition, over-expression of ΔFosB in the NAc of sexually naïve female and male rodents leads to increased sexual performance and motivation. In contrast, blocking the transcription of

genes normally induced by ΔFosB through the over-expression of ΔJunD, which competitively binds ΔFosB, leads to decreased sexual performance and motivation (Hedges et al., 2009; Pitchers et al., 2010; Wallace et al., 2008). Therefore, ΔFosB induction within the mPfC and NAc appears to be an element of the restructuring of neural circuits that underlie long-term behavioral adaptations with sexual experience, presumably by regulating transcription of downstream target genes related to synaptic and behavioral plasticity.

The purpose of experiments in this Chapter was two-fold. Experiment 1 first investigated whether a deficiency in ΔFosB expression in the mPfC and/or NAc is correlated with the behavioral deficits and/or differences seen in NoT@P males compared to T@P males. Experiment 2 next determined whether over-expression of ΔFosB in the mPfC is sufficient for NoT@P males to acquire the ability to adapt their behavior with experience, effectively behaving similarly to T@P males.

Methods.

Animals: For both Experiments 1 and 2, prepubertal (P21-26) sexually naïve male Syrian hamsters were ordered from a Harlan in 2 separate groups (arriving 4 weeks apart) and individually housed upon arrival in clear polycarbonate cages (30.5 x 10.2 x 20.3 cm) with *ad libitum* access to food and water in a 14:10 light/dark cycle (lights out at 1400 h). Male hamsters were used as experimental animals and ovariectomized female Syrian hamsters from our colony (also from Harlan) were used as stimulus animals in behavioral tests. All animals were treated in accordance with the NIH Guide for the Care

and Use of Laboratory Animals and protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

Stimulus Females: Behavioral receptivity was induced in ovariectomized female hamsters by treatment with estradiol benzoate (10 µg in 0.05 mL sesame oil, sc injection) and progesterone (500 µg in 0.1 mL sesame oil, sc injection) 52 hours and 4-5 hours, respectively, prior to using the females in sexual behavior tests with males. Each receptive female was used only once per sexual behavior test and was never paired with the same male more than once throughout the experiments.

Sexual behavior testing: Testing began 1 hour into the dark phase of the light-dark cycle under dim red light. Following a 2 minute acclimation period in a clean large glass aquarium, each male was allowed to interact with a receptive female until the male achieved the sexual behavior criteria for that trial, or after 30 minutes had passed. Sexual behavior was kept equivalent (instead of absolute amount of time with the female) across all of the males to ensure that any behavioral differences between groups were not due to different sensory experience, as previous data from the lab found that during timed-tests, NoT@P males achieve fewer intromissions and ejaculations than T@P males, and thus, a lesser amount of rewarding sensory feedback (Schulz et al., 2004; Schulz & Sisk, 2006). The behavior criteria that ended each trial were: 1 ejaculation for trial 1, 1 ejaculation plus 2 intromissions for trial 2, 2 ejaculations for trial 3, 1 ejaculation plus 2 intromissions for trial 3, and 1 ejaculation for trial 5. The behavioral criteria were varied so the male hamsters would not associate a specific

sexual behavior with being removed from the aquarium. Behavior testing was conducted once a week for five weeks (one of the five above-described trials/week). Males were excluded if they did not meet the sexual behavior criteria in at least 3 out of the first 4 trials. Behavior was recorded for later quantification.

Sexual behavior quantification: The behaviors investigated for both experiments were: rate of ectopic mounting (male grips female tightly and displays fast thrusting, but the mount is not vaginally oriented), latency to mount (male orients himself up on the female's hind flanks, grips her tightly with his forepaws, and displays fast thrusts), latency to intromit (male is vaginally-oriented and makes a long-lasting thrust resulting in vaginal penetration), latency to ejaculate (occurring after a series of intromissions followed by the male self-grooming and showing no sexual interest in the female for at least 20 seconds), and number of intromissions to reach ejaculation. Ejaculation latency and intromissions to ejaculate reflect sexual performance, whereas latencies to mount or intromit are measures of sexual motivation (Hull, Meisel, & Sachs, 2002). Latencies to mount and intromit were timed from the moment the female was introduced to the male in the aquarium, and latency to ejaculate was defined as the amount of time that passed between the first intromission and ejaculation. Rate of ectopic mounting was used because males reached behavioral criteria to end the trial within varying times; therefore, a rate was calculated by dividing the frequency of ectopic mounts displayed by the total test time per male per trial. If a male displayed no mounts of any kind within 30 minutes, then his ectopic mounting data were not included in the statistical analysis (i.e., zeroes

were not recorded), because inclusion would skew the data to reflect more efficient behavior than actually displayed.

Behavioral outliers and sample sizes: For each behavior, a box-plot utilizing stem-and-leaf descriptives was used to identify the extreme data points within each experimental group for both experiments. Dixon's Q-test was then used to determine if the extreme was a single statistical outlier. If the extreme was identified as an outlier for any behavior, the data for that animal were taken out of the analysis. One T@P male was an extreme high outlier for intromissions to ejaculation after sexual experience in Experiment 1 and one NoT@P-ΔFosB male was an extreme high outlier for ectopic mounts per minute after sexual experience in Experiment 2. Final sample sizes for each behavior are reflected in the tables and figures.

Sexual behavior statistical analysis: Multilevel modeling (MLM) was used to provide an integrated assessment of experimental group (respectively for each experiment) and/or sexual experience (trial 1: naïve vs. trial 5: experienced) on the measures of behavior described above. The model treated the animal as the upper-level sampling unit and sexual experience as the lower-level sampling unit. Experimental groups (between subjects variable) and sexual experience (trials 1 and 5, within subject variable) were independent variables. The error structure was modeled to impose the traditional homoscedasticity assumption used in analysis of variance (ANOVA). MLM provides a more powerful analysis than a traditional repeated measures ANOVA because it integrates non-independence between samples from the same subject in the model, and

allows unequal sample sizes within the repeated measures. The nature of the main effect of experimental group for Experiment 2 was determined using a Bonferroni correction, and interactions were followed up by one-way ANOVAs for between subject measures or MLM for repeated measures within a subset of animals, as appropriate. $p \leq 0.05$ was considered significant. For Experiment 1, analysis was performed separately for GDX and sham groups.

Experiment 1 Methods:

Animal model (Figure 8): A total of 111 males was used in this experiment, which was conducted in 3 cohorts (see Table 2 for distribution of sample sizes within each group). Four to five weeks after the first group of prepubertal males arrived and 2-7 days after the second group of prepubertal males arrived, half of the first group (now adults, P56) and half of the second group of prepubertal (P28) males were either gonadectomized (GDX; T@P and NoT@P, respectively) or received sham surgeries (sham-T@P and sham-NoT@P, respectively). The sham groups were used as age-matched controls to confirm that behavioral differences found between T@P and NoT@P males were due to the presence or absence of testosterone during puberty, and not to age at the time of surgery or to age at the time of behavior testing. Four weeks later, when all of the males were in adulthood, the T@P and NoT@P males received two T capsules (one 21 mm and one 13 mm; inner diameter 1.98 mm; outer diameter 3.18 mm) and the sham-T@P and sham- NoT@P males received blank capsules of matched size. Thus, this design yielded four experimental groups: T@P, NoT@P, sham-T@P, and sham-NoT@P. Two weeks later, approximately half of the males from each group were used for sexual

behavior testing, while the other half of each group remained sexually naïve. The sexually naïve males were placed in an empty aquarium for 5 minutes before sexual behavior testing began for the sexually experienced males. This was done to ensure that the sexually naïve males were not exposed to female pheromones that could have been present in the behavior testing room following sexual behavior testing, and eliminated any confound of handling and being placed in an aquarium, each of which could potentially influence the expression of ΔFosB in brain regions of interest.

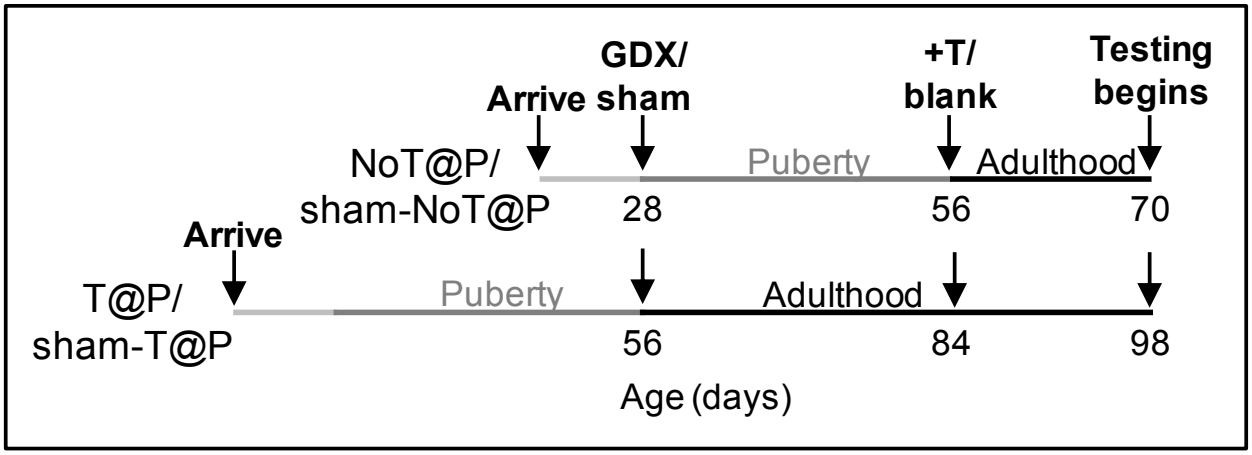

Figure 8. Animal model for Experiment 1. T@P and sham-T@P males arrived 4 weeks prior to NoT@P and sham-NoT@P males to control for the age of shipping and environment during puberty. After the NoT@P and sham-NoT@P arrived, males were either gonadectomized (GDX) or sham-GDX during adulthood (P56; T@P and sham-T@P) or prepubertally (P28; NoT@P and sham-NoT@P). Four weeks later, T@P and NoT@P males received testosterone (T)-filled capsules and sham males receive empty capsules of the same size. Sexual behavior testing began two weeks later.

Group	Sexually naïve	Sexually experienced	Total
T@P	14	15	29
NoT@P	14	15	29
sham-T@P	13	14	27
sham-NoT@P	14	12	26

Table 2. Sample sizes per group for Experiment 1.

Tissue collection: Twenty-four hours after the final behavior test described above, 64 males (n = 8 per group) were deeply anesthetized with an overdose of sodium pentobarbital (130mg/kg, ip). Although the pan-FosB primary antibody detects both FosB and ΔFosB, previous studies have confirmed that FosB is degraded within 18-24 h post-stimulus (in this case either sexual behavior or being placed in an empty aquarium); therefore, the ΔFosB immunoreactive (-ir) cells are specifically ΔFosB positive when examined at the chosen time of tissue collection relative to the behavioral test (Perrotti et al., 2005; Perrotti et al., 2004; Perrotti et al., 2008; Pitchers et al., 2010; Wallace et al., 2008). Blood was collected via cardiac puncture, and the animals were perfused with 100 ml of buffered saline rinse and 150 ml of 4% paraformaldehyde. Brains were collected, post-fixed over-night in 4% paraformaldehyde, and then stored in 20% sucrose until sectioning. Sections were cut (40μm) into four coronal series using a cryostat; one series was used for ΔFosB/FosB immunohistochemistry staining to identify, trace, and count cells in the cingulate (Cg1), prelimbic (PrL), and infralimbic (IL) cortices of the mPfC and shell and core of NAc (described below).

The rest of the males (n = 47, with n = 5-7 per group) were decapitated 18-24 hours after the final behavior test and trunk blood was collected. Their brains were rapidly removed and allowed to cool in saline (4ºC) for 7 minutes. The right and left mPfC and NAc were dissected out using a dissecting block that measures 2 mm sections starting with the front of the forebrain to identify specific brain regions. The mPfC was identified using the midline and corpus callosum as reference. The NAc was identified using the anterior commissure (AC) and lateral ventricle (LV), as the NAc

wraps around the AC and is lateral-ventral to the LV. The sections were immediately frozen on dry ice and kept at -80 ºC until processing for future protein assays.

Plasma from both blood collections was used to determine testosterone concentrations by radioimmunoassay. Plasma concentrations of testosterone were determined from duplicate 50 µl samples in a single assay using the Coat-A-Count Total Testosterone Kit (Diagnostic Products, Los Angeles, CA). The intra-assay coefficient of variance was 3.5% and the minimum limit of detectability was 0.12 ng/ml.

ΔFosB Immunohistochemistry: Free floating sections were rinsed 3 times for 5 minutes with 0.05 M Tris-buffered saline (TBS; pH 7.6) between all incubations with reagents. Sections were exposed to 0.1% hydrogen peroxide for 10 minutes at room temperature to destroy endogenous peroxidases. The sections were then blocked in TBS containing 20% normal goat serum (NGS) and 0.3% Triton X-100 for 60 minutes. Sections were then incubated overnight at 4ºC in 2% NGS and 0.3% Triton X-100 and the pan-FosB rabbit polyclonal antibody (1:10,000 dilution for a final concentration of 0.02 µg/ml; sc-48 Santa Cruz Biotechnology, Santa Cruz, CA, USA). The pan-FosB antibody was raised against an internal region shared by FosB and ΔFosB (Hedges et al., 2009). After primary antibody incubation, the sections were washed in TBS, and then incubated for 1 hour in goat anti-rabbit secondary antibody (1:500 dilution) containing 2% NGS and 0.3% Triton X-100 in TBS. Then, the sections were incubated in Vectastain ABC Elite kit (Vector, Burlingame, CA) for 1 hour at room temperature before visualizing the immunoreactivity with diaminobenzidine (DAB, 0.5 mg/ml plus NiCl with 0.025% H_2O_2). The sections were rinsed in TBS 4 times before mounting them onto glass slides. The

mounted sections were then put through a series of ethanols and xylene before coverslipping.

Immunohistochemistry analysis: Tissue was analyzed using Neurolucida software (Microbrightfield, Inc) for ΔFosB-ir cells in 3 anatomically matched sections for both PfC and NAc. For the mPfC, a 450 x 450 μm box was placed in each subregion (Cg1, Prl, IL) relative to brain midline and corpus callosum landmarks, and for the NAc, two 250 x 250 μm boxes were placed in the NAc core and a 250 x 250 μm box in the NAc shell relative to anterior commissure and lateral ventricle (Figure 9). The Morin and Wood (2001) hamster atlas was also used as a reference. Box placements were determined bilaterally under a 4x objective.

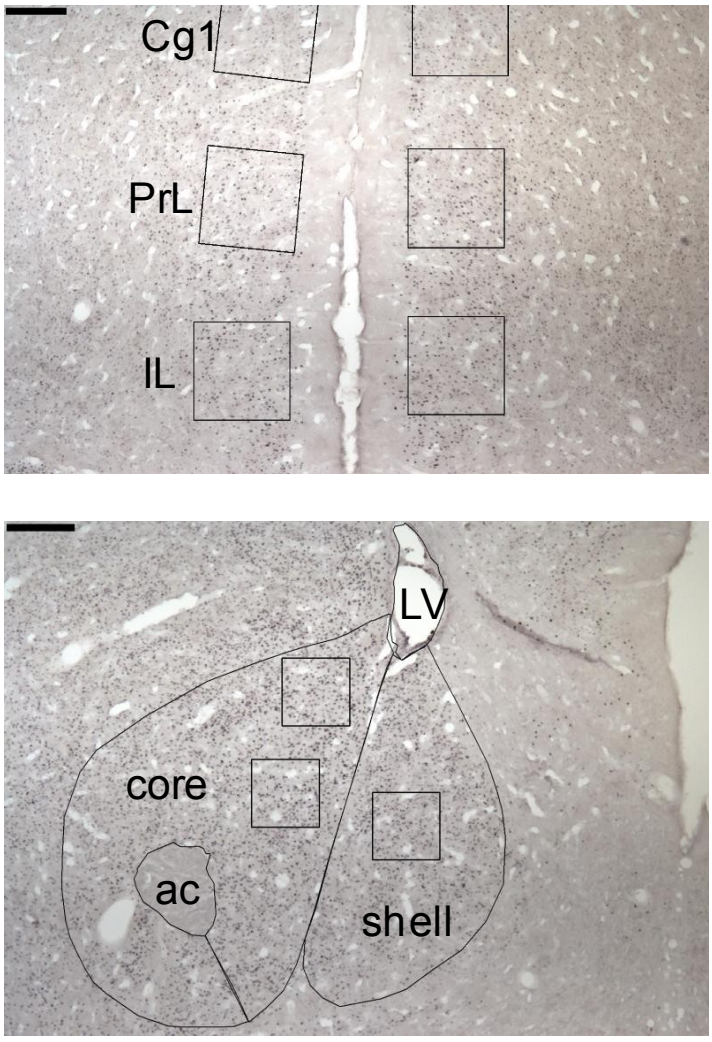

Figure 9: Photomicrographs of drawn contours of the medial prefrontal cortex (mPfC; top panel) and nucleus accumbens (NAc; bottom panel) onto immunohistochemically-treated tissue sections. The mPfC included the anterior cingulate (Cg1), prelimbic (PrL), and infralimbic (IL) cortex. The NAc included the shell and core. LV = lateral ventricle; ac = anterior commissure. Scale bars = 250 μm.

Cell counts were made within each contour by an experimenter blind to treatment group with an UPlanSApo 40x (0.9NA) objective on IHC tissue. Cells were considered ΔFosB-ir if they had a distinct nucleus with visible puncta stained dark purple-blue. All analyses were performed on an Olympus BX51 microscope under brightfield illumination using Neurolucida (version 9; Microbrightfield, Williston, VT). The average

number of ΔFosB-ir cells from each tissue section per subregion per hamster was used in statistical analysis (described below).

Statistical Analysis for ΔFosB-ir: To provide an integrated assessment of pubertal testosterone and sexual experience on ΔFosB-ir expression within the mPfC and NAc, multilevel modeling (MLM) was used. For the analysis, the model treated the animal as the upper-level sampling unit and tissue section as the lower-level sampling unit, with pubertal testosterone and sexual experience as independent variables and ΔFosB-ir cell number as the dependent variable. Interactions were followed up by MLMs within a subset of animals, as appropriate. $p \leq 0.05$ was considered significant. Due to poor tissue quality for some males, the sample sizes varied between groups of animals. Final sample sizes for each brain region are provided in the data figures.

Experiment 2 methods:

Animal model and viral vector injections (Figure 10): A total of 34 male hamsters were used for this experiment, which was conducted in 2 cohorts. Four-five weeks after the first group of prepubertal males arrived and 2-7 days after the second group of prepubertal males arrived, half of the first group (now adults P56; T@P) and half of the second group (prepubertal P28; NoT@P) males were GDX. Four weeks later, all of the T@P (P84) and NoT@P (P56) males received two T capsules (one 21 mm and one 13 mm; inner diameter 1.98 mm; outer diameter 3.18 mm) and bilateral microinjections aimed at the IL of recombinant adeno-associated viral (rAAV) vectors encoding either green fluorescence protein (GFP) or GFP and wild-type ΔFosB. Thus, there were three

groups: T@P-GFP, NoT@P-GFP, and NoT@P-ΔFosB. It is important to note that the vectors only infect neurons and are no more toxic than vehicle alone (Zachariou et al., 2006). For the microinjections, a small hole was drilled in the skull and a 5 µL Hamilton syringe (26-gauge, Hamilton) was lowered at a 20° angle to the level of the IL (3.3 mm rostral, ± 1.6mm lateral, and 4.5 mm ventral relative to bregma) based on Morin and Wood (2001). The syringe was kept in place for 2 minutes prior to injections and then either rAAV-ΔFosB or rAAV-GFP (1.0 µl per hemisphere) was injected into the IL over 10 minutes, with the syringe being kept in place for an additional 5 minutes after injection was complete. This procedure was done on both hemispheres. These AAV vectors reach maximal expression around 10 days and sustain expression indefinitely (Hedges et al., 2009; Perrotti et al., 2005; Perrotti et al., 2004; Perrotti et al., 2008; Pitchers et al., 2010; Wallace et al., 2008). Two weeks later (P70 and P98, respectively), all of the males from each group were used for sexual behavior testing.

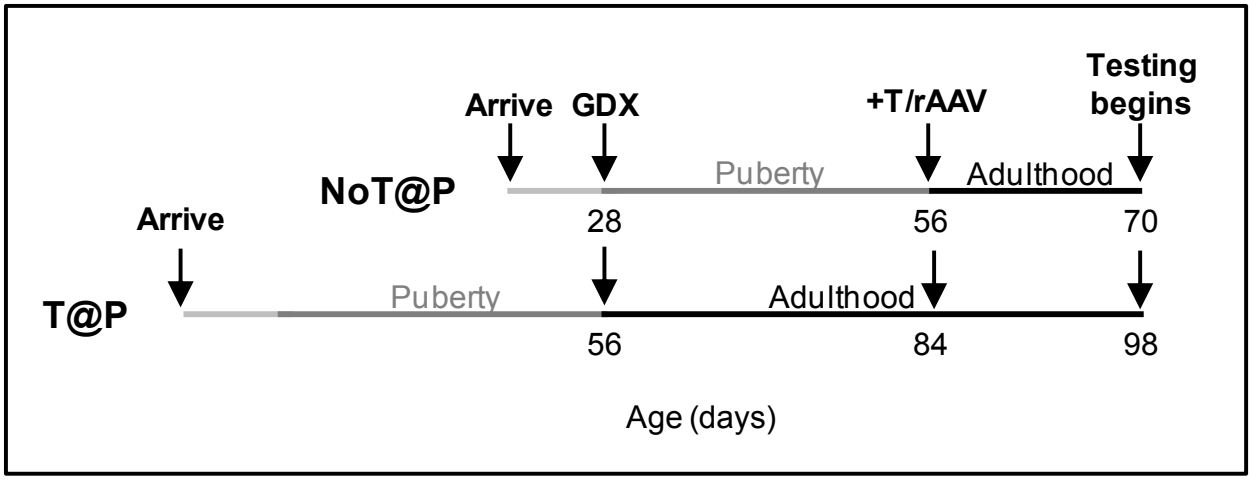

Figure 10. Animal model and viral vector injections for Experiment 2. T@P males arrived 4 weeks prior to NoT@P males to control for the age of shipping and environment during puberty. After the NoT@P males arrived, all males were either gonadectomized (GDX) during adulthood (P56; T@P) or prepubertally (P28; NoT@P). Four weeks later, all males received testosterone (T)-filled capsules and bilateral microinjections of recombinant adeno-associated viral (rAAV) vectors encoding wild-type ΔFosB or green fluorescence protein (GFP) into the IL. All of the T@P males and half of the NoT@P males received rAAV-GFP and the other half of the NoT@P males received rAAV-ΔFosB. Sexual behavior testing began two weeks later.

Tissue processing: As previously described, 18 to 24 hours after the final behavior test, all of the males were deeply anesthetized with an overdose of sodium pentobarbital (130 mg/kg, ip). Blood was collected via cardiac puncture, and the animals were perfused with 100 ml of buffered saline rinse and 150 ml of 4% paraformaldehyde. Brains were collected, post-fixed overnight in 4% paraformaldehyde, and then stored in 20% sucrose until sectioning. Sections were cut (40μm) into coronal sections using a cryostat to verify the correct placement of the injection using GFP as a marker. The ΔFosB vector contains a segment expressing GFP, allowing for the injection site and extent of infection of cells to be verified by GFP visualization. The sections were washed in TBS, mounted, and coverslipped while still wet with Vectashield hard set mounting medium (Vector Laboratories, Burlingame, CA). Plasma from the blood collection was used to determine

testosterone concentrations by radioimmunoassay. The intra-assay coefficient of variance was 5.4% and the minimum limit of detectability was 0.11 ng/ml.

Exclusion criteria: Males were excluded from behavioral analysis if they did not have adequate injection placement or expression of viral vector (n = 8) or displayed abnormal behavior following stereotaxic surgery that may interfere with sexual behavior (n = 2). A total of 10 males were excluded from behavioral analysis yielding a total of 24 males with T@P-GFP having n = 7, NoT@P-GFP having n = 10, and NoT@P- ΔFosB having n = 7.

Results.

Experiment 1 Results: Effects of pubertal testosterone and sexual experience on sexual behavior and ΔFosB expression in the mPfC and NAc

Physiological measures

All sexually naïve and sexually experienced males within each experimental group had adult physiological concentrations of circulating testosterone (Table 3).

Plasma Testosterone (ng/ml)

Group	Sexual Experience	
	Naïve	Experienced
T@P	2.77 ± 1.01	3.54 ± 1.66
NoT@P	2.77 ± 1.21	3.68 ± 1.30
sham-T@P	1.71 ± 0.72	2.59 ± 0.78
sham-NoT@P	1.67 ± 0.88	2.57 ± 0.72

Table 3. Concentrations of circulating plasma testosterone (ng/ml) per group.

Rate of ectopic mounting is dependent on pubertal testosterone

MLM revealed a significant main effect of pubertal testosterone on ectopic mounts per minute [$F(1, 28) = 6.88$, $p = 0.014$; Figure 11] with T@P males displaying overall fewer ectopic mounts per minute than NoT@P males. There was no main effect of sexual experience [$F(1, 28) = 0.75$, $p = 0.394$], nor a pubertal testosterone x sexual experience interaction [$F(1, 28) = 0.58$, $p = 0.451$]. Although no statistically significant interaction was found, T@P males do show a clear decrease in the rate of ectopic mounting after sexual experience, whereas NoT@P males do not.

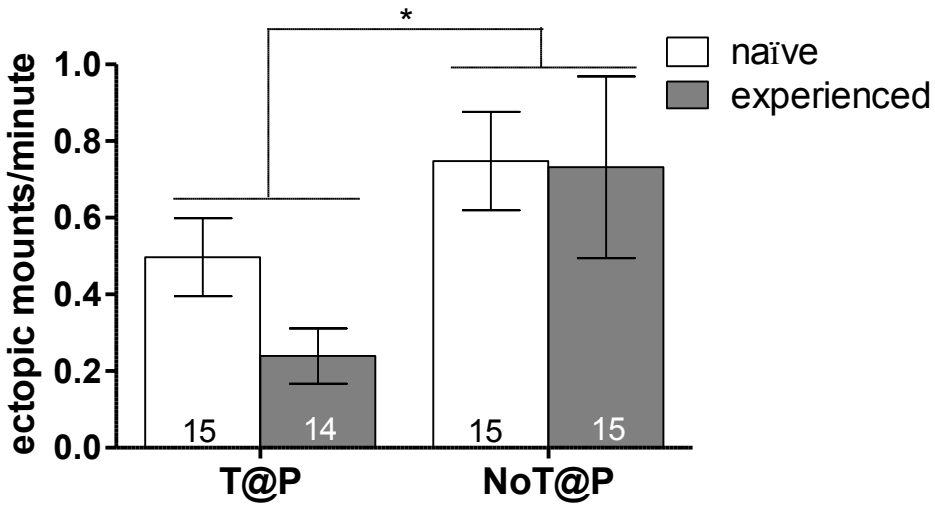

Figure 11: Rate of ectopic mounting is dependent on pubertal testosterone. T@P males had significantly fewer ectopic mounts per minute compared to NoT@P males. Numbers on bars indicate sample size. *Main effect of pubertal testosterone, $p \leq 0.05$.

Number of intromissions to ejaculation is dependent on sexual experience

MLM revealed a significant main effect of sexual experience on number of intromissions to ejaculation [$F(1, 27) = 25.25$, $p < 0.001$; Figure 12A], with sexually experienced males displaying fewer intromissions to reach ejaculation than sexually naïve males. There

was no main effect of pubertal testosterone [$F(1, 27) = 0.57$, $p = 0.458$], nor a pubertal testosterone x sexual experience interaction [$F(1, 27) = 3.00$, $p = 0.095$].

Latency to mount is dependent on sexual experience

MLM revealed a significant main effect of sexual experience on latency to mount [$F(1, 23) = 18.16$, $p < 0.001$; Figure 12B], with sexually experienced males having shorter latencies to mount than sexually naïve males. There was no main effect of pubertal testosterone [$F(1, 23) = 0.49$, $p = 0.490$], nor a pubertal testosterone x sexual experience interaction [$F(1, 23) = 0.01$, $p = 0.942$].

Latency to intromit is dependent on sexual experience

MLM revealed a significant main effect of sexual experience on latency to intromit [$F(1, 27) = 17.371$, $p < 0.001$; Figure 12C], with sexually experienced males having shorter latencies to intromit than sexually naïve males. There was no main effect of pubertal testosterone [$F(1, 27) = 0.632$, $p = 0.434$], nor a pubertal testosterone x sexual experience interaction [$F(1, 27) = 1.87$, $p = 0.182$].

Latency to ejaculate is dependent on pubertal testosterone and sexual experience

MLM revealed significant main effects of sexual experience [$F(1, 28) = 15.98$, $p < 0.001$] and pubertal testosterone [$F(1, 27) = 4.31$, $p = 0.047$] on latency to ejaculate (Figure 12D). These main effects were qualified by a pubertal testosterone x sexual experience interaction [$F(1, 28) = 5.78$, $p = 0.023$]. A follow-up one-way ANOVA found a main effect of pubertal testosterone [$F(1, 25) = 5.73$, $p = 0.025$] within sexually naïve males,

with sexually naïve T@P males having shorter latencies to ejaculate than sexually naïve NoT@P males. This effect of pubertal testosterone was not seen in sexually experienced males [$F(1, 27) = 0.28$, $p = 0.604$].

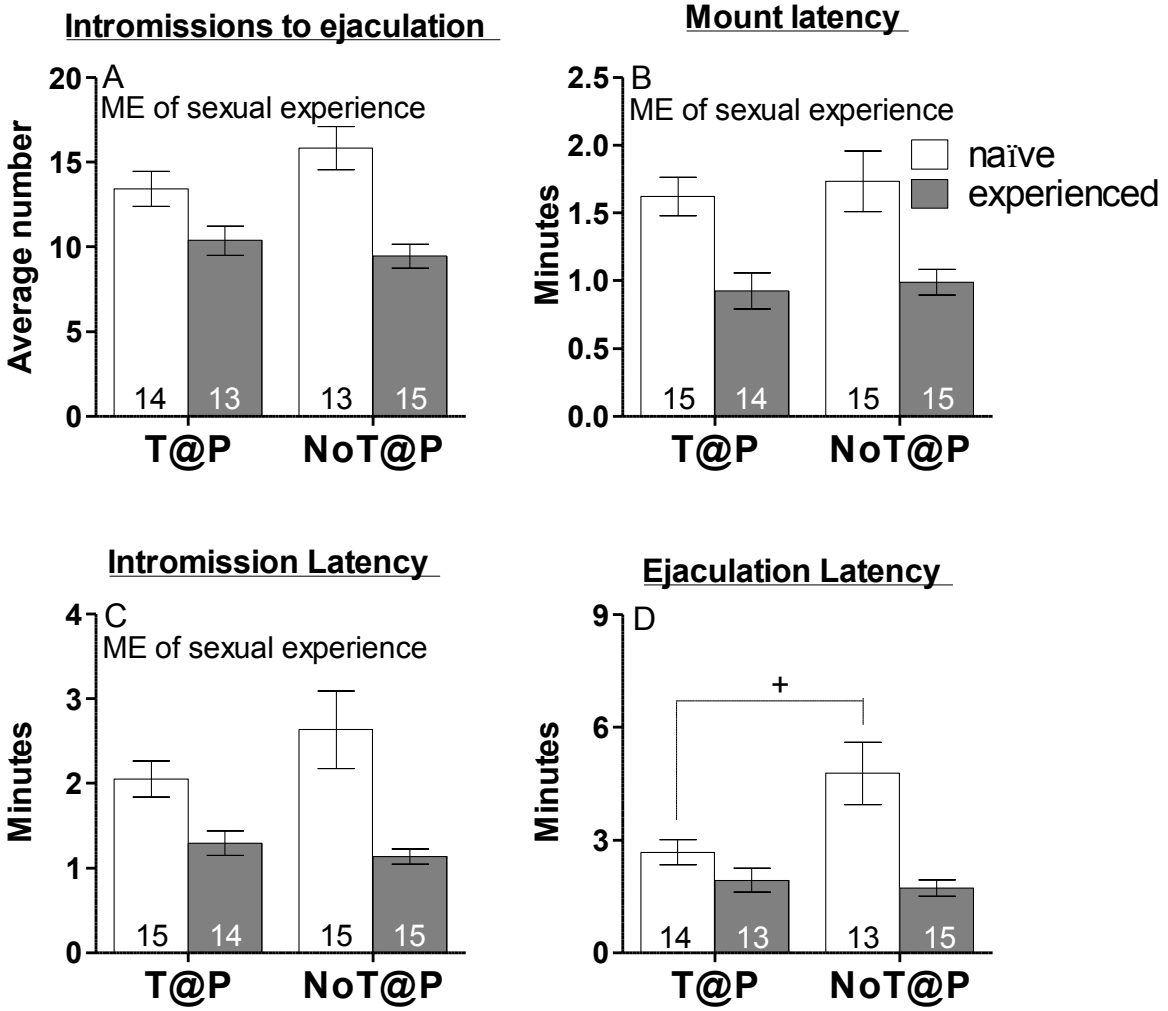

Figure 12: The effects of pubertal testosterone and sexual experience on number of intromissions to ejaculation and latency to mount, intromit, and ejaculate. A: There was a main effect (ME) of sexual experience for intromissions to ejaculate with sexually experienced males having less intromissions to achieve ejaculation compared to sexually naïve males. B, C: There was a main effect of sexual experience on mount latency and intromission latency with sexually experienced males having shorter latencies to mount and intromit compared to sexually naïve males. D: There was a pubertal testosterone x sexual experience interaction on ejaculation latency with sexually naïve NoT@P males having a longer latency to ejaculate compared to sexually naïve T@P males. This effect was not seen in sexually experienced males. Numbers on bars indicate sample size. +Interaction between pubertal testosterone and sexual experience, $p \leq 0.05$.

ΔFosB expression in the IL is dependent on pubertal testosterone and sexual experience

MLM revealed a significant interaction between pubertal testosterone x sexual experience [$F(1, 24) = 9.408$, $p = 0.005$, Figure 13]. A follow-up MLM found a significant main effect of sexual experience in the T@P males [$F(1, 13) = 12.11$, $p = 0.004$], with sexually experienced T@P males showing more ΔFosB expression in the IL compared to sexually naïve T@P males. This effect of sexual experience was not found in NoT@P males [$F(1, 11) = 0.71$, $p = 0.416$]. There were no main effects of pubertal testosterone [$F(1, 24) = 0.952$, $p = 0.339$] or sexual experience [$F(1, 24) = 3.64$, $p = 0.068$].

ΔFosB expression in the PrL is not dependent on pubertal testosterone or sexual experience

There were no main effects or interactions of pubertal testosterone or sexual experience [$F(1, 24) = 0.01$, $p = 0.934$; $F(1, 24) = 1.32$, $p = 0.262$; $F(1, 24) = 1.75$, $p = 0.198$, Figure 14A] on ΔFosB expression in the PrL.

ΔFosB expression in the Cg1 is not dependent on pubertal testosterone or sexual experience

There were no main effects or interactions of pubertal testosterone or sexual experience [$F(1, 24) = 0.01$, $p = 0.973$; $F(1, 24) = 0.01$, $p = 0.914$; $F(1, 24) = 0.42$, $p = 0.521$, Figure 14B] on ΔFosB expression in the Cg1.

ΔFosB expression in the NAc shell is not dependent on pubertal testosterone or sexual experience

There was a trend toward a main effect of sexual experience [$F(1, 24) = 4.06$, $p = 0.055$, Figure 15A], with sexually experienced males having more ΔFosB expression in the NAc shell compared to sexually naïve males. There was no main effect of pubertal testosterone [$F(1, 24) = 0.524$, $p = 0.476$] nor a pubertal testosterone x sexual experience interaction [$F(1, 24) = 1.14$, $p = 0.296$].

ΔFosB expression in the NAc core is not dependent on pubertal testosterone or sexual experience

There were no main effects of pubertal testosterone [$F(1, 24) = 1.29$, $p = 0.267$, Figure 15B], or sexual experience [$F(1, 24) = 3.26$, $p = 0.084$], or an interaction [$F(1, 24) = 2.69$, $p = 0.114$] on ΔFosB expression in the NAc core.

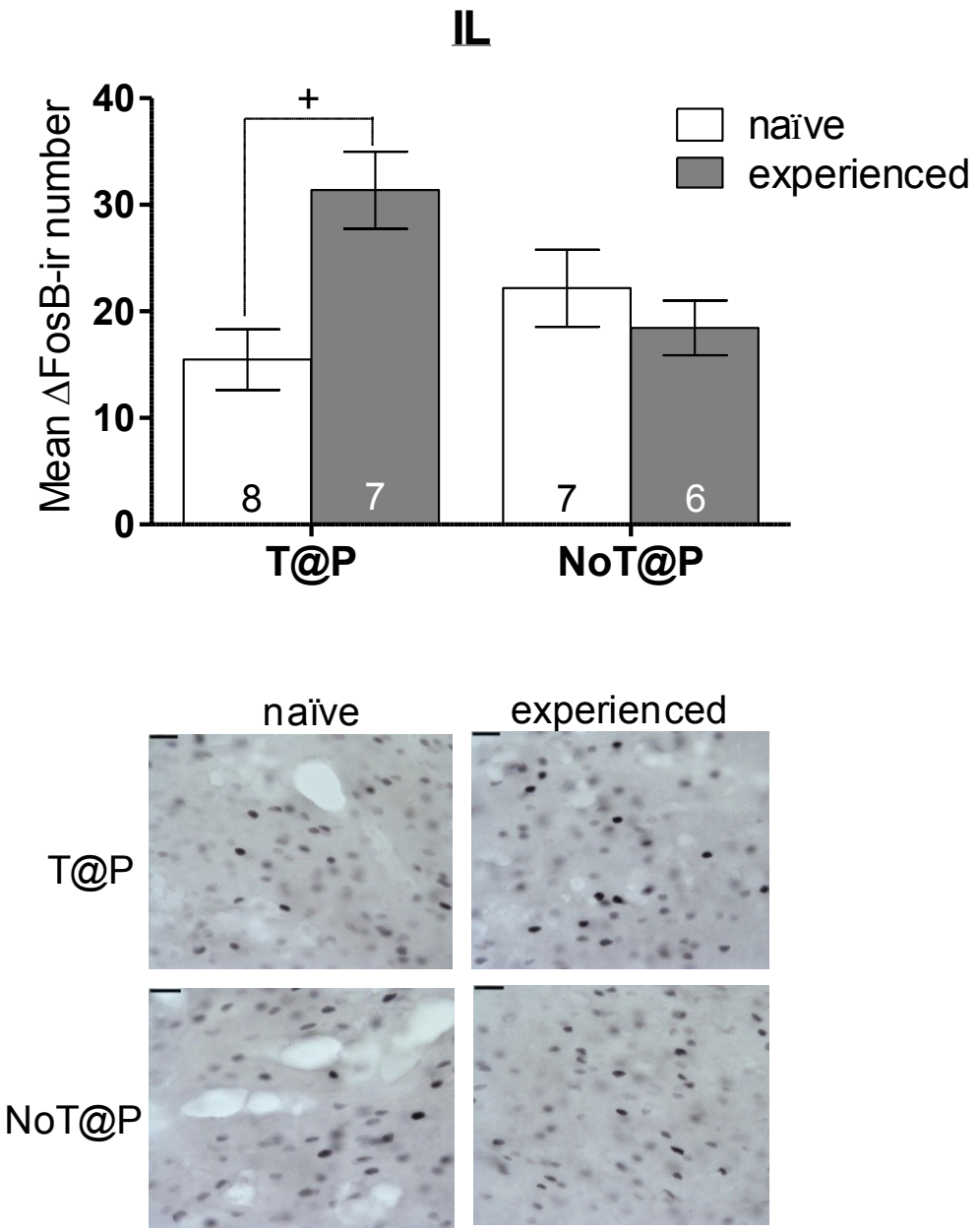

Figure 13: Mean (±SEM) number of ΔFosB-ir cells in infralimbic cortex (IL) of the medial prefrontal cortex is dependent on pubertal testosterone and sexual experience. There was an interaction between pubertal testosterone and sexual experience in the IL, where sexual experienced T@P males had significantly more ΔFosB-ir cells compared to sexually naïve T@P males. There were no significant differences in ΔFosB-ir cells as a function of sexual experience within NoT@P males. Numbers on bars indicate sample sizes. +Interaction between pubertal testosterone and sexual experience, $p \leq 0.05$. The 2 x 2 panel of photomicrographs below the bar graph are representative images of ΔFosB-ir at 40x objective for the specified group of males; scale bars = 25 μm.

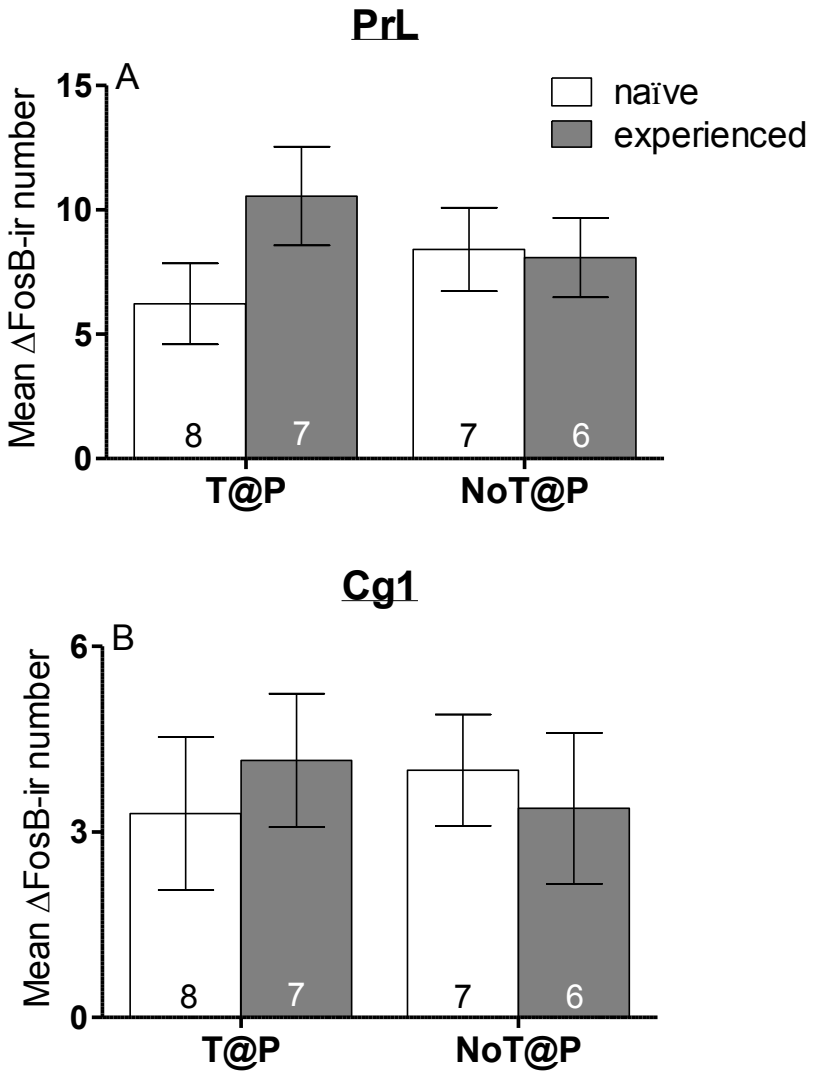

Figure 14: Mean (±SEM) number of ΔFosB-ir cells in the prelimbic and cingulate cortex of the mPfC is not dependent on pubertal testosterone or sexual experience. A, B: There were no effects or interactions of pubertal testosterone and sexual experience on ΔFosB-ir expression in the PrL or CgL. Numbers on bars indicate sample sizes.

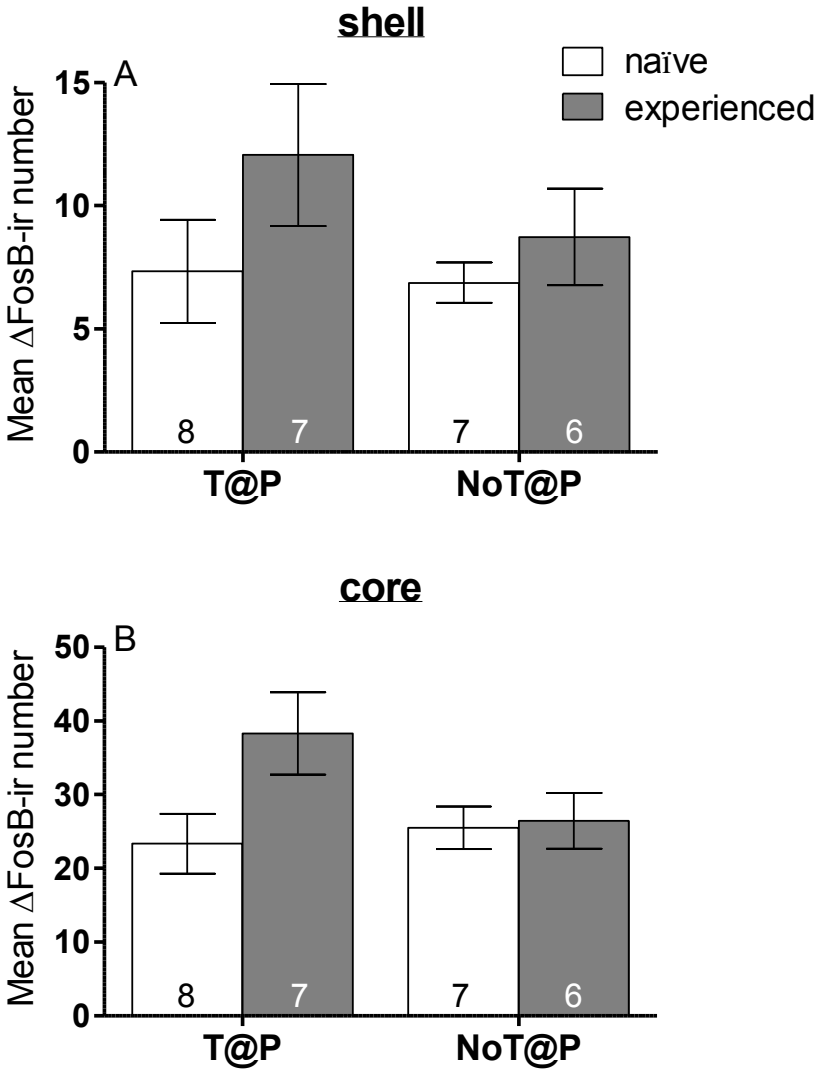

Figure 15: Mean (±SEM) number of ΔFosB-ir cells in the nucleus accumbens (NAc) shell and core is not dependent on pubertal testosterone or sexual experience. A, B: There were no effects or interactions of pubertal testosterone and sexual experience on ΔFosB-ir expression in the NAc shell or core. Numbers on bars indicate sample sizes.

Sham controls

The sham-T@P and sham-NoT@P males were tested at the same time as the T@P and NoT@P males. The two sham groups were compared to assess whether age at the time of surgery or age at the time of behavior testing explained behavioral or neural

differences seen between T@P and NoT@P. There were no significant differences between the sham groups for any of the behaviors analyzed with the exception of number intromissions to ejaculation (Table 4). MLM revealed a sham group x sexual experience interaction [$F(1, 17) = 4.82$, $p = 0.042$]. A follow-up one-way ANOVA found a main effect of sexual experience [$F(1, 20) = 11.50$, $p = 0.003$] within sham-NoT@P males, with sexually experienced males having fewer intromissions to reach ejaculation than sexually naïve males. This effect of sexual experience was not seen in sham-T@P males [$F(1, 27) = 1.00$, $p = 0.328$]. Because we did not find a difference between T@P and NoT@P on this specific measure, the difference between the sham groups will not be further discussed.

The IHC procedure for the sham controls used the same protocol and antibodies as the T@P and NoT@P groups; however, it was done separately. There were no differences found between the sham groups in expression of ΔFosB in any of the subregions of the mPfC (Table 5).

Sexual behavior of sham controls

	sham-T@P		sham-NoT@P		
Behavior	Naïve (trial 1)	Experienced (trial 5)	Naïve (trial 1)	Experienced (trial 5)	Effects
Ectopic mounts/minute	0.28 ± 0.29	0.39 ± 0.44	0.40 ± 0.32	0.36 ± 0.39	none
Intromissions to ejaculation	9.79 ± 3.66	8.57 ± 2.71	13.27 ± 4.88	7.91 ± 1.92	Group x experience, $p = 0.042$
Mount latency (minutes)	2.92 ± 1.28	0.87 ± 0.37	2.55 ± 3.92	0.91 ± 0.78	ME of experience, $p = 0.001$
Intromission latency (minutes)	4.06 ± 2.16	1.19 ± 0.52	2.80 ± 4.16	1.14 ± 0.77	ME of experience, $p = 0.001$
Ejaculation latency (minutes)	2.02 ± 0.86	1.22 ± 0.46	2.64 ± 1.08	1.27 ± 0.56	ME of experience, $p < 0.001$

Table 4. Effect of sexual experience on mean (± SD) of sexual behaviors observed in sham groups. There were no differences between groups for rate of ectopic mounting or any of the latencies. There was a main effect (ME) of sexual experience for latency to mount, intromit, and ejaculate with sexually naïve males having longer latencies to show all three behaviors. There was a group x experience interaction for intromissions to ejaculate, with sexual experience only having a main effect in sham-NoT@P males.

ΔFosB expression in the mPfC of sham controls

	sham-T@P		sham-NoT@P		
Region	Naïve	Experienced	Naïve	Experienced	Effects
IL	14.61 ± 9.60	20.40 ± 8.54	10.81 ± 8.99	18.74 ± 11.76	ME of experience, $p = 0.046$
PrL	6.39 ± 7.07	6.24 ± 5.85	6.50 ± 7.78	8.17 ± 8.92	none
Cg1	2.72 ± 4.77	3.62 ± 8.31	3.19 ± 5.43	6.85 ± 13.53	none

Table 5. Effect of sexual experience on mean (± SD) number of ΔFosB-ir cells in the mPfC observed in sham groups. There were no differences between groups for ΔFosB expression within any of the subregions. There was a main effect (ME) of sexual experience for the IL with sexual experienced males having more ΔFosB expression than sexually naïve males.

Experiment 2 Results: Effects of ΔFosB over-expression in the IL and sexual experience on the sexual behavior of NoT@P male hamsters

Physiological measures

All males had adult physiological concentrations of circulating testosterone, with T@P-GFP males having an average concentration of 4.07 ± 1.16 ng/ml, NoT@P-GFP males having an average concentration of 4.76 ± 0.85 ng/ml and NoT@P-ΔFosB males having an average concentration of 3.84 ± 0.73 ng/ml.

Viral vector injection placement

All males used in the behavioral analysis showed evidence of viral-mediated gene over-expression. However, the GFP control males were included in analyses if the over-expression was located within any subregion of the mPfC, whereas NoT@P-ΔFosB were included only if the over-expression was located in the IL (Figure 16). It should be noted that in 4 out of the 7 NoT@P-ΔFosB group, over-expression of ΔFosB occurred in both the IL and the ventral region of the PrL.

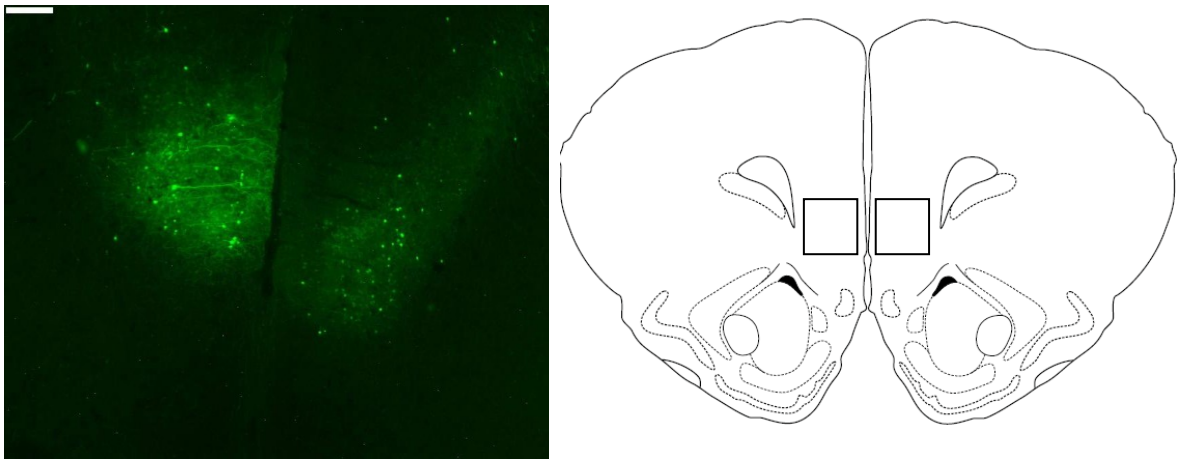

Figure 16. Visualization of GFP to verify injection site and extent of infected cells in the infralimbic cortex (IL). Photomicrograph of GFP over-expression in a NoT@P-ΔFosB male and boxes of representative injection sites in the IL for NoT@P-ΔFosB males over coronal atlas diagram (Morin and Wood, 2001). Scale bar = 250 μm. For interpretation of the references to color in this figure, the reader is referred to the electronic version of this dissertation.

Over-expression of ΔFosB in the IL decreases the rate of ectopic mounting in NoT@P males

MLM revealed a significant main effect of experimental group on ectopic mounts per minute [$F(2, 22) = 3.94$, $p = 0.035$; Figure 17] with NoT@P-ΔFosB males displaying overall less ectopic mounts per minute than NoT@P-GFP males ($p = 0.043$). T@P-GFP males did not significantly differ from NoT@P-GFP ($p = 0.201$) or NoT@P-ΔFosB ($p = 1.00$). It is noteworthy that sexually naïve NoT@P-ΔFosB males had similar rates of ectopic mounting (0.16 ± 0.14) as sexually experienced T@P males (0.17 ± 0.33). There was no main effect of sexual experience [$F(1, 21) = 1.25$, $p = 0.277$] nor an experimental group x sexual experience interaction [$F(2, 21) = 1.20$, $p = 0.322$].

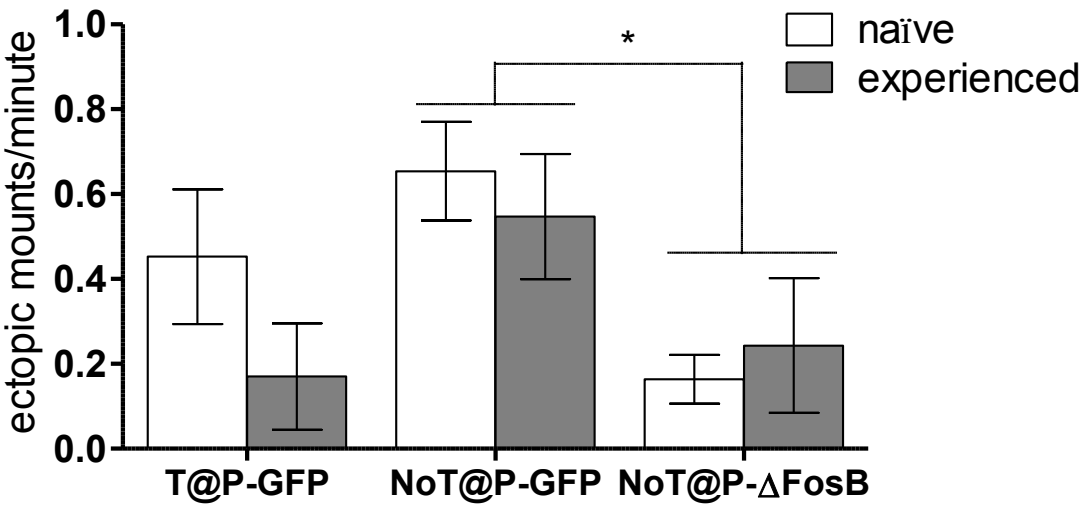

Figure 17: Over-expression of ΔFosB in the IL decreases the rate of ectopic mounting in NoT@P males. NoT@P-ΔFosB males (n = 6) had significantly less ectopic mounts per minute compared to NoT@P-GFP males (n = 9). T@P-GFP males (n = 7) did not differ from either group in rate of ectopic mounting. *Main effect of experimental group, $p \leq 0.05$.

Number of intromissions to ejaculation is dependent on sexual experience

MLM revealed a significant main effect of sexual experience on number of intromissions to ejaculation [$F(1, 20) = 11.70$, $p = 0.003$; Figure 18A], with sexually experienced males displaying fewer intromissions to reach ejaculation than sexually naïve males. There was no main effect of experimental group [$F(2, 21) = 0.87$, $p = 0.434$] nor an experimental group x sexual experience interaction [$F(2, 20) = 0.06$, $p = 0.940$].

Latency to mount is dependent on sexual experience for males with over-expression of ΔFosB in the IL

MLM revealed a significant main effect of sexual experience on latency to mount [$F(1, 19) = 11.12$, $p = 0.003$; Figure 18B], which was qualified by an experimental group x sexual experience interaction [$F(2, 19) = 3.61$, $p = 0.047$]. A follow-up MLM revealed a

main effect of sexual experience [$F(1, 6) = 11.00$, $p = 0.015$] within NoT@P-ΔFosB males, with sexually naïve NoT@P-ΔFosB males having longer latencies to mount than sexually experienced NoT@P-ΔFosB males. This effect of pubertal testosterone was not seen in T@P-GFP males [$F(1, 6) = 1.61$, $p = 0.252$] or NoT@P-GFP males [$F(1, 7) = 0.61$, $p = 0.459$]. There was no main effect of experimental group [$F(2, 19) = 1.80$, $p = 0.192$].

Latency to intromit is dependent on sexual experience

MLM revealed a significant main effect of sexual experience on latency to intromit [$F(1, 20) = 14.09$, $p = 0.001$; Figure 18C], with sexually experienced males having shorter latencies to intromit than sexually naïve males. There was no main effect of experimental group [$F(2, 20) = 1.12$, $p = 0.346$] nor an experimental group x sexual experience interaction [$F(2, 20) = 3.06$, $p = 0.070$].

Latency to ejaculate is dependent on sexual experience

MLM revealed a significant main effect of sexual experience on latency to ejaculate [$F(1, 21) = 15.20$, $p < 0.001$; Figure 18D], with sexually experienced males having shorter latencies to ejaculate than sexually naïve males. There was no main effect of experimental group [$F(2, 20) = 2.23$, $p = 0.134$], nor an experimental group x sexual experience interaction [$F(2, 20) = 1.64$, $p = 0.218$].

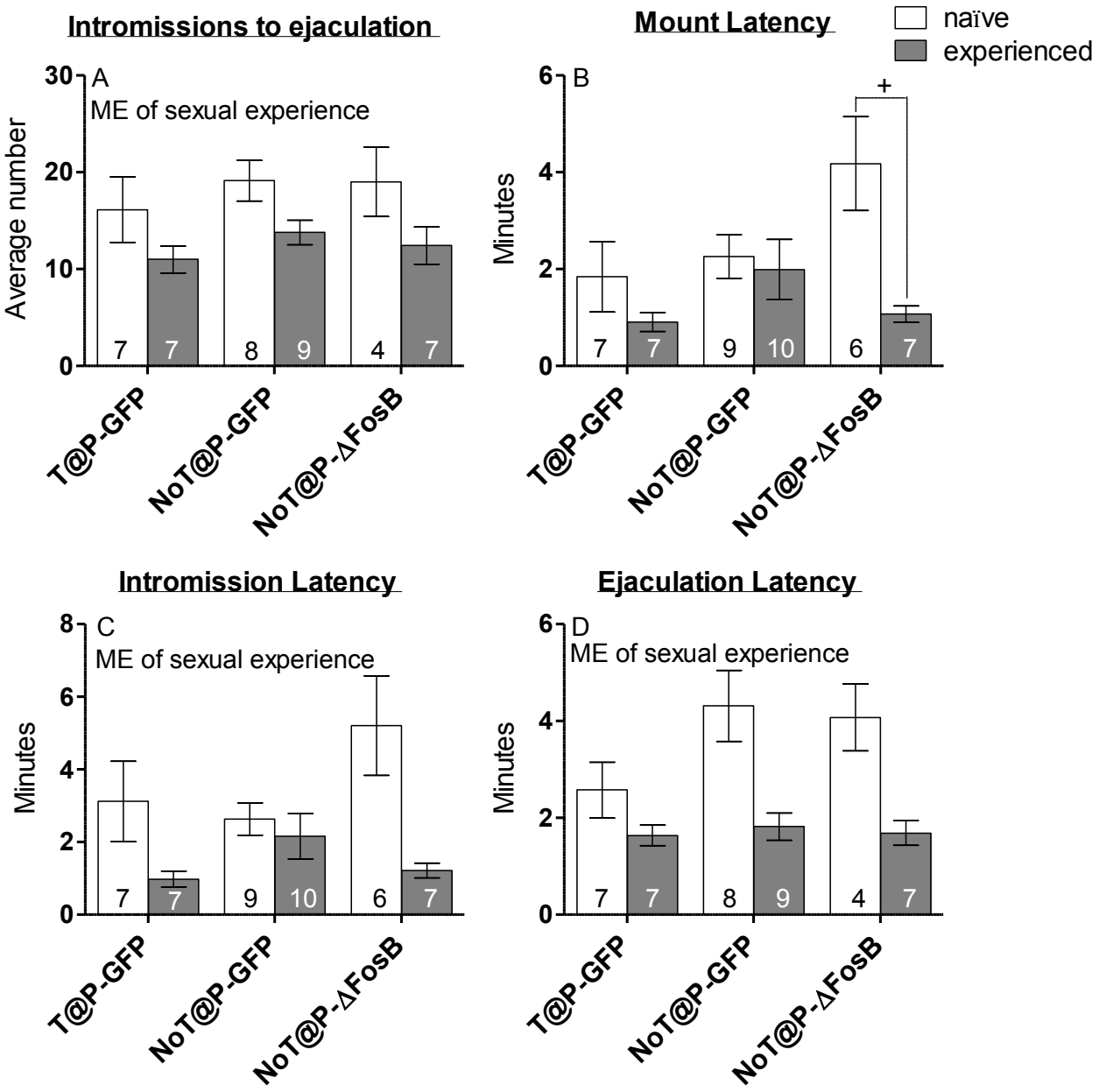

Figure 18. The effects of ΔFosB over-expression in the ventral mPfC and sexual experience on number of intromissions to ejaculation and latency to mount, intromit, and ejaculate. A: There was a main effect (ME) of sexual experience for intromissions to ejaculate with sexually experienced males having less intromissions to achieve ejaculation compared to sexually naïve males. B: There was a pubertal testosterone x sexual experience interaction on mount latency with sexually naïve NoT@P-ΔFosB males having a longer latency to mount compared to sexually experienced NoT@P-ΔFosB males. This effect of sexual experience was not found in T@P-GFP or NoT@P-GFP males. C, D: There was a main effect of sexual experience on intromission latency and ejaculation latency with sexually experienced males having shorter latencies to mount and intromit compared to sexually naïve males. Numbers on bars indicate sample size. +Interaction between experimental group and sexual experience, $p \leq 0.05$

Discussion.

The purpose of these studies was to determine the role of ΔFosB in the adolescent maturation of sexual proficiency that is gained as a function of experience. We first replicated our previous finding that testosterone during puberty is necessary for male hamsters to learn to adapt certain behaviors (i.e., ectopic mounting) with sexual experience. Then, using immunohistochemistry, we found that increased expression of the transcription factor ΔFosB in the IL is correlated with the ability to reduce ectopic mounting with sexual experience, suggesting that the failure of NoT@P males to make this behavioral adjustment is related to dysregulation of ΔFosB induction by the absence of testosterone during puberty. To test this hypothesis, NoT@P males were injected with a viral vector to over-express ΔFosB in the IL before sexual behavior testing. We discovered that the induction of ΔFosB in the IL enhance the ability of NoT@P males to gain sexual proficiency. These data provide evidence that testosterone during puberty regulates the induction of ΔFosB in the IL by sexual experience to program sexual proficiency in adulthood.

Compared to sexually naïve male rodents, sexually experienced males have shorter latencies to mount, intromit, and ejaculate, and they show fewer intromissions to reach ejaculation as a result of learning from experience and making behavioral adaptations accordingly (Bialy et al., 2000; Dewsbury, 1969; Miller et al., 1977). In addition, ectopic mounting decreases with sexual experience and represents a behavioral adaptation that increases sexual competency. We have previously reported that NoT@P males do not display the expected decrease in ectopic mounting with experience. It was not clear whether NoT@P males are less sensitive to rewarding sensory feedback during intromissions, or alternatively, whether NoT@P males

experience less reward than T@P males in timed trials because they achieve fewer intromissions (Schulz et al., 2004; Schulz & Sisk, 2006), The present study suggests that NoT@P males are not less sensitive to rewarding sexual stimuli, as they appeared to be just as sexually proficient as T@P males after sexual experience in regards to behaviors that reflect both sexual motivation (i.e., latency to mount and intromit) and sexual performance (i.e., intromissions to ejaculation, ejaculation latency). This is consistent with previous data showing that both T@P and NoT@P males show a conditioned place preference for mating with a sexually receptive female, indicating that NoT@P males perceive sexual behavior as rewarding (De Lorme, Bell, & Sisk, 2012).

NoT@P males may perceive sexual stimuli as rewarding and adjust certain sexual behaviors with experience, just as T@P males do. However, NoT@P males show other types of deficits in sexual performance. For example, sexually naïve NoT@P males had longer latencies to ejaculate compared to sexually naïve T@P males. Interestingly, with sexual experience, NoT@P males were able to achieve ejaculation latencies similar to those of T@P males. Thus, we hypothesize that pubertal testosterone programs initial sexual performance, and in the absence of pubertal testosterone, initial deficits can be compensated for with sufficient sexual experience. Indeed, there have been many studies investigating the power of experience on neural plasticity from the motor system to cognition (e.g., Jones et al., 2013; Park & Bischof, 2013). However, this may be the first report of sexual experience compensating for deficits in sexual behavior caused by the lack of organizational effects of pubertal testosterone.

Sexual proficiency is gained through learning from experience and making behavioral adaptations in part through the inhibition of behaviors that become

maladaptive with experience such as ectopic mounting. The major behavioral difference found between T@P and NoT@P males was rate of ectopic mounting. NoT@P males showed initial high rates of ectopic mounting and, even after sexual experience, continued to show high rates. Conversely, sexually naïve T@P males had lower rates of ectopic mounting compared to NoT@P males, and also showed a decrease in ectopic mounting with sexual experience. Being able to inhibit behaviors that are maladaptive is imperative to successful social interactions. Therefore, these data indicate that pubertal testosterone programs the ability to inhibit behaviors that are not rewarding or advantageous, and thus, maladaptive.

Although sexually experienced NoT@P males show high rates of ectopic mounting, they still ejaculate at similar latencies to T@P males. One could argue that ectopic mounting is not maladaptive as it did not deter NoT@P males from achieving ejaculation in this context. However, in a natural setting, exhibiting inefficient behaviors such as ectopic mounting would be detrimental to reproductive success of that male if he is competing with other, more sexually proficient males. Additionally, when a male ectopically mounts a female, she is presumably not receiving the rewarding aspect of the sexual encounter, and may not remain receptive to that male if given another mate choice.

ΔFosB is a transcription factor involved in stable, long-term behavioral adaptations, and thus, a possible key factor in the adolescent maturation of sexual proficiency. Postnatal developmental changes in ΔFosB expression have been reported. For example, adolescent male mice have overall lower protein levels of ΔFosB in the NAc and caudate putamen compared to adults (Ehrlich, Sommer, Canas, & Unterwald,

2002). Additionally, when preadolescent, adolescent, and adult male mice are administered a psychostimulant, only the adolescent mice show a significant induction of ΔFosB in the NAc and caudate putamen relative to age-matched controls (Ehrlich et al., 2002). These data indicate that not only are there developmental changes in baseline ΔFosB *expression*, but also fine-tuning of adult-typical ΔFosB *induction* across adolescence.

In the present study, we found that induction of ΔFosB in the IL occurred after sexual experience in only T@P males, suggesting a possible site of action in which pubertal testosterone programs the ability to inhibit maladaptive behaviors. The ventral mPfC, which includes the IL and PrL, is critical for behavioral inhibition in sexual contexts. Male rats with ventral mPfC lesions continue to mate with females even when sexual behavior is paired with aversive consequences (Davis et al., 2010). Furthermore, ΔFosB is integral in inducing neural plasticity in response to both natural and drug rewards by regulating gene expression (McClung et al., 2004; Nestler et al., 1999; Pitchers et al., 2013). Therefore, NoT@P males may not have the capacity for behavioral inhibition as a result of lacking this crucial plasticity within the IL. Indeed, we found that NoT@P males over-expressing ΔFosB show low rates of ectopic mounting over-all compared to control NoT@P males. Although NoT@P-ΔFosB males did not show a decrease in ectopic mounting with sexual experience, this may be due to floor effect as they had very low rates as sexually naïve males. In fact, sexually naïve NoT@P-ΔFosB males had ectopic mounting rates similar to sexually experienced T@P males, suggesting that ΔFosB over-expression reduces this maladaptive behavior even in the absence of experience.

Here we have demonstrated that pubertal testosterone exerts a nuanced organization of sexual proficiency. NoT@P males are as sexually proficient as T@P males when given the same sexual experience in certain measures of sexual motivation and sexual performance, suggesting that NoT@P males do not have decreased sensitivity to sexual reward. Furthermore, deficits found in sexually naïve NoT@P males were negated by sexual experience, which highlights the importance of experience-induced neural plasticity. However, even after sexual experience similar to T@P males, NoT@P males still showed high rates of ectopic mounting suggesting that pubertal testosterone programs the ability to inhibit maladaptive or socially inappropriate behavior. NoT@P males also did not show an experienced-induced increase in ΔFosB in the IL, which was found in T@P males. Over-expression of ΔFosB in the IL of NoT@P males is sufficient to reverse the persistence in ectopic mounting normally observed in NoT@P males. Therefore, pubertal testosterone programs the ability to inhibit inappropriate or maladaptive behavior through the regulation of ΔFosB in the IL.

Chapter 5: General Discussion

The focus of this research was to investigate the interaction between pubertal gonadal hormones and the developing brain during adolescent maturation of social cognition. Specifically, we sought to determine whether pubertal testosterone has organizational effects on the maturation of social reward and social proficiency, using the male Syrian hamster as a model system. The current set of experiments provide evidence that the adolescent acquisition of social reward does not depend on organizational effects of pubertal testosterone, whereas the adolescent gain in certain aspects of social proficiency does depend on organizational effects of pubertal testosterone. Gaining social proficiency involves both facilitation of appropriate behaviors and inhibition of socially inappropriate behaviors. Here we found that pubertal testosterone does not have an effect on facilitation of behavior, but rather behavioral inhibition. Furthermore, pubertal testosterone may organize the adolescent gain in the ability to inhibit inappropriate behavior through the regulation of the transcription factor ΔFosB in the IL of the mPfC.

Social Reward

Almost all social cues are motivationally relevant, inducing an animal either to seek out potential rewards or to avoid aversive stimuli. The male Syrian hamster is an ideal model for studying the motivational properties of social cues because neural processing and integration of pheromones contained in vaginal secretions are obligatory for expression of adult male sexual behavior (Wood, 1998). Juveniles are not attracted to female pheromones, as are adults (Johnston & Coplin, 1979), nor do juveniles show

adult-typical neuroendocrine and neurochemical responses to pheromones (Romeo et al., 1998; Schulz et al., 2003). Furthermore, unlike adult males, prepubertal male hamsters fail to show a CPP to pheromones contained in vaginal secretions, demonstrating that pheromones are not an unconditioned reward in prepubertal males and that rewarding properties of female pheromones are acquired over adolescent development (M. R. Bell, De Lorme, Figueira, Kashy, & Sisk, 2012). Thus, female sexual stimuli have different motivational properties in prepubertal and adult male hamsters, leading us to hypothesize that neuroendocrine events during male adolescence result in an increase in the rewarding properties of vaginal secretions.

We first explored whether maturation of social reward is due to organizational effects of pubertal testosterone by comparing the ability of NoT@P and T@P males to form a CPP to pheromones. Both groups of males showed a robust and long-lasting CPP to vaginal secretions and a receptive female, and we therefore concluded that organizational effects of pubertal testosterone are not necessary for the adolescent gain in the perception of female sexual stimuli as rewarding (De Lorme et al., 2012). Subsequent experiments in our lab demonstrated that activational effects of testosterone are both necessary and sufficient for finding vaginal secretions as rewarding in prepubertal and adult male hamsters. First, when males were tested several weeks after castration in adulthood, they no longer showed a CPP to female pheromones (M. R. Bell & Sisk, 2013). Second, when prepubertal hamsters were treated with testosterone, they did show a CPP to pheromones, whereas vehicle-treated prepubertal males did not (M. R. Bell et al., 2013; M. R. Bell & Sisk, 2013). Thus, the perception of female sexual

stimuli as a rewarding social stimulus is the result of activational effects of pubertal testosterone that does not require organizational effects of testosterone to be discerned.

Social Proficiency: Behavioral Inhibition and ΔFosB

Behavioral responses in a particular social setting are generated in the context of prior social experience, and may require adjustments or fine-tuning in the current social situation to increase social proficiency. Because social spheres are changing during adolescence and the valence of certain social cues shifts, a new suite of social behaviors must also develop during adolescence. Sexual behavior gradually emerges during adolescence, and in adulthood, male hamsters must appropriately display sexual behaviors to increase chances of reproductive success. Sexual experience is highly rewarding and has a profound effect on subsequent sexual behavior in male rodents. As a result of social learning and behavioral adaptation, the facilitation and motivation of sexual behavior increases with sexual experience. Thus, the adolescent acquisition of sexual proficiency is important for reproductive success in adulthood.

We sought to investigate whether the ability to gain sexual proficiency in adulthood is dependent on organizational effects of pubertal testosterone. In Chapter 3, T@P and NoT@P males were given the same sexual experience over multiple sexual trials, but not allowed to ejaculate. NoT@P males had overall higher rates of ectopic mounting than T@P males and did not decrease their rate of ectopic mounting with sexual experience. Furthermore, NoT@P males had consistent rates of mounts and intromission across the 4 trials, whereas T@P males showed an inverted U-shape pattern possibly because, without ejaculations, mounts and intromissions were no longer

rewarding. Therefore, NoT@P males did not efficiently adapt their behavior. This finding is consistent with previous data that compared agonistic behaviors of NoT@P and T@P males during repeated male-male interactions in a neutral arena. NoT@P males used inefficient behavioral strategies to maintain dominant-subordinate relationships with one another, whereas T@P males displayed adult-typical patterns of behavior (De Lorme & Sisk, 2013). Taken together, we conclude that testosterone during puberty programs the ability to adapt behaviors via inhibition in a social context-dependent manner.

Pubertal testosterone does not exert long-term effects on motivation or behavioral flexibility related to rewarding sensory feedback. For Chapter 4, we again equated sexual experience for T@P and NoT@P males, but allowed them to ejaculate to further explore sexual proficiency. After sexual experience, T@P and NoT@P males did not differ in a variety of behaviors that are indicative of sexual motivation and performance. Additionally, sexual experience negated the deficits observed for ejaculation latency in sexually naïve NoT@P males. This may explain why we did not find a difference in the induction of ΔFosB in the NAc between T@P and NoT@P males. Indeed, ΔFosB in the NAc is obligatory for experience-induced facilitation of sexual motivation and performance (Pitchers et al., 2010). Therefore, the lack of organizational effects of pubertal testosterone on ΔFosB expression in the NAc may protect the facilitation of sexual motivation and performance, which are crucial for successful sexual interactions, from being negatively affected by potential hormonal disturbances during adolescence.

We discovered that pubertal testosterone does not have a global effect on sexual proficiency, but instead it exerts a more nuanced effect through the regulation of ΔFosB

in the IL. The most striking behavioral deficit shown by NoT@P males was related to their rate of ectopic mounting. NoT@P males showed high rates of ectopic mounting, which did not decrease with sexual experience as observed in T@P males. Decreasing ectopic mounting with sexual experience may be indicative of behavioral flexibility through the ability to inhibit inappropriate behaviors. These behavioral data were then correlated with ΔFosB induction in the IL. We found that T@P, but not NoT@P males showed upregulation of ΔFosB in the IL after sexual experience. Over-expression of ΔFosB in the IL of NoT@P males prior to sexual behavior testing was sufficient to reverse the deficits in behavioral inhibition found in NoT@P males. The neural plasticity of the PfC is not only important for behavioral inhibition during sexual encounters, but for a variety of complex social behaviors. For example, female rats with PfC lesions have deficits in pup retrieval and licking, both of which require a complex behavioral pattern during pup interaction (Afonso, Sison, Lovic, & Fleming, 2007). Additionally, mPfC lesions in juvenile rats resulted in a disruption of social play behavior such that they were not able to temporally sequence responses properly to their partner (H. C. Bell, McCaffrey, Forgie, Kolb, & Pellis, 2009). Although we found that ΔFosB in the IL is sufficient to enhance behavioral inhibition, we do not know if it is necessary. Thus, future studies will focus on blocking the induction of ΔFosB in the IL in T@P males by over-expressing ΔJunD, which competitively binds to ΔFosB. If ΔFosB in the IL is necessary for the ability to inhibit inappropriate behavior, then T@P males over-expressing ΔJunD would show similar patterns of ectopic mounting as NoT@P males.

Pubertal Testosterone and ΔFosB

The mechanism by which pubertal testosterone organizes the induction of ΔFosB in the IL that mediates this sexual experience-induced plasticity remains unknown. One possible mechanism is through pubertal organization and experience-induced activation of the dopamine D1 receptor (D1R). There are sexually dimorphic developmental changes in D1R expression in the striatum and prefrontal cortex with D1R density peaking and then decreasing across adolescence in male, but female, rats suggesting a role of gonadal hormones in the ontogeny of D1R (Andersen, Rutstein, Benzo, Hostetter, & Teicher, 1997; Andersen & Teicher, 2000; Andersen, Thompson, Rutstein, Hostetter, & Teicher, 2000). Although this overproduction of D1R during adolescence is not due to the rise in gonadal hormones at pubertal onset , the sex differences in D1R overproduction may be due to organizational effects of pubertal testosterone (Andersen, Thompson, Krenzel, & Teicher, 2002). This possible organization would have a direct effect on ΔFosB induction as it occurs only in medium spiny neurons that express D1R (Nestler, 2008). Furthermore, blocking D1R in the NAc of male rats prevent the induction of ΔFosB after sexual experience, and D1R mutant mice show reduced ΔFosB induction in the NAc after repeated cocaine exposure compared to wild-type mice (Pitchers et al., 2013; Zhang et al., 2002). Therefore, ΔFosB upregulation in the NAc is dependent on the activation of the D1R. Whether this is also true in the IL has not been well-studied. Dopamine activation of the D1R in the mPfC mediates a variety of behaviors. For example, the IL is involved in inhibiting impulsive behavior, and dopamine release in the IL enhances the control of impulsive behavior through the D1R (Chudasama et al., 2003; Loos et al., 2010; E. R. Murphy, Dalley, & Robbins, 2005; Tsutsui-Kimura et al., 2013). Thus, induction of the ΔFosB in the IL via D1R activation

as a possible mechanism by which pubertal testosterone programs experience-induced ΔFosB expression in adulthood should be further explored.

Pubertal testosterone may affect the potential of epigenetic mechanisms of ΔFosB in the IL in response to sexual experience. Broadly, epigenetics refers to transcriptional mechanisms by which cellular traits can be inherited without changing the DNA sequence. Indeed, ΔFosB activates specific genes through histone modification (via acetylation) and these changes in histone acetylation may underlie behavioral adaptations. For example, ΔFosB binds to the cyclic-dependent kinase-5 (*Cdk5*) gene promoter and recruits co-activators including CREB binding protein, which leads to an increase in histone acetylation, and brahma-related gene 1, which is involved in remodeling chromatin (Kumar et al., 2005). This results in gene activation and increased expression of CDK5. CDK5 regulates cytoskeletal proteins and neurite outgrowth, and thus, experience-induced ΔFosB expression may increase spine density and neural plasticity through activation of CDK5 (Taylor et al., 2007). Indeed, ΔFosB overexpression in the NAc increases immature dendritic spines (Grueter, Robison, Neve, Nestler, & Malenka, 2013). Specific to the IL, repeated social stress increases ΔFosB expression in glutamatergic pyramidal neurons and H3 histone acetylation (Hinwood, Tynan, Day, & Walker, 2011). Taken together, it is tempting to speculate that NoT@P males are not learning to inhibit certain behaviors due to the lack of ΔFosB-induced formation of immature spines that could mature into glutamatergic spines with experience in the IL. Therefore, without this potential for *neural* plasticity of the IL with experience, NoT@P males also have a deficit in *behavioral* plasticity with experience.

Considerations

Here we provide evidence that pubertal testosterone organizes the ability to inhibit inappropriate behavior. However, there are some limitations to the present set of experiments. Although the male Syrian hamster is an excellent model to study both sexual reward and sexual proficiency, hamsters are a solitary species. Thus, the generalizability of our results to more social species is limited. Perhaps pubertal testosterone plays a bigger role in organizing the brain in solitary species compared to social species as solitary animals do not have as much exposure to social experience to help shape the brain. To address this, a similar experiment could be conducted using more social species such as rats. Additionally, sexual behavior is only one small aspect of social behavior and social cognition. This needs to be considered when generalizing to other social behaviors; however, we have shown similar deficits in social cognition during male-male agonistic encounters of NoT@P males (De Lorme & Sisk, 2013; Schulz et al., 2006). Thus, we hypothesize that the social ineptness of NoT@P males is not unique to a specific social context, but reflects a more global dysfunction in the expression of context-appropriate social behavior.

Relevance to Human Mental Health

Given that social contexts are ever changing, behavioral inhibition is crucial for competent social development across the life-span as it allows an individual to interact appropriately with others in social situations. The PfC and orbitofrontal cortex (OFC) are both involved in emotion regulation and behavioral inhibition, and continue to develop well into young adulthood (Nelson & Guyer, 2011; van Wingen, Ossewaarde,

Backstrom, Hermans, & Fernandez, 2011). The neurodevelopment of these regions are particularly sensitive to adverse experiences and gonadal hormone concentrations during adolescence, which may contribute to adolescents' sensitivity to certain psychological disorders (Nelson & Guyer, 2011; van Wingen et al., 2011). Furthermore, the PfC and OFC develop much sooner in girls compared to boys, most likely related to pubertal onset, which may account for the increased risk of psychopathologies characterized by impaired inhibitory control (i.e., substance abuse) in adolescent boys (Raznahan et al., 2010). Many sexually dimorphic psychological disorders that emerge during puberty are characterized in part by a dysfunction in behavioral inhibition, e.g., schizophrenia, chronic substance abuse, anxiety disorders, depression, and eating disorders (Hayward & Sanborn, 2002). These data suggest that gonadal hormones during puberty influence the neurodevelopment of brain regions involved in the regulation of the development of important social behaviors such as behavioral inhibition. Here we demonstrated that the ability to inhibit certain behaviors that are socially inappropriate or maladaptive is influenced by testosterone during puberty through the organization of the mPfC. Taken together, these data indicate that the ability to gain social proficiency in humans may be similarly influenced by organizational effects of hormones during puberty. Lastly, we highlight the importance of investigating pubertal onset and exposure to exogenous hormones in order to further understand their role in the development of certain psychological disorders.

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