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Pubertal testosterone programs context-appropriate agonistic behavior and associated neural activation patterns in male Syrian hamsters

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Abstract

Pubertal testosterone programs the level of aggressive behavior displayed by male Syrian hamsters during resident-intruder interactions. To further explore the pubertal programming of adult male agonistic behaviors, the current study investigated the formation, stability, and maintenance of dominant-subordinate relationships in males that either did (T@P) or did not (NoT@P) experience testicular hormones during adolescent development. NoT@P males were gonadectomized prepubertally and T@P males were gonadectomized in adulthood. Four weeks after gonadectomy, all males received testosterone-replacement. Two weeks later, two males of the same hormonal history were given a 60 min introductory trial in a neutral arena, followed immediately and again 24 h later by three 5-min trials. During the introductory trial, each male was deemed dominant, subordinate, or no-status. Brains were collected 1 h after the last trial and sections were stained for Fos-immunoreactivity. Dominant T@P males flank marked more frequently than subordinate and no-status T@P males; this difference was not found in NoT@P males. NoT@P males showed an increase in the number of offensive postures the day after the first series of tests, whereas T@P males did not. Dominant T@P males had significantly more Fos expression than no-status T@P males in anterior cingulate cortex; this relationship was not observed in NoT@P males. Additionally, dominant T@P males had higher Fos expression than dominant NoT@P males in lateral septum. Thus, pubertal testosterone does not organize the formation or stability of male-male relationships, but does program the behavioral strategies used to maintain these relationships over time and the neural correlates of status.

Keywords

puberty; testosterone; fos; agonistic behavior; cingulate cortex; lateral septum

1. Introduction

The ability to appropriately process social information and adapt behavior in social settings becomes increasingly important during adolescent maturation, when the successful

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transition to adulthood entails learning to interact proficiently with conspecifics. One example of context-appropriate social behavior is seen during male-male interactions between adult Syrian hamsters. Dominant-subordinate relationships are normally established rather quickly after an aggressive encounter, and thereafter, maintenance of social status is accomplished not by overt aggression, but by chemosensory communication via flank gland secretions, called flank marking (Ferris et al., 1987). Both males flank mark, but the dominant male does so more than the subordinate male. The stability of this social relationship depends on the expression of flank marking according to an individual's status and on the appropriate interpretation of this social information by the other conspecific. Hormonal regulation of flank marking behavior interacts with age: adults flank mark more than juveniles (Johnston, 1981, Ferris et al., 1996) and testosterone activates flank marking in adult, but not juvenile, males (Johnston, 1981, De Lorme et al., 2012). Thus, flank marking behavior matures during adolescence and may be due to the organization of relevant neural circuits via pubertal testosterone.

To determine whether adolescent maturation of flank marking and the underlying testosterone-sensitive neural circuits is due to organizational effects of pubertal testosterone, we studied behavioral and neural activation patterns after repeated agonistic encounters in male hamsters that either did or did not experience testosterone during adolescent development. We used an experimental model in which male Syrian hamsters deprived of testicular hormones during puberty (no testes/testosterone at puberty: NoT@P) are compared to males deprived of testicular hormones for an equivalent amount of time in adulthood (testes/testosterone at puberty: T@P) after normal pubertal development (Schulz et al., 2004). We've previously shown that NoT@P males do not express adult-typical agonistic behaviors as compared to T@P males (Schulz et al., 2006, Schulz and Sisk, 2006). Specifically, during resident-intruder tests, resident NoT@P males display low levels of aggression and flank marking, while displaying high levels of submissive behavior (Schulz et al., 2006, Schulz and Sisk, 2006). We've also found that pubertal testosterone organizes both the volume and neuron number of the medial amygdala (Newman et al., 1997), a region that regulates social behaviors, and vasopressin receptor binding in the lateral septum, a region that mediates flank marking behavior (Schulz et al., 2006). Therefore, pubertal testosterone appears to organize male agonistic behaviors that depend on social information processing and the underlying neural circuitry.

To determine whether pubertal testosterone programs male agonistic behaviors in social contexts other than defense of territory (as assessed by our previous work using resident-intruder tests), the current study assessed male-male interactions in a neutral arena and addressed four questions: 1) Do NoT@P males form dominant-subordinate relationships with other NoT@P males? 2) If so, do these dominant-subordinate relationships remain stable, as indicated by the dominant male remaining dominant over repeated encounters? 3) Do NoT@P males employ behavioral strategies similar to those used by T@P males to actively maintain stable relationships? 4) Do neural activation patterns in response to male-male encounters differ between NoT@P and T@P males according to their individual status?

2. Materials and methods

2.1 Animals

Twenty-four prepubertal (P20-22) and 26 young adult (P52-56) male Syrian hamsters were ordered from Harlan Sprague-Dawley laboratories (Madison, WI) and individually housed upon arrival in clear polycarbonate cages (30.5 × 10.2 × 20.3 cm) with *ad libitum* access to food and water with a 14:10 light/dark cycle (lights out at 1300 h). 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.

2.2 Testosterone Treatments

Both prepubertal and adult male hamsters were gonadectomized at P21-23 and P53-57 to form NoT@P and T@P groups, respectively. Four weeks later the hamsters in each group received 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). After two weeks of testosterone replacement, behavior testing began during adulthood (Figure 1). All behavioral testing occurred under dim red light 1 hour into the dark phase.

2.3 Behavior Testing

All males were weight-matched with another male of the same pubertal hormone history, yielding 12 NoT@P pairs and 13 T@P pairs. All pairs were within 10g of each other's weight, with the exception of one T@P pair that had a 24.6g difference in weight. The behavioral patterns of this T@P pair were not noticeably different from those of other T@P pairs, nor were levels of their behaviors statistical outliers for the group. An initial 'formation test' was used to determine each male's status, in which the pairs were placed in a neutral arena (51 × 26 × 31.5 cm glass aquarium) that neither male had occupied prior to testing. Criteria for determining social status were adapted from previous work in male Syrian hamsters (Albers et al., 2002, Bath and Johnston, 2007). A male was deemed subordinate if he made 3 attempts to escape the aquarium after being attacked by the other male. The male that made the initial attack was then deemed dominant. Every pair was given up to 60 minutes to form a dominant-subordinate relationship; if a dominant-subordinate relationship was not formed within 60 minutes, then it was determined that neither male of that pair acquired dominant or subordinate status. After the initial formation test, the same two hamsters were put together again in the same arena for an additional 6 trials: 3 trials started 5 minutes after the formation test and 3 trials started 24 hours after the formation test. All trials lasted 5 minutes with 5 minutes between each trial on both test days.

Stability was assessed by determining if there were dominant-subordinate reversals during the 6 trials (i.e. the dominant male showed more instances of dominant behavior compared to subordinate males). Behavioral strategies for active maintenance of the dominant-subordinate relationship were determined from the pattern of the behaviors displayed during the 6 trials. Behaviors during the 6 trials were quantified using the following definitions: flank-marking (rubbing a flank gland against another surface), social contact (investigating/sniffing or touching noses with the other male), defensive posture (standing on hind legs with paws out or on-back), escape dash (after a social or agonistic encounter, quickly running from the other male), tail-up (when a male is on all four paws walking slowly with his tail sticking straight in the air), offensive posture (pinning the other male or standing on hind legs bending to the side of the other male), and attack (bite or an attempt to bite the other male).

2.4 Tissue and blood collection

One hour after the sixth trial, all the males were deeply anesthetized with an overdose of sodium pentobarbital (150 mg/kg, ip), had a terminal blood sample taken via cardiac puncture, and then perfused intracardially with 4% paraformaldehyde. The brains were collected and stored at 4°C in 30% sucrose until further analysis. Plasma concentrations of testosterone were determined from duplicate 50 µl samples using the Coat-A-Count Total testosterone Kit (Diagnostic Products, Los Angeles, CA). The intra-assay CV was 1.5% and the minimum limit of detectability was 0.1 ng/ml.

2.5 Histological procedures

Brains were sectioned with a cryostat into 4 series of 40 μm thick sections and stored in cryoprotectant at -20°C until histological processing. One series was thaw-mounted onto glass slides. Sections were allowed to dry, then thionin-stained, and cover-slipped for Nissl-based identification of the subregions of interest. A second series of sections was used for visualization of cFos immunoreactivity by free-floating immunohistochemistry (IHC) for identification of transcriptional activation in neurons (Sheng and Greenberg, 1990, Hughes and Dragunow, 1995).

All IHC work occurred at room temperature unless otherwise noted, rinses in Trizma buffered saline (TBS, 0.05M, pH = 7.6) occurred initially and between steps, and all antibodies were diluted in 2% of normal goat serum (Pel-Freez Biologicals, Rogers, AR) and 0.3% Triton-X in TBS. To visualize cFos, residual aldehydes were removed using 0.1% sodium borohydride and endogenous peroxidase activity was quenched using 1% hydrogen peroxide before tissue was blocked and made permeable with 20% goat serum and 0.3% Triton-X TBS. Tissue was then consecutively incubated in the cFos primary antibody (c-Fos (4): rabbit, sc-52. 1:10,000, 0.02 μg IgG/ml solution, Santa Cruz Biotech, Santa Cruz, CA) for 48 hours at 4°C , secondary antibody (biotinylated goat anti-rabbit IgG (H+L), 1:500, 3 μg IgG/ml solution, Vector Laboratories, Burlingame, CA) for one hour, and avidin-biotin complex (ABC Kit PK-6100, Vector Laboratories, Burlingame, CA) for one hour. Then, tissue was reacted with Ni-3,3'-Diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO) to produce a dark purple-blue reaction product in the nucleus of Fos-immunoreactive (Fos-ir) cells. Primary and secondary antibody deletion control studies were run on separate sections with minimal non-specific background staining detected in these sections. Tissue sections were mounted onto glass slides and dehydrated with a series of ethanols before coverslipping.

2.6 Microscopic analysis

Regions of interest (ROI) included the medial prefrontal cortex (mPFC), intermediate lateral septum (LSi), anterior hypothalamus (AH), central amygdala (Ce), and ventromedial hypothalamus (VMH) as they mediate flank marking and other agonistic behaviors (Ferris et al., 1990, Irvin et al., 1990, as reviewed by Ferris and Delville, 1994, Bamshad and Albers, 1996, Bamshad et al., 1997, Kollack-Walker and Newman, 1997, Kollack-Walker et al., 1999). Some ROI were further subdivided according to the hamster brain atlas (Morin and Wood, 2001) as indicated by distinct functional and anatomical characteristics of the subregions. The mPFC included the anterior cingulate (Cg1), prelimbic (PrL), and infralimbic (IL); the Ce included medial (CeM) and central (CeC); and the VMH included medial (VMHM) and lateral (VMHL) portions.

Three anatomically matched tissue sections throughout the extent of each ROI were selected at 4x objective. In the LSi, AH, Ce, and VMH, contours were manually traced bilaterally according to the atlas and cytoarchitecture in Nissl-stained sections. In the mPFC, contours were 600 $\times$ 600 μm boxes placed in the mPFC relative to medial brain outline and corpus callosum landmarks. The contours were then overlaid onto corresponding IHC-treated tissue sections for cell counting (Figure 2).

Cells counts were made within a contour by an experimenter blind to hamster treatment with an UPlanSApo 40 $\times$ (0.9NA) objective on IHC tissue. Cells were considered Fos-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). Fos-ir number from each tissue section per subregion per hamster was used in statistical analysis (described below).

2.7 Statistical analysis

2.7.1 Behavioral outliers and sample sizes—For each behavior, a box-plot utilizing stem-and-leaf descriptives was used to identify the extremes within each pubertal testosterone group for each status (no status, subordinate, dominant). 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 animal was taken out of the study. Two males were identified as outliers (one subordinate T@P and one no-status T@P), and thus, removed from the study. In addition, due to poor tissue quality in some sections, the sample sizes for each brain region sometimes varied within a group of animals. Final sample sizes for each behavior and brain region are reflected in the table and figures.

2.7.2 Percentage to form—A Fischer's exact test was used to compare the proportions of pairs that did or did not form a dominant-subordinate relationship within the NoT@P and T@P groups.

2.7.3 Behavior and brain statistical analysis—To provide an integrated assessment of pubertal testosterone and status on behavior and Fos expression within all brain regions studied, multilevel modeling (MLM) was used. For behavior analysis, the model treated animal as the upper-level sampling unit and trial as the lower-level sampling unit, with pubertal testosterone, status (no status, subordinate, or dominant), and trial as independent variables and each behavior as the dependent variable. This analysis was first done including all 6 trials, and again including only the last 3 trials in order to better relate Fos expression to behavior, as the last three behavioral trials occurred 60–90 minutes before tissue collection. For Fos-ir analysis, the model treated animal as the upper-level sampling unit and tissue section as the lower-level sampling unit, with pubertal testosterone, status, and hemisphere as independent variables and Fos-ir cell number as the dependent 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. Significant interactions were followed up by MLMs using a Bonferroni correction within a subset of animals, as appropriate. For all analyses, $p < 0.05$ was considered significant.

3. Results

3.1 Physiological measures

All males had adult physiological concentrations of circulating testosterone, with NoT@P males having an average concentration of 4.69 ± 1.16 ng/ml and T@P males having an average concentration of 4.45 ± 0.86 ng/ml. All males had normal adult flank gland length, with NoT@P males having an average length of 5.77 ± 1.47 mm and T@P males having an average length of 6.07 ± 1.36 mm.

3.2 Formation and stability of a dominant-subordinate relationship is not dependent on pubertal testosterone

Six of 12 NoT@P pairs and 9 of 13 T@P pairs formed a dominant-subordinate relationship. These proportions are relatively low, and are most likely due to weight-matching the pairs and/or using a neutral arena. Fischer's exact test revealed that the proportion of pairs forming a dominant-subordinate relationship was not significantly different between the NoT@P and T@P groups ($p = 0.43$). In addition, dominant males remained dominant across all 6 trials regardless of pubertal testosterone.

3.3 Behavioral strategies for maintaining a dominant-subordinate relationship are dependent on pubertal testosterone and status

MLM revealed a pubertal testosterone x status interaction on number of flank marks across all 6 trials [$F(2, 42) = 3.91, p = 0.028$; Figure 3A] and across the last 3 trials [$F(2, 42) = 3.661, p = 0.034$, Figure 3B]. A follow-up MLM found a significant effect of status on flank mark frequency within T@P males [$F(2, 21) = 9.92, p = 0.001$; $F(2, 21) = 11.47, p < 0.0001$, respectively] with dominant T@P males flank marking more frequently than subordinate and no-status T@P males. In contrast, status did not significantly affect flank marking frequency within NoT@P males [$F(2, 21) = 1.20, p = 0.320$; $F(2, 21) = 1.25, p = 0.308$, respectively].

MLM revealed a pubertal testosterone x trial interaction on number of offensive postures across the 6 trials [$F(5, 210) = 2.73, p = 0.021$; Figure 4]. A follow-up MLM revealed a significant effect of trial on frequency of offensive posturing within NoT@P males [$F(5, 115) = 3.11, p = 0.011$] with NoT@P males showing significantly more offensive posturing in trial 4 compared to trial 3 (24 h separated trials 3 and 4). In contrast, trial did not significantly affect frequency of offensive posturing within T@P males [$F(5, 115) = 2.02, p = 0.081$]. For the last 3 trials, MLM revealed a main effect of pubertal testosterone [$F(1, 42) = 5.87, p = 0.020$] on the frequency of offensive posturing, with NoT@P males showing significantly more offensive posturing than T@P males.

MLM revealed a status x pubertal testosterone x trial interaction across the last 3 trials [$F(4, 84) = 3.16, p = 0.018$, Table 1] on the frequency of escape dashes. Follow-up MLMs did not reveal significant effects that could explain this 3-way interaction; therefore, the exact nature of this interaction is unclear. However, the data suggest that within subordinate males there was slight decrease in frequency of escape dashes across the 3 trials for T@P males [$F(2, 14) = 2.31, p = 0.136$], whereas this same decrease was not seen in NoT@P males [$F(2, 10) = 0.73, p = 0.507$].

3.4 Males of differing status employ different behavioral strategies during interactions over time

As expected, MLM revealed a trial x status interaction across all 6 trials for flank marks, initiating social contact, attacks, escape dashes, tail-up walking, and defensive postures as well as a main effect of status for offensive posturing. For the last 3 trials, MLM revealed a trial x status interaction for flank marks and a main effect of status for initiating social contact, attacks, tail-up walking, offensive postures, and defensive postures. Because none of these main effects or interactions involved pubertal testosterone, these data will not be further discussed.

3.5 Fos-ir number within specific brain regions is dependent on pubertal testosterone and/or status

MLM revealed a pubertal testosterone x status interaction on Fos-ir number in the Cg1 [$F(2, 84) = 4.77, p = 0.011$; Figure 5] and LSi [$F(2, 88) = 4.44, p = 0.015$; Figure 6A]. For Cg1, a follow-up MLM found an effect of status within T@P males [$F(2, 43) = 3.57, p = 0.037$] with dominant T@P males having more Fos-ir cells compared to no-status T@P males. In contrast, status did not have an effect within NoT@P males [$F(2, 41) = 1.61, p = 0.212$]. For LSi, a follow-up MLM found an effect of pubertal testosterone within dominant males [$F(1, 28) = 5.11, p = 0.032$] with dominant T@P males having more Fos-ir cells compared to dominant NoT@P males. In contrast, pubertal testosterone did not have an effect with no-status or subordinate males [$F(1, 36) = 3.42, p = 0.073$ and $F(1, 24) = 0.11, p = 0.738$, respectively].

MLM revealed a main effect of status in the AH approaching statistical significance [$F(2, 89) = 3.03$, $p = 0.054$; Figure 6B] and a significant main effect of status in the VMHL [$F(2, 81) = 5.83$, $p = 0.004$; Figure 6C] on Fos-ir cell number, such that subordinate males had more Fos-ir cells than no-status males for both regions, regardless of pubertal testosterone. No other main effects or interactions were revealed for these regions.

There were no main effects or interactions on Fos-ir cell number in the PrL, IL, CeC, CeM, or VMHM (data not shown).

4. Discussion

The current study sought to determine what aspects of species-typical social behaviors during male-male interactions are organized by testicular hormones during puberty. We asked whether hormones program 1) the ability to form stable dominant-subordinate relationships, 2) the behavioral mechanisms by which these relationships are kept stable, and 3) the neural activation patterns associated with context-appropriate behaviors. We found that pubertal testicular hormones, most likely testosterone or an active metabolite, are not key for whether or not two male Syrian hamsters form a dominant-subordinate relationship or for the stability of the relationship. However, pubertal testicular hormones do program certain behavioral strategies used to maintain the relationship and the pattern of neural activation correlated with status within the relationship. When testosterone is present during puberty, adult males rely on flank marking to maintain dominant-subordinate relationships, and dominant males robustly flank mark more than subordinate males. In contrast, in the absence of pubertal testosterone, the level of flank marking during male-male interactions is generally low and not directly correlated with status. In addition, overt aggression is characteristic during re-encounters between NoT@P males, suggesting that either memory of the previously established relationship is impaired in the absence of pubertal testosterone, or that pubertal testosterone programs the behavioral mechanisms through which stability of the relationship is reinforced.

The behavioral strategies shown by T@P males were similar to the patterns previously found in gonad-intact adult males in which dominant males flank marked significantly more than subordinate males and also showed a gradual decrease in offensive aggression over repeated interactions with the subordinate male (Ferris et al., 1987). Although dominant NoT@P males tended to flank mark more than subordinate NoT@P males, overall there was not an effect of status on flank marking in NoT@P males. Furthermore, dominant and no-status NoT@P males showed similar levels of flank marking. NoT@P males also showed a significant increase in offensive posturing after the 24 h separation between trials 3 and 4, and showed overall more offensive postures than T@P males during the last 3 trials, regardless of social status. Thus, NoT@P males may be more reliant on aggressive posturing (although not actual attacks) than on chemosensory communication. Flank marking is an effective strategy in active maintenance as it does not require further one-on-one contact with the other conspecific after the dominant-subordinate relationship is established. The combination of low flank marking and high offensive posturing by NoT@P males is an inefficient behavioral strategy that may increase the probability of overt aggression.

The behavioral differences between T@P and NoT@P males during social interactions provide clues about the aspects of social behavior that are organized by pubertal testosterone. It may be that pubertal testosterone organizes neural circuits involved in response selection in social situations, such that NoT@P males use inappropriate behaviors (for the species) to communicate status. Alternatively, NoT@P males may not appropriately perceive signals from another male, leading to incorrect evaluation of their own status. A third possibility is that pubertal testosterone organizes neural circuits involved in social

recognition or memory, so that NoT@P males rely on aggression to reestablish relationships over time. These facets of social cognition and proficiency are not mutually exclusive, and additional research is needed to determine which of these (or others) are programmed by pubertal testosterone.

The relative lack of social proficiency in NoT@P males may be due to organizational differences in the neural circuitry that regulates social behaviors. Cg1 activation has not been well-studied with regard to male-male agonistic interactions in adult hamsters, but it has been implicated in emotion regulation, response selection, and appropriate social behavior during social interactions in primates and rodents (as reviewed in Devinsky et al., 1995). Therefore, Cg1 may be involved in effective maintenance of dominant status through the regulation of overt aggression and communication of status through chemosensory cues. Increased neural activation in the Cg1 was associated with dominant status in T@P males, whereas no such relationship was observed in NoT@P males. This suggests that pubertal testosterone organizes the Cg1 to promote behavioral flexibility of dominant males in order to proficiently regulate male-male relationships and maintain their dominant status. Additionally, dominant T@P males had greater neural activation in the LS compared to dominant NoT@P males. The LS suppresses aggression and reactivity in mammals, including hamsters (Potegal et al., 1981, Albert and Walsh, 1984, David et al., 2004), and is also part of the neural circuitry that regulates flank marking in hamsters (Ferris et al., 1990, Irvin et al., 1990). Therefore, the inappropriate low displays of flank marking and high displays of offensive posturing seen in NoT@P males could reflect insufficient activation of the LS in dominant NoT@P males to inhibit aggression and promote flank marking. Taken together, these data suggest a role for pubertal testosterone in organizing both the Cg1 and LS to promote appropriate neural processing of social information during male-male interactions.

Testosterone has been linked to flank marking and aggression through the modulation of arginine vasopressin. For example, microinjections of arginine vasopressin into the LS of castrated and sham-castrated adult male hamsters lead to a dose-dependent increase in flank marks for both groups, but the sham-castrates display more flank marks overall than the castrated group (Albers and Cooper, 1995). Organizational effects of testosterone on V1a receptors have also been demonstrated, with NoT@P males showing significantly increased V1a receptor binding in the LS compared to T@P males (Schulz et al., 2006). This effect seems counterintuitive given the behavioral data; however, it is possible that incomplete synaptic pruning that typically occurs during puberty may account for this discrepancy. Taken together, these results imply that testosterone facilitates agonistic behaviors through the modulation of arginine vasopressin within the LS. Therefore, further examination into the mechanisms by which pubertal testosterone organizes this neurotransmitter system could give insight into the maturation of social information processing during male-male interactions.

Pubertal testosterone did not affect neural activation patterns within the AH or VMHL. However, subordinate males had more activated cells than no-status males in these regions, a finding that may be related to the regulation of defensive behavior by the AH and VMH. For example, subordinate males that are either acutely or chronically defeated by unfamiliar dominant males both show higher levels of *c-fos* mRNA within neurons in the AH and VMHL compared to handling-control males (Kollack-Walker et al., 1999). Furthermore, in greater long-tailed hamsters, subordinate males have higher density of Fos-ir cells than dominant and control males in the AH and control males in the VMH (Pan et al., 2010). Therefore, the current study extends previous findings on the importance of both the AH and VMHL in the regulation of defensive behavior in subordinate males, and is the first to

highlight neural activation in these regions after repeated encounters with a familiar dominant male.

Our previous work showed that NoT@P males are atypically submissive when confronted by with an unfamiliar male in a resident-intruder test, indicating that pubertal testosterone programs defensive aggression in adulthood (Schulz et al., 2006). Using repeated pairings of males in the neutral arena paradigm in the current study provides insight into the contribution of pubertal testosterone in programming male agonistic behavior in other social contexts. Specifically, we found that pubertal testosterone programs the behavioral strategies used for active maintenance of male-male relationships over time. Because males were matched based on hormonal history in the current study, an important consideration in interpreting our behavioral data is that the nature of the initial social interaction between NoT@P and T@P pairs may be different and possibly influence how the individuals relate to each other in subsequent encounters. However, because our previous studies used intact males as the intruder for both NoT@P and T@P males and yet also found atypical behavioral responses by NoT@P males, this is most likely not the case (Schulz et al., 2006). Despite using different approaches, our current and previous studies point to the importance of pubertal testosterone in programming adult-typical agonistic behavior, and it appears 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 agonistic behavior.

In addition to pubertal testosterone, other variables may have contributed to the behavioral differences seen between NoT@P and T@P males in adulthood. One such variable is housing history: in this experiment, NoT@P males experienced a longer period of being singly-housed compared with T@P males. Social isolation does tend to increase aggression in male hamsters compared to males that are allowed to interact with other males over a three week period (Albers et al., 2006). However, the most robust behavioral difference between T@P and NoT@P males in the current study was the pattern of flank marking, which is not affected by social history (Albers et al., 2006). Thus, we think it is unlikely that differences in housing history account for the behavioral differences observed in this study.

5. Conclusions

The formation of stable dominant-subordinate relationships in male hamsters is not dependent on organizational effects of pubertal testosterone, but the behavioral strategy used to maintain status during repeated encounters is programmed by pubertal testosterone. Because flank marking does not differ by status in NoT@P males, we conclude that it is not a situation-dependent behavior for these males as it is for intact and T@P males. In addition, unlike T@P males, NoT@P males react more aggressively towards one another after a 24 h separation, which is indicative of an inefficient strategy for maintenance of a stable relationship. The neural activation data is reflective of the behavioral outputs observed, as the Cg1 is involved in the regulation of social behaviors and response selection, while the LS suppresses aggression and regulates flank marking. These data suggest that the differences between NoT@P and T@P males in selection of behavioral strategies for maintaining status is related to organizational effects of pubertal testosterone on either structure or functional connectivity of Cg1 and LS.

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Highlights

- Formation of stable male-male relationships is pubertal testosterone-independent
- Maintenance of male-male relationships is programmed by pubertal testosterone
- Pubertal testosterone organizes the cingulate cortex to program agonistic behavior
- Pubertal testosterone organizes the lateral septum to regulate agonistic behavior

Figure 1.

Experimental Design. Prepubertal (P21-23; NoT@P) and adult (P53-57; 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 behavior testing began.

Figure 2.

Photomicrographs of drawn contours of regions of interest (ROI) overlaid onto immunohistochemically-treated tissue sections. The ROI included: the anterior cingulate (Cg1), prelimbic (PrL), and infralimbic (IL) cortex; intermediate lateral septum (LSi), anterior hypothalamus (AH), medial (CeM) and capsular (CeC) central amygdala; and medial (VMHM) and lateral (VMHL) ventromedial hypothalamus (VMH). Scale bar = 250 μ m.

Figure 3. Mean ($\pm$ SEM) number of flank marks across all 6 trials (A) and only the last 3 trials (B) is dependent on an interaction between pubertal testosterone and status and main effect of trial. Status only affected number of flank marks in T@P males, with dominant T@P ($n = 9$) males flank marking significantly more than no-status ($n = 7$) and subordinate ($n = 8$) T@P males (+, $p < 0.05$). There were no differences between no-status ($n = 12$), subordinate ($n = 6$), or dominant ($n = 6$) NoT@P males.

Figure 4.

Mean ($\pm$ SEM) number of offensive postures is dependent on an interaction between pubertal testosterone and trial across all 6 trials and a main effect of pubertal testosterone across the last 3 trials. Trial only affected number of offensive postures only in NoT@P males ($n = 24$ per trial), with NoT@P males showing significantly less offensive postures on trial 3 compared to trial 4 (+, $p < 0.05$). There was no difference between trials within T@P males ($n = 24$ per trial). NoT@P males showed overall more offensive postures across the last 3 trials compared to T@P males. Hash marks represent 24 hours between trial 3 and 4.

Figure 5.

Mean ($\pm$ SEM) number of Fos-ir cells in anterior cingulate cortex (Cg1) is dependent on pubertal testosterone and status. There was an interaction between pubertal testosterone and status in the Cg1, where dominant T@P males had significantly more Fos-ir cells compared to no-status T@P males (+, $p < 0.05$). There were no significant differences in Fos-ir cells as a function of status within NoT@P males. Photomicrographs to the right of the bar graph are representative images of Fos-ir at 40x objective for specified group of males; scale bar = 50 μ m.

Figure 6.

Mean ($\pm$ SEM) number of Fos-ir cells in specific regions of interest is dependent on pubertal testosterone and/or status. A: There was an interaction between pubertal testosterone and status in the intermediate lateral septum (LSi), where dominant T@P males had significantly more Fos-ir cells compared to dominant NoT@P males (+, $p < 0.05$). B: There was a trend ($p = 0.054$) for subordinate males to have more Fos-ir cells compared to no-status males in the anterior hypothalamus (AH). C: Subordinate males had significantly more Fos-ir cells compared to no-status males in the lateral ventromedial hypothalamus (VMHL; *, $p < 0.05$). The two photomicrographs to the right of each ROI bar graph are representative images of Fos-ir at 40x objective for specified group of males; scale bar = 50 μ m.

Table 1Mean ($\pm$ SD) number of escape dashes during the last 3 trials

<u>Pubertal Testosterone</u>	<u>Status</u>	<u>Trial 4</u>	<u>Trial 5</u>	<u>Trial 6</u>
T@P	None (n = 7)	0	0	0
	Subordinate (n = 8)	3.75 $\pm$ 2.76	3.13 $\pm$ 2.30	2.00 $\pm$ 1.85
	Dominant (n = 9)	0	0	0
NoT@P	None (n = 12)	0	0.08 $\pm$ 0.29	0
	Subordinate (n = 6)	2.17 $\pm$ 1.83	2.17 $\pm$ 3.06	2.83 $\pm$ 2.64
	Dominant (n = 6)	0	0	0