

Sexual experience and testosterone during adolescence alter adult neuronal morphology and behavior



John S. Morris, Zachary M. Weil, Randy J. Nelson*

Department of Neuroscience, The Ohio State University, Wexner Medical Center, Columbus, OH 43210, USA
 Department of Psychology, The Ohio State University, Columbus, OH 43210, USA

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ABSTRACT

Steroid hormones released immediately before and after birth provoke sexual differentiation of neural circuits. Further, steroid hormones secreted during adolescence also exert long lasting effects on the nervous system. Hormones secreted during development may act through two distinct pathways: (1) hormones can directly affect neuron and synapse elimination and (2) endocrine changes in the nervous system may occur secondary to changes in social behaviors. Therefore, a critical period for organization of the nervous system by steroid hormones during adolescence may also be a sensitive period for the effects of social experience. The overall goal of this experiment was to determine whether the opportunity to mate with a sexually receptive female during this adolescent critical period would have enduring effects on behavior and neuronal morphology into adulthood. A second question was to determine the extent to which testosterone mediated the effects of these social interactions on adult outcomes. Compared to sexually inexperienced hamsters and those that experienced sex for the first time in adulthood, hamsters that experienced adolescent sexual experience displayed increased anxiety- and depressive-like behavioral responses. Adolescent sexual experiences decreased the complexity and length of dendrites on prefrontal cortical neurons and increased the expression of the pro-inflammatory cytokine interleukin 1 β (IL-1 β) in the PFC. In a second experiment, administration of testosterone during the adolescent period largely recapitulated the effects of adolescent sexual experience. These data support the overall hypothesis that a sensitive period extends into adolescence and that salient social stimuli during this time can significantly and persistently alter adult phenotype.

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Introduction

Steroid hormones released immediately before and after birth provoke sexual differentiation of neural circuits. Steroid exposure during prenatal and neonatal life organizes the brain into its sexually differentiated state, as well as determines the potential for behavioral responses to steroid hormones later in life (i.e., activational effects of hormones).

The distinction between organizational and activational effects has eroded with the accumulation of evidence that organizational effects of steroid hormones persist well after early neuronal development (Arnold and Breedlove, 1985). The two-stage model of neurodevelopmental programming of brain and behavior posits the existence of two sensitive periods for steroid dependent organization of brain and behavior (Phoenix et al., 1959; Sisk and Zehr, 2005). More recently, experimental attention has focused on adolescence as a time when steroid hormones can exert long lasting effects on the nervous system (Romeo et al., 2002; Schulz and Sisk, 2006). For example, hamsters deprived of androgens during adolescence show reduced

sexual and aggressive behaviors in adulthood even when treated with androgens (Schulz and Sisk, 2006; Schulz et al., 2004, 2006).

The transitions from immaturity to fully developed adult that occur during adolescence presumably require significant adjustments in behavior and neural circuitry for future reproductive success (Romeo et al., 2002; Sisk and Foster, 2004). Adolescence is a transitional phase when juvenile animals shift into adult typical behavioral and neurological phenotypes (Buwalda et al., 2011). Marked plasticity occurs during adolescence that alters both the structure and function of the brain (Cunningham et al., 2002). Regions of particular interest during this period are the frontal cortical areas because these nuclei undergo structural remodeling during adolescence and early adulthood (Andersen and Teicher, 2008; Asato et al., 2010; Kolb and Nonneman, 1976).

Hormones during development may act through two distinct pathways. First, hormones can directly affect neuron and synapse elimination and neuronal morphology (McEwen, 1999; Morris et al., 2004; Simerly, 2002). However, endocrine changes in the nervous system may occur secondary to changes in social behaviors. For instance, androgenic hormones promote aggressive behaviors in many species (Beeman, 1947; Wingfield et al., 1990) and the experience of fighting can both alter neuronal function and future circulating androgen concentrations (Delville et al., 1998; Faruzzi et al., 2005; Trainor et al., 2004). Therefore,

* Corresponding author at: The Ohio State University, Wexner Medical Center, Department of Neuroscience, 333 West 10th Avenue, Columbus, OH 43210 USA.
 E-mail address: Randy.nelson@osumc.edu (R.J. Nelson).

a critical period for organization of the nervous system by steroid hormones may also be a sensitive period for the effects of social experience. The overall goal of these experiments was to determine whether the opportunity to mate with a sexually receptive female during this adolescent critical period would have enduring effects on behavior and neuronal morphology into adulthood. In a second experiment, we sought to determine whether testosterone, a hormone that increases during mating behavior (Kamel and Frankel, 1978), could mimic the effects of early sexual experience if administered during adolescence.

Materials & methods

Animals

Siberian hamsters (*Phodopus sungorus*) used in this study were bred in our colony at The Ohio State University from a wild-bred stock obtained from Dr. K. Wynne-Edwards (Kingston, Ontario, Canada). Hamsters were housed in polypropylene cages (28 × 17 × 12 cm) with a nestlet and 1 cm of corncob bedding. Hamsters were weaned at approximately 21 days of age in a long photoperiod (16:8 LD; with lights-off at 1500 Eastern Standard Time [EST] in the room) and housed within this room for the duration of the study. All hamsters had ad libitum access to food (Harlan Teklad Rodent Diet 8640, Indianapolis, IN, USA) and filtered tap water. Animal rooms were maintained at an unvarying temperature and humidity (21 ± 2 °C and 50 ± 10%, respectively). All procedures were conducted in accordance with the US National Institute of Health (1986) Guide for the Care and Use of Laboratory Animals, The Ohio State University Institutional Animal Care and Use Committee, and the international ethical standards described previously (Portaluppi et al., 2008).

Siberian hamsters were selected for these experiments for three reasons. First, Siberian hamsters exhibit significant morphological and behavioral shifts in response to other environmental perturbations (e.g. day length, food restriction, etc.). Secondly, using non-traditional laboratory species allows us to (largely) avoid the effects of long term breeding in captivity and domestication that could potentially obscure some of these phenomena. Third, although they used a different species of hamsters, Sisk and colleagues conducted many of the foundational studies for this work in hamsters (Schulz and Sisk, 2006; Schulz et al., 2009).

Experiment 1

Sexual experience

Adult male Siberian hamsters were housed individually from weaning. At 40 or 80 days of age males were paired with an ovariectomized, hormonally primed female or kept in isolation; mating behavior was recorded to ensure that copulation occurred and females were removed after six hours. In order to control for both age at the time of mating and the time between mating and behavioral testing hamsters were tested either 40 or 80 days after mating. This produced 5 experimental groups, (1) a control group that never mated (control) and were tested at 80 days, (2) hamsters that mated at 40 days and were tested 80 days later at 120 days (40 × 80), (3) hamsters that mated at 40 days and were tested 40 days later (40 × 40), (4) hamsters that mated at 80 days and were tested 40 days later (80 × 40), or (5) hamsters that mated at 80 days and were tested 80 days later (80 × 80). The comparison of developmental age across species produces only rough equivalencies. However, 40 days of age in Siberian hamsters correspond to approximately 13.2 years in humans and 80 days corresponds to approximately 16.4 years in humans (Flurkey et al., 2007).

All behavioral tests were administered between 15:00 and 18:00 h and hamsters were given 30 min to habituate to the test room before initiation of testing. Tests were performed in the following order: (1) elevated-plus maze, (2) forced swim test, and (3) sucrose anhedonia.

Behavioral tests

Elevated plus maze

At 80, 120, and 160 days of age Siberian hamsters were tested in the elevated plus maze. The closed arms are enclosed by 15 cm tall black Plexiglas. All arms were covered with contact paper to prevent the hamsters from sliding off, and all surfaces were wiped with 70% alcohol between animals. Hamsters that fell off the maze into compartments below were placed back on the maze. An observer uninformed about experimental conditions scored the videotapes with The Observer software (version 5, Noldus Software, Leesburg, VA, USA) for (a) percentage of entries into open arms (b) and total entries into all arms. Hamsters were considered to have entered an arm when all four paws crossed onto an arm of the maze.

Forced swim test

Hamsters were examined for cessation of attempting to escape water by placing them in 17 cm of room-temperature water (22 ± 1 °C) in a cylindrical pool (24 cm diameter, 53 cm height) with opaque walls. Behavior was recorded for 7 min and scored by an uninformed observer to determine (a) swimming (i.e., climbing or scratching directed at the wall of the tank and horizontal movement in the tank), or (b) floating (i.e., minimal movement required to maintain head above the surface of the water).

Sucrose anhedonia

Hamsters were first presented with a modified water bottle containing tap water and an empty modified water bottle for 5 h over the course of three consecutive nights (15:00–21:00 h). The drinking water was measured each night in order to establish a baseline measurement of overall water consumption. On the fourth day, hamsters were presented with a bottle containing water and a bottle containing sucrose solution for 5 h over 6 consecutive nights (15:00–21:00 h). To control for possible side preferences, placement of the bottles in the cage was counterbalanced. Sucrose consumption was normalized to the average pretesting water consumption.

Following behavioral testing hamsters were anesthetized, decapitated and reproductive tissues were extracted (testes, epididymal fat pads, seminal vesicles, and epididymides), cleaned of connective tissue, and weighed to the nearest 0.1 mg. Half brains were submerged in Golgi-Cox solution and stored for 14 days in the dark. Brains were then flash frozen with dry ice and 100 μm coronal sections were sliced on a cryostat and collected onto gelatin-coated glass slides. The stain was developed in NH₄OH for 10 min and sections were counterstained with cresyl violet. The other half of the brain was used for analysis of gene expression.

Polymerase chain reaction

RNA was extracted from dissected PFC. cDNA was created via reverse transcription of 2 μg of RNA from each sample with MMLV Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Interleukin IL-1β gene expression was quantified with a premade kit (Applied Biosystems). A TaqMan 18S rRNA primer and probe set, labeled with VIC dye (Applied Biosystems), were used as a control gene for relative quantification. Amplification was performed on an ABI 7000 Sequence Detection System.

Analysis of brain morphology

Histology

Neurons impregnated with the Golgi-Cox solution were chosen within layer III of the PFC. A Rapid Stain Golgi kit (FD Neurotechnologies, Columbia, MD) was used according to the manufacturer's instructions. We have previously used this kit successfully with Siberian hamsters (Bedrosian et al., 2011). The layer 3 cortical neurons were chosen because they display robust aging and stress-related plasticity and because they are corticocortical projection neurons that receive dense inputs

from forebrain association and limbic cortices and thus are intimately involved in the integrative and regulatory functions of prefrontal cortex (Bloss et al., 2010, 2011; Hoover and Vertes, 2007). Only fully impregnated neurons that were not obscured by neighboring neurons and had no obviously truncated dendrites were chosen for analysis. An investigator blind to the conditions of the animals conducted all scoring. For each animal, six randomly chosen, representative neurons were completely traced at 20 \times (N.A. 0.75) using NeuroLucida 8 software (MicroBrightField, Williston, VT, USA) for PC and a Nikon Eclipse E800 brightfield microscope. Morphological characteristics were analyzed using NeuroLucida Explorer software (MicroBrightField, Williston, VT, USA) and consisted of: (1) basilar dendritic length, (2) apical dendritic length, (3) basilar dendritic intersections, and (4) apical dendritic intersections.

Statistical analyses

Data analyses were conducted using analysis of variance (ANOVA). Mean differences were considered statistically significant if $p < 0.05$. Significant differences were followed by LSD post-hoc tests.

Results

Experiment 1

Several behavioral measures were used to assess anxiety-like and depressive-like responses well after sexual contact (i.e., at ages P80, P120, P160). Total arm entries did not differ among groups ($p > 0.05$ see Fig. 1A). However, there was a significant reduction in percentage of time spent in the open arm among all sexually experienced males compared to control hamsters ($p < 0.05$ see Fig. 1B). In contrast, sexual experience during early adolescence, but not during other developmental epochs, increased time spent floating in the forced swim test when tested 80 days later ($p < 0.05$ see Fig. 1C). Sexual experience reduced voluntary sucrose intake with 40 \times 80, 40 \times 40 and 80 \times 40 groups all consuming less sucrose than unmated control hamsters ($p < 0.05$; see Fig. 1D).

Paired testis mass was reduced in the 40 \times 80 group as compared to other groups ($p < 0.05$; see Fig. 2A). Similarly, other steroid-sensitive tissue masses were reduced in 40 \times 80 hamsters with smaller seminal vesicles (Fig. 2B) and epididymides ($p < 0.05$ in both cases; see Fig. 2C). There were no differences in body mass detected ($p > 0.05$ see Fig. 2D).

Neuronal morphology

Sexual experience during adolescence significantly altered the morphology of layer three pyramidal neurons in the prefrontal cortex. Sexual experience during adolescence (40 day groups) reduced the total length (Figs. 3A–B) and number of intersections (Figs. 3C–D) of apical and basilar dendrites compared to all other groups ($p < 0.05$ in all cases). There were no significant differences observed between 80 \times 80 or 80 \times 40 and sexually naïve control hamsters.

Gene expression analysis of the prefrontal cortex in hamsters that engaged in a first sexual encounter during adolescence (killed at 120 days of age) revealed increased mRNA expression of IL-1 β as compared to all other experimental groups ($p < 0.01$) (Fig. 4).

Experiment 2

A separate cohort of hamsters was subcutaneously injected with testosterone at a concentration of 250 $\mu\text{g}/\text{kg}$ in olive oil, at either 40 or 80 days of age and then tested 40 or 80 days later. Control animals were injected with the oil vehicle.

Animals that were exposed to T during adolescence displayed increased anxiety- and depressive-like behavioral responses, compared

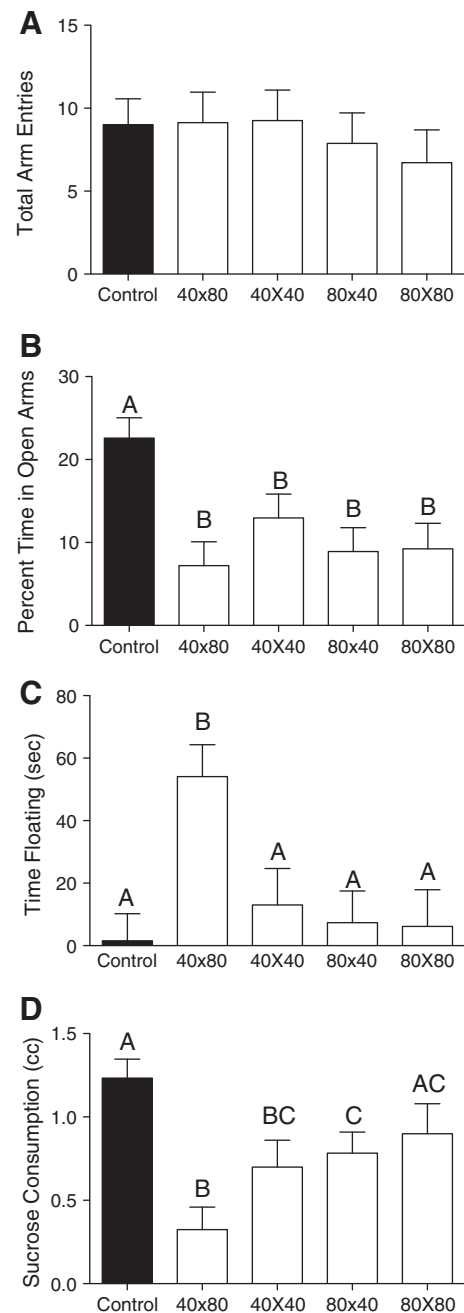


Fig. 1. Early sexual experience increases depressive like behaviors. In the elevated plus maze there was no effect of sexual experience on (A) total arm entries; however sexual experience, regardless of age, decreased (B) the percentage of time spent in the open arms. In the forced swim test, (C) the 40 \times 80 group spent significantly more time floating than all other groups. (D) Sucrose intake was also reduced by sexual experience particularly when it occurred early in life. Data are presented as means (\pm SEM). Bars with different letters are significantly different from one another. Data were considered statistically significant if $p < 0.05$.

to the non-T and adult-T injected hamsters. In the forced swim test, the P40 \times 80 and hamsters significantly increased the percentage of time spent floating ($p < 0.01$) (Fig. 5A). In the elevated plus maze, the P40 \times 80 and P40 \times 40 hamsters spent significantly less time in the open arms than did sexually naïve hamsters ($p < 0.05$; Fig. 5B) and there was no difference among groups in regard to total arm entries.

Paired testis mass was reduced in the 40 \times 80 group as compared to other groups ($p < 0.05$; see Fig. 6A). The other steroid-sensitive tissue masses including seminal vesicles (Fig. 6B) and epididymides ($p > 0.05$

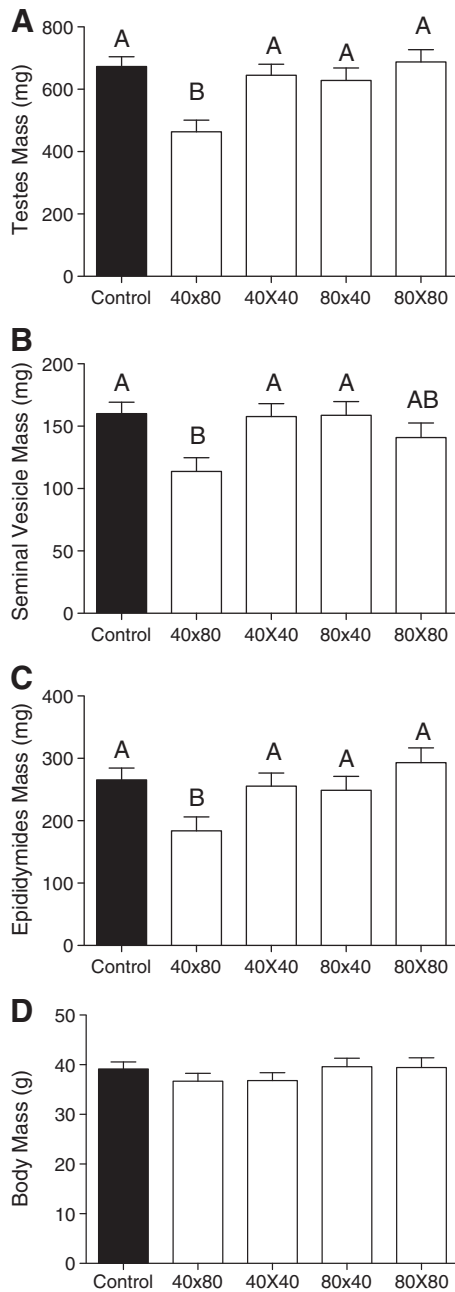


Fig. 2. Early sexual experience reduced reproductive tissue masses. (A) Testes, (B) seminal vesicle, and (C) epididymal mass were all reduced in hamsters from the 40 × 80 group. Data are presented as means (\pm SEM). Bars with different letters are significantly different from one another. Data were considered statistically significant if $p < 0.05$.

in both cases; see Fig. 6C) were not altered by adolescent testosterone administration. There were no differences in body mass detected ($p > 0.05$ see Fig. 6D).

Discussion

Exposure to sexual contact during adolescence altered anxiety- and depressive-like behaviors, expression of the gene encoding the proinflammatory cytokine IL-1 β , and neuronal morphology. Administration of testosterone during a similar developmental epoch largely recapitulated the behavioral phenotype of sexually experienced hamsters. These data support the overall hypothesis that a sensitive period extends into adolescence and that salient social stimuli during this time can significantly and persistently alter adult phenotype (Romeo et al.,

2002; Schulz and Sisk, 2006; Schulz et al., 2004, 2009). The results are somewhat counterintuitive because sexual activity is typically interpreted as a rewarding experience (Leuner et al., 2010). In rodents sexual experience is a powerful motivator with the potential to impose organizational change in neuro-circuitry. For instance, chronic sexual experience enhances cell propagation, adult neurogenesis, and dendritic spine numbers in emotion and memory related areas in the brains of adult male rats (Leuner et al., 2010). Importantly, the large changes in anxiety- and depressive-like behaviors and neuronal morphology occur only after sexual experience during adolescence and not after sexual experience during adulthood.

Adolescent brain development consists in large part of the organization of neural circuits in the brain that then promote rapid neural and gonadal development (Sisk and Foster, 2004). The enhancement of plasticity during adolescence and puberty may impose increased susceptibility to neurological and behavioral disturbance. Substantial remodeling takes place during this period in brain areas involved in emotion and learning such as the prefrontal cortex (PFC) (Spear, 2000). The PFC is highly sensitive to the effects of environmental perturbations, and is associated with socio-emotional disturbances and increased risk for psychopathologies later in life (Heim and Nemeroff, 2001; Heim et al., 2004; Spear, 2000). The prefrontal cortex is an area of the brain involved in executive function that has a protracted maturational trajectory, with this region not reaching full maturity until well into adulthood. Adolescent sexual experience reduced both the numbers of intersections and overall length of both basilar and apical dendrites on pyramidal cells in the PFC. Although it is not currently possible to causally link the depressive- and anxiety-like behaviors to changes in prefrontal morphology, there is strong evidence for reduced prefrontal dendritic complexity in various animal models of stress and depression (Cook and Wellman, 2004; Cotter et al., 2002).

An additional potential mediator of the effects of adolescent sexual experience on adult phenotype is the inflammatory system. Inflammation has been strongly linked to depression (Capuron and Miller, 2011). The presence of inflammatory factors predicts the onset of depressive disorders (Dantzer et al., 2008). Patients with major depression have increased peripheral blood inflammatory biomarkers (Miller et al., 2009). Cytokines interact with nearly every neuronal domain pertinent to the onset of depression, including the metabolic processes of neurotransmission, the function and communication of neuroendocrine systems, and plasticity in the brain (Raison et al., 2006). Early life experiences are important modulators of immune function, but the presence of inflammatory mediators has yet to be assessed following exposure to a prominent social event, viz., sexual intercourse, during adolescent life. In addition to their role in immune signaling, pro-inflammatory cytokines are potent modulators of behavior and affect (Dantzer et al., 2008). Both exogenous and endogenous proinflammatory cytokines (e.g., IL-1 β) induce depressive-like behavioral responses in nonhuman animals (Dantzer et al., 2008).

Adult behavioral, hormonal, and immunologic responsivity can be largely modified by early experiences; thus, any events that disrupt development early on might lead to a maladaptive stress response system and increased susceptibility to disease (O'Mahony et al., 2009). Childhood maltreatment predicts inflammatory bias in adults, possibly due to early life adverse experiences programming stress-responses later in life (Danese et al., 2007). Taken together, these data indicate that enhanced prefrontal cytokine gene expression may underlie the depressive-like behaviors induced by early sexual experience. However, IL-1 β gene expression was only elevated in the 40 × 80 group suggesting that the inflammatory signaling may only occur during specific developmental periods in response to social stimuli. The cellular source, proximal stimuli, and causal relationship among prefrontal cortical morphology, proinflammatory cytokines, and affective behaviors all remain unspecified. Additional research also needs to address the role of the hypothalamic–pituitary–adrenal (HPA) responses to these adolescent experiences as glucocorticoids have been implicated in the

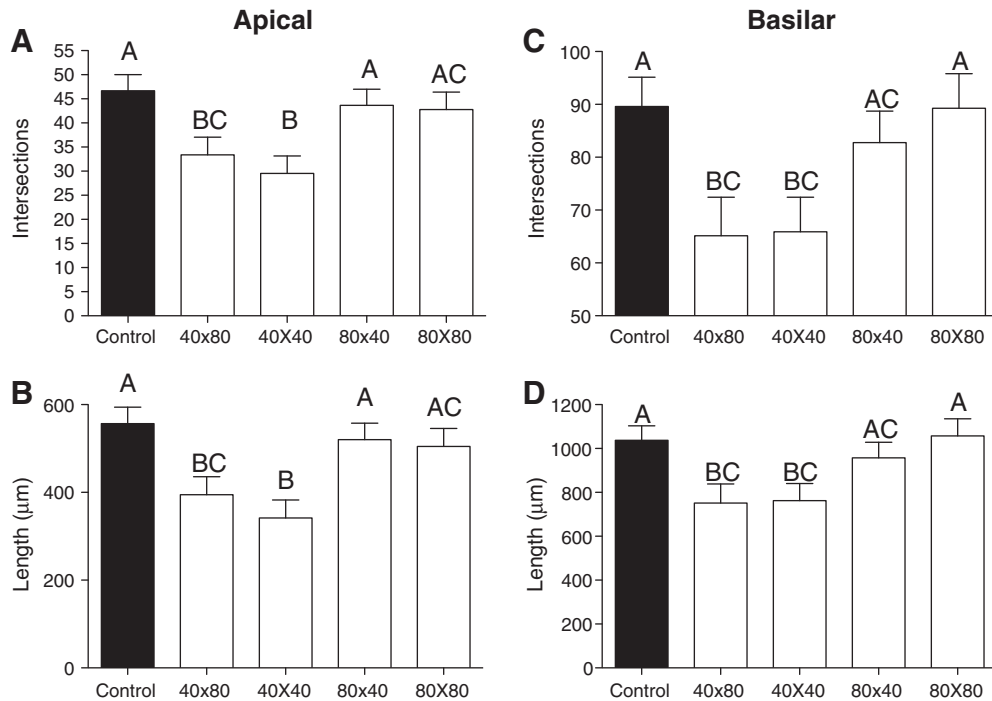


Fig. 3. Early sexual experience reduces dendritic length and complexity in the prefrontal cortex. Early sexual experience reduced the number of intersections and total length of both apical (A and B) and basilar dendrites (C and D). Data are presented as means (\pm SEM). Bars with different letters are significantly different from one another. Data were considered statistically significant if $p < 0.05$.

key outcome measures here including anxiety- and depressive-like behavior, proinflammatory cytokine gene expression, and altered neuronal morphology (McEwen, 1999).

The objective of the second experiment was to test the hypothesis that elevated testosterone during adolescence could recapitulate the effects of early sexual experience on anxiety- and depressive-like behaviors in adulthood. The major aim of this experiment was to evaluate the effects of exposure to elevated concentrations of testosterone during adolescence and the potential effects it could have on behavioral development and adult phenotype. When administered to Syrian hamsters early during puberty, T treatment led to greater sex behavior in adulthood including mounts, intromissions, and ejaculations (Sisk and Zehr, 2005). Furthermore, a correlation exists between testosterone and anxiety and depressive-related behavior in this species (Sisk and Zehr, 2005). The behavioral analyses of both sexual experience and testosterone-treated hamsters mirrored each other in a way that suggests that testosterone plays a crucial role in mediating this process. High concentrations of testosterone during mid-adolescence coinciding with changes in neural circuitry are sufficient to alter adult affective behaviors.

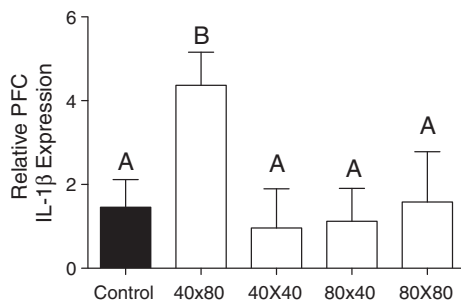


Fig. 4. Early sexual experience elevates prefrontal IL-1 gene expression. Data are presented as means (\pm SEM). Bars with different letters are significantly different from one another. Data were considered statistically significant if $p < 0.05$.

Accessory reproductive tissues were removed and weighed at the conclusion of this experiment. Hamsters that engaged in sexual activity in adolescence had decreased overall weights of testes and accessory reproductive tissue. These data suggest that in addition to the time of sexual exposure, this manipulation also appears to have produced long-term suppression of gonadal activity at least in the 40×80 group. Reductions in circulating androgens could result from the smaller testes and lead to involution in the accessory reproductive tissues that are highly androgen-dependent. The effects of early sexual experience on testes mass (but not seminal vesicles) were recapitulated by early testosterone administration. This allows for the possibility that HPG responses to sexual stimuli cause an overall reduction in HPG activity later in life although why there was no difference in the size of the seminal vesicles remains unspecified. In any case, the role of reduced HPG axis activity in depression and altered neuronal morphology remains speculative, but could be a contributing factor (Lund et al., 1999).

One unexpected result of this study is that some of the effects of sexual experience and testosterone in adolescence, specifically related to depressive- and anxiety-like behaviors and IL-1 gene expression, were observed 80, but not 40, days after sexual experience. A similar pattern of responses (i.e., phenotypic shifts after early manipulations that are not apparent until later in life) has been reported in response to neonatal hippocampal lesions, obstetric complications, nicotine and alcohol administrations, and neonatal stress (Andersen et al., 2002; Fried, 1982; Kosofsky and Hyman, 2001; Lillrank et al., 1995). In contrast, prefrontal cortical morphology was altered by adolescent sexual experience regardless of when tissue was collected. It is not obvious why these results are not convergent. It is possible that the linkage among these variables (cytokine gene expression, neuronal morphology, and affective behaviors) is not directly causal though there are examples of transient cytokine expression having enduring effects on behavior (Bilbo et al., 2005). The behavioral and inflammatory changes occur later than the morphological changes suggesting an interaction and possibly developmental process between the age of sexual encounter and the age at which the animals are tested (Andersen, 2003).

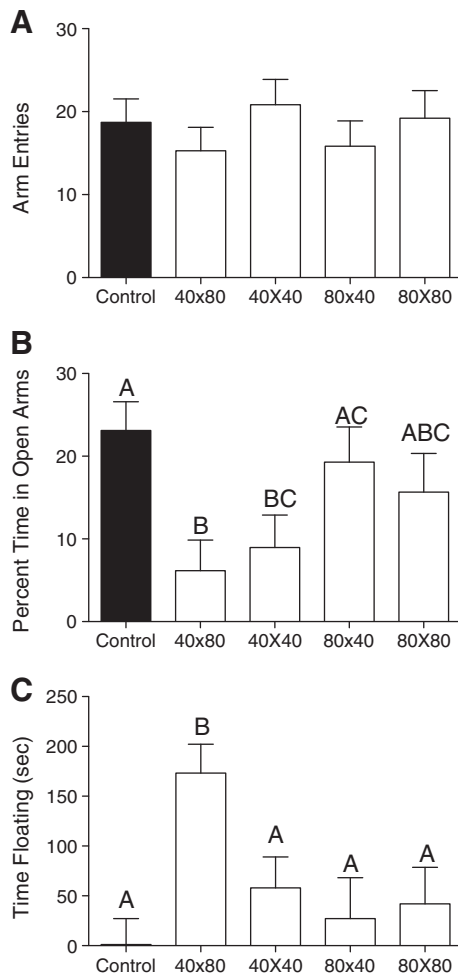


Fig. 5. Testosterone administered during adolescence induces anxiety- and depressive-like behaviors. A separate cohort of hamsters was treated with testosterone at either 40 or 80 days of age and then tested 40 or 80 days later. (A) Time spent floating was significantly elevated in the 40 × 80 group relative to all other groups, (B) the percentage of time spent in the open arms was reduced in hamsters that were injected with testosterone at 40 days of age, but there were no differences in (C) total arm entries. Data are presented as means (± SEM). Bars with different letters are significantly different from one another. Data were considered statistically significant if $p < 0.05$.

Sexual experience regardless of developmental timing reduced sucrose intake compared to sexually naïve animals. The specific mechanisms that underlie this phenomenon remain unspecified, but apparently sexual experience produces long lasting changes in reward salience.

These studies have potential implications beyond basic biology; evidence from human studies shows that male and female early maturers reported engaging in more sexual activity and are more susceptible to negative outcomes in adulthood than late maturers (Flannery et al., 1993). Epidemiological studies have demonstrated that there is a positive relationship between early sexual intercourse and development of depression in adult humans (Jamieson and Wade, 2011; Kaltiala-Heino et al., 2003; Waller et al., 2006). These data demonstrate that in a controlled laboratory environment sexual experience has a long lasting effect on adult phenotype and indicates that natural variation in pubertal onset and first sexual experience could play a critical role as a risk factor for psychiatric disease.

The studies presented here add to the growing body of literature linking events during the pubertal period to adult phenotype. Specifically, sexual experience during adolescent development has an enduring effect on neuronal development, inflammatory signaling, and behavior — an effect that is largely recapitulated by exogenous testosterone.

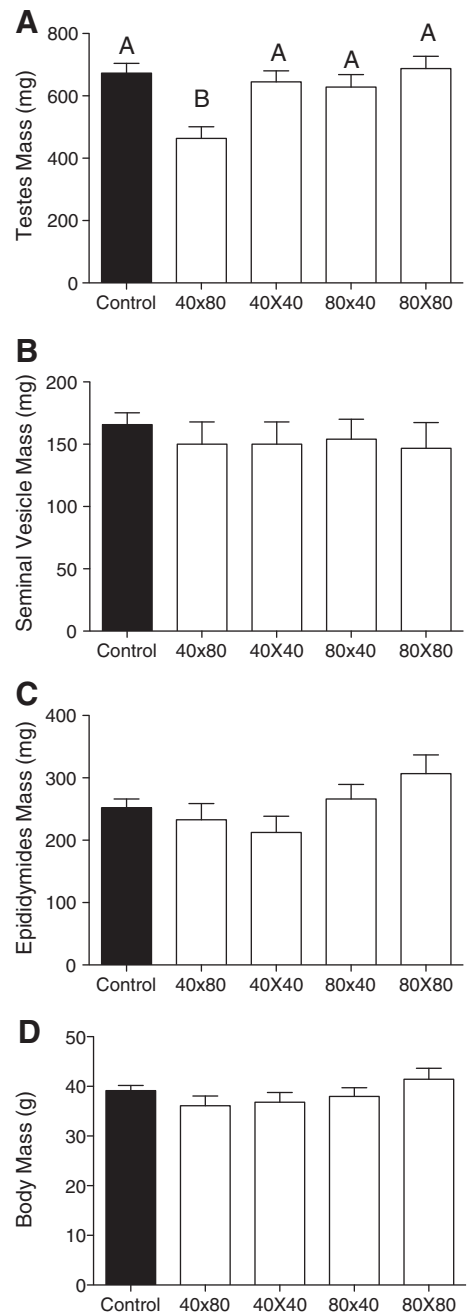


Fig. 6. Early testosterone administration reduces testicular but not accessory reproductive tissue masses. (A) Testes masses were reduced in hamsters from the 40 × 80 group. However, (B) seminal vesicle, and (C) epididymal masses did not vary. Data are presented as means (± SEM). Bars with different letters are significantly different from one another. Data were considered statistically significant if $p < 0.05$.

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