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Pubertal testosterone programs adult behavioral adaptations to sexual experience through infralimbic cortex ΔFosB

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Abstract

Acquisition of social proficiency entails behavioral adaptations to social experience, including both behavioral flexibility and inhibition of behaviors inappropriate in specific social contexts. Here, we investigated the contributions of testosterone and ΔFosB, a transcription factor linked to experience-dependent neural plasticity, to the adolescent maturation of social proficiency in male-female social interactions. To determine whether pubertal testosterone organizes circuits underlying social proficiency, we first compared behavioral adaptations to sexual experience in male Syrian hamsters that were deprived of testosterone during puberty (prepubertal castration; NoT@P) to those of males deprived of testosterone for an equivalent period of time in adulthood (postpubertal castration; T@P). All males were given testosterone replacement in adulthood for two weeks before sexual behavior testing, where males were allowed to interact with a receptive female once per week for five consecutive weeks. T@P males showed the expected decrease in ectopic (mis-directed) mounts with sexual experience, whereas NoT@P males did not. In addition, sexual experience induced *FosB* gene products expression in the infralimbic cortex (IL) in T@P, but not NoT@P, males. Over-expression of ΔFosB via an adeno-associated viral vector in the IL of NoT@P males prior to sexual behavior testing was sufficient to produce a behavioral phenotype similar to that of experienced T@P males. Finally, over-expression of ΔFosB in IL increased the density of immature spines on IL dendrites. Our findings provide evidence that social proficiency acquired through sexual experience is organized by pubertal testosterone through the regulation of ΔFosB in the IL, possibly through increasing synaptic lability.

Significance Statement

Social proficiency is the ability to make experience-dependent behavioral adaptations that enhance the success of subsequent social interactions. In male rodents, social proficiency in adulthood is programmed by the pubertal rise in testosterone, but neuroendocrine mechanisms underlying this behavioral plasticity are not understood. We show that pubertal testosterone is necessary for both sexual proficiency and experience-dependent induction of ΔFosB in the infralimbic (IL) medial prefrontal cortex in adulthood. Furthermore, over-expression of ΔFosB in the IL increases immature dendritic spines on IL neurons and is sufficient to restore a socially proficient phenotype in males that lacked testosterone during puberty. Hormonal programming of experience-dependent regulation of prefrontal ΔFosB is a novel mechanism of adolescent development of behavioral and neural plasticity in adulthood.

Introduction

A vital aspect of adolescent development is the acquisition of social behaviors and skills that prepare an individual for successful adult social interactions and promote evolutionary fitness. During adolescence, the primary social sphere transitions from family to peers, resulting in new social experiences and competencies (Nelson et al., 2005). Social proficiency is the ability of an individual to make experience-dependent behavioral adaptations that enhance the success of subsequent social interactions, and this proficiency involves behavioral flexibility and inhibition of maladaptive behaviors. Adolescent maturation of behavioral inhibition and social proficiency necessarily involves circuits underlying executive control of social motivation and learning (De Lorme et al.,

2013), but the neural and endocrine mechanisms of this developmental change are largely unexplored; the present experiments were designed to identify these potential mechanisms.

Many of the behavioral changes related to the adolescent maturation of social proficiency have been attributed to puberty, which defines the onset of adolescence and is characterized by an increase in gonadal hormone secretion as reproductive maturation begins. The single-most important social interaction for evolutionary fitness is sexual behavior that leads to the production of offspring. Adolescent maturation of male sexual behavior is achieved in part through organizational effects of testosterone on the developing brain to program sexual proficiency, as shown in studies using male Syrian hamsters. For example, during a first sexual encounter with a receptive female, sexually naïve adult male hamsters typically display a high rate of ectopic (mis-directed) mounts in addition to vaginally oriented mounts. With sexual experience, however, the number of ectopic mounts decreases, thereby improving sexual proficiency and reproductive success (Schulz and Sisk, 2006). Notably, the acquisition of sexual proficiency is not observed in adult hamsters deprived of testosterone during puberty via prepubertal castration; such males continue to show high rates of ectopic mounts even after repeated sexual encounters (Schulz and Sisk, 2006; De Lorme and Sisk, 2016). These data suggest that testosterone programs social proficiency by organizing neural circuitry involved in behavioral inhibition.

The medial prefrontal cortex (mPfC) and nucleus accumbens (NAc) are key components of the neural circuitry that regulates motivated behaviors. The mPfC is involved in behavioral flexibility and inhibition, whereas the NAc is critical for processing

and evaluating social information and then generating a behavioral response (Sesack and Grace, 2010; Euston et al., 2012). Sexual experience induces long-term expression of the transcription factor ΔFosB within both the mPfC and NAc of male and female rodents, and experimental over-expression of ΔFosB in the NAc of sexually naïve female and male rodents increases sexual performance and motivation (Wallace et al., 2008; Hedges et al., 2009; Pitchers et al., 2010). 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. One possible mechanism by which the induction of ΔFosB mediates experience-dependent plasticity is through the formation of immature dendritic spines, as ΔFosB over-expression in the NAc increases immature dendritic spines (Grueter et al., 2013; Robison et al., 2013; Eagle et al., 2015). Thus, ΔFosB may regulate behavioral plasticity by modulating transcription of downstream target genes related to synaptic plasticity.

Here, we investigated the neural mechanisms by which pubertal testosterone programs sexual proficiency in adulthood. First, to determine whether pubertal testosterone affects regulation of ΔFosB, the effects of sexual experience on ΔFosB expression in the mPfC and NAc were compared in male hamsters that underwent adolescent development in either the presence or absence of testosterone during puberty. Next, to link ΔFosB with behavioral flexibility and inhibition, we determined whether over-expression of ΔFosB in the infralimbic cortex (IL) of the mPfC is sufficient to restore sexual proficiency in males that were deprived of testosterone during puberty. Finally, we asked whether sexual experience or over-expression of ΔFosB in the IL of sexually naïve males induce similar changes in IL dendritic spines. We discovered that

pubertal testosterone programs behavioral adaptability through the regulation of ΔFosB in the IL, and our data also suggest that ΔFosB in the IL may exert its behavioral effects through changes in glutamatergic synapse formation and/or stability.

Materials and Methods

General Methods

Animals

To exclude age at shipment as a potentially confounding variable, and to ensure that post-weaning housing conditions were similar for all experimental subjects in Experiments 1 and 2, gonad-intact weanling (postnatal day (P)21-26) male Syrian hamsters were ordered from Harlan Laboratories (Madison, WI). In addition, so that prepubertal and postpubertal gonadectomies, stereotaxic injections, behavioral testing, and tissue collection could be performed at the same time for all groups in Experiments 1 and 2, the males that were gonadectomized prepubertally were shipped and received 4 weeks later than males that were gonadectomized postpubertally (described below; see Figure 1). For Experiment 3, sexually naïve, gonad-intact adult male hamsters were ordered from Harlan. For all experiments, hamsters were individually housed 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) upon arrival. Sample sizes and experimental manipulations are described for each experiment below. Ovariectomized, sexually experienced female Syrian hamsters (4-7 months of age) from our colony (also originally obtained 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.

Gonadectomy

Gonadectomies were performed on male hamsters under isoflurane anesthesia and aseptic conditions. Before the procedure, ketoprofen analgesic (5 mg/kg, sc) was administered to each male. For the surgery, a bilateral scrotal incision 8-10 mm in length was made, the testes were then gently pulled through the incision, and the testicular veins and arteries were tied with suture silk (3-0) before removal of the testes. For prepubertal animals, testes were removed via cauterization of the testicular veins and arteries. For sham surgeries in Experiment 1, a bilateral scrotal incision 8-10 mm in length was made, the testes were then gently pulled through the incision, and then placed back into the incision. After removal of the testes (gonadectomy) or replacement of testes back into the incision (sham surgery), the incisions were closed with suture silk (5-0).

Testosterone replacement

Two silastic pellets (13 mm and 5 mm length with 4 mm of sealing glue on both ends; inner diameter 1.98 mm; outer diameter 3.18 mm) that contained either testosterone or nothing were inserted subcutaneously through a 5 mm incision made on the dorsal midline between the scapulae of the animal while anesthetized with isoflurane. The pellets are constructed by loading silastic tubing with powdered testosterone (or left empty) and sealing each end of the tube with surgical grade adhesive.

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 testing

In all experiments, 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, whichever came first. Previous experiments showed that during fixed-time tests, males deprived of testosterone during puberty achieve fewer intromissions and ejaculations than males deprived after puberty, and consequently, less sensory feedback that is potentially important for behavioral adaptations (Schulz et al., 2004; Schulz and Sisk, 2006). To address this possible confound, instead of absolute amount of time with the female, sexual behavior was equated for all males to ensure that any behavioral differences between groups were not due to different sensory experience. The behavioral criteria that ended each trial were: one ejaculation for trial 1, one ejaculation plus two intromissions for trial 2, two ejaculations for trial 3, one ejaculation plus two intromissions for trial 4, and one ejaculation for trial 5. The behavioral criteria were varied because we found in a previous study that when the same criterion was applied for each sexual behavioral test (achieving five intromissions) both gonad-intact and T@P male

161 hamsters would decrease their sexual behavior after trial 3 (De Lorme and Sisk, 2016).
162 Behavior testing was conducted once a week for five consecutive weeks (one of the five
163 above-described trials/week). Males were excluded from further study if they did not
164 meet the behavioral criteria in at least 3 out of the first 4 trials. Behavior during each trial
165 was digitally recorded for later quantification.

166 *Sexual behavior quantification*

167 The behaviors investigated for Experiments 1 and 2 were: rate (instances/minute)
168 of ectopic mounts (male grips female tightly and displays fast thrusting, but the mount is
169 not vaginally oriented), latency to mount (male orients himself on the female's hind
170 flanks, grips her tightly with his forepaws, and displays fast thrusts), latency to intromit
171 (male is vaginally-oriented and makes a long-lasting thrust resulting in vaginal
172 penetration), latency to ejaculate (occurring after a series of intromissions followed by
173 the male self-grooming and showing no sexual interest in the female for at least 20
174 seconds), and number of intromissions to reach ejaculation. Ejaculation latency and
175 intromissions to ejaculate reflect sexual performance, whereas latencies to mount or
176 intromit are measures of sexual motivation (Hull et al., 2002). Latencies to mount and
177 intromit were timed from the moment the female was introduced to the male in the
178 aquarium, and latency to ejaculate was defined as the amount of time that passed
179 between the first intromission and ejaculation. Rate of ectopic mounting was used
180 because males reached behavioral criteria to end the trial within varying times; rate was
181 calculated by dividing the frequency of ectopic mounts displayed by the total test time
182 per male per trial. All behavioral statistical analyses were performed using IBM SPSS
183 software (version 19).

184 Experiment 1: Determine the effects of pubertal testosterone on sexual proficiency and
185 ΔFosB expression in the mPfC and NAc.

186 *Animals* (Figure 1)

187 This experiment was conducted in three consecutive cohorts, with all
188 experimental and control groups represented in each cohort. A total of 109 male
189 hamsters were used in the experiment. Four to five weeks after the first group of
190 weanling males arrived and 2-7 days after the second group of weanling males arrived
191 (see above for age at shipping), half of the first group (now adults, P56) and half of the
192 second group (prepubertal, P28) males were either gonadectomized (GDX; T@P and
193 NoT@P, respectively) or received sham surgeries (sham-T@P and sham-NoT@P,
194 respectively). The sham groups were used as age-matched methodological controls to
195 confirm that behavioral differences found between T@P and NoT@P males were due to
196 the presence or absence of testosterone during puberty, and not to age at the time of
197 surgery or at the time of behavior testing. Four weeks after surgery, when all males
198 were in adulthood, the T@P and NoT@P males received two testosterone-filled
199 capsules and the sham-T@P and sham-NoT@P males received blank capsules of
200 matched size. Two weeks later, approximately half of the males from each group began
201 sexual behavior testing, while the other half of each group remained sexually naïve.
202 The sexually naïve males were placed in an empty aquarium for 5 minutes before
203 sexual behavior testing began for the sexually experienced males. This was done to
204 ensure that the sexually naïve males were not exposed to female pheromones that
205 could have been present in the behavior testing room following sexual behavior testing,
206 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. Thus, this design yielded eight experimental groups: 1) naïve T@P, 2) experienced T@P, 3) naïve NoT@P, 4) experienced NoT@P, 5) naïve sham-T@P, 6) experienced sham-T@P, 7) naïve sham-NoT@P, and 8) experienced sham-NoT@P.

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. 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. Final sample sizes for each behavior analyzed are provided in the respective figures or figure legends.

Sexual behavior statistical analyses:

Sexual behavior was analyzed using multilevel modeling (MLM), which provides an integrated assessment of experimental group (T@P, NoT@P) 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. Analyses were performed separately for experimental and sham groups. $p \leq 0.05$ was considered significant.

Tissue collection

Twenty-four hours after the final behavior test, 64 males (n = 8 per group, randomly chosen) were deeply anesthetized with an overdose of sodium pentobarbital (150mg/kg, ip). The pan-FosB primary antibody used here for immunohistochemistry (rabbit α-FosB, sc-48 Santa Cruz Biotechnology, Santa Cruz, CA, USA) detects both FosB and ΔFosB; however, previous studies have confirmed that most full-length FosB is degraded within 18-24 hours post-stimulus (in this case either sexual behavior or being placed in an empty aquarium). Thus, the FosB immunoreactive cells are specifically enriched for ΔFosB when examined at the chosen time of 24 hours relative to the behavioral manipulation (Perrotti et al., 2004; Perrotti et al., 2005; Perrotti et al., 2008; Wallace et al., 2008; Pitchers et al., 2010). 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 and stored in cryoprotectant at -20°C until staining; one series was used for ΔFosB immunohistochemistry 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).

Radioimmunoassay

In addition to cardiac blood collection from hamsters used for ΔFosB immunohistochemistry (described above), trunk blood was collected from the rest of the

hamsters (n = 5-7 per group), which were not perfused, via rapid decapitation 24 hours after the final behavior test. 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. All sexually naïve and sexually experienced males within each experimental group had adult physiological concentrations of circulating testosterone (Table 1).

ΔFosB Immunohistochemistry

Free floating sections were first rinsed 4 times for 5 minutes with 0.05 M Tris-buffered saline (TBS; pH 7.6) to remove cryoprotectant, and subsequently rinsed 3 times for 5 minutes with TBS 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). After primary antibody incubation, the sections were washed in TBS, and then incubated for 1 hour in goat anti-rabbit horseradish peroxidase-conjugated 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

The number of ΔFosB-ir cells was quantified in 3 anatomically matched sections for both mPfC 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. The Morin and Wood (2001) hamster atlas was also used as a reference. Box placements were determined bilaterally under a 4x objective.

Cell counts were made within each contour by an experimenter blind to treatment group with an UPlanSApo 40x (0.9NA) objective. Cells were considered ΔFosB-ir if they had a distinct nucleus with visible puncta stained opaque, dark purple-blue; cells that had translucent, lighter stained nuclei were not counted. Sample images of stained cells were also used as a guide to determine which cells met the criteria described above. All analyses were performed on an Olympus BX51 microscope under brightfield illumination using Neurolucida (version 9; Microbrightfield, Williston, VT). The number of ΔFosB-ir cells from the three tissue sections per subregion per hamster was used in statistical analysis (described below).

ΔFosB-immunoreactive (-ir) expression statistical analysis:

298 To provide an integrated assessment of pubertal testosterone and sexual
299 experience on ΔFosB-ir expression within the mPfC and NAc, multilevel modeling
300 (MLM) was used. For the analysis, the model treated the animal as the upper-level
301 sampling unit and tissue section as the lower-level sampling unit, with pubertal
302 testosterone and sexual experience as independent variables and ΔFosB-ir cell number
303 as the dependent variable. Interactions were followed up by MLMs within a subset of
304 animals, as appropriate. All statistical analyses were performed using IBM SPSS
305 software (version 19). Analysis was performed separately for experimental and sham
306 groups; $p \leq 0.05$ was considered significant. Due to poor tissue quality for some males,
307 the sample sizes varied between groups of animals. Final sample sizes for each brain
308 region are provided in the data figures.

309 Experiment 2: Determine whether over-expression of ΔFosB in the IL is sufficient to
310 restore sexual proficiency in NoT@P males

311 Experiment 1 showed that the absence of testosterone during puberty impaired sexual
312 proficiency: NoT@P males continued to show high rates of ectopic mounts even after
313 sexual experience. In addition, sexual experience led to an increase in ΔFosB
314 expression in the IL mPfC and NAc core only in T@P males. Experiment 2 was
315 designed to probe the role of ΔFosB in sexual proficiency by asking whether over-
316 expression of ΔFosB in the IL of NoT@P males is sufficient to instate sexual proficiency.
317 We chose to overexpress ΔFosB in the IL instead of NAc core because the impairment
318 in sexual proficiency seen in NoT@P males appears to be related to impairment in
319 behavioral inhibition, which is more closely linked to IL function than to NAc core function
320 (Vertes, 2006; Euston et al., 2012).

Animals

Thirty-four male hamsters were used for this experiment; they arrived as gonad-intact weanlings in two groups 4 weeks apart, as in Experiment 1. Four to five weeks after the first group arrived and 2 to 7 days after the second group arrived, the first group (now adults P56; T@P) and the second group (prepubertal P28; NoT@P) males were GDX. Four weeks later, under isoflurane anesthesia, all of the T@P (P84) and NoT@P (P56) males received two testosterone-filled capsules and bilateral microinjections of recombinant adeno-associated viral (rAAV) vectors encoding either green fluorescence protein (GFP) or GFP and wild-type ΔFosB aimed at the IL. Thus, there were three groups: T@P-GFP, NoT@P-GFP, and NoT@P-ΔFosB. 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, ± 1.6 mm lateral, and 4.5 mm ventral relative to bregma) based on the Morin and Wood (2001) atlas. 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 kept in place for an additional 5 minutes after injection was complete. AAV2/5 viral vectors were purchased from the University of North Carolina Viral Vector Core, and titres were approximately $1.0x10^{13}$ transducing particles per µL. These AAV vectors reach maximal expression around 10 days and sustain expression indefinitely (Vialou et al., 2010; Eagle et al., 2015; Sarno and Robison, 2018). These vectors only infect neurons and are no more toxic than vehicle alone (Zachariou et al., 2006). This AAV overexpression methodology provides the advantages of temporal (in this case, adulthood) and spatial (in this case, IL neurons) specificity, and the disadvantages of a

344 greater level of expression than is typically seen in neurons and expression throughout
345 the neuron, rather than specifically in the nucleus (Vialou et al., 2010; Robison et al.,
346 2013; Eagle et al., 2015). As no other strategy for testing the role of ΔFosB in hamsters
347 is currently available, we feel the advantages of viral overexpression outweigh the
348 disadvantages, and this approach can yield critical new understanding of the molecular
349 mechanisms of hamster behavior. Two weeks later (P70 and P98, respectively), all of
350 the males from each group underwent sexual behavior testing over the next 5 weeks, as
351 described in General Methods above.

352 *Viral vector placement, exclusion criteria, and sample sizes*

353 Based on the previously described behavioral outlier criteria for Experiment 1,
354 one NoT@P-ΔFosB male was an extreme high outlier for ectopic mounts per minute
355 after sexual experience. Additionally, males were excluded from behavioral analysis if
356 injections were misplaced or the viral vector was not expressed (n = 8), or if they
357 displayed impaired motor behavior (e.g., difficulty walking or slowed movement)
358 following stereotaxic surgery (n = 2). The GFP control males were included in analyses
359 if the over-expression was located within any subregion of the mPfC, whereas NoT@P-
360 ΔFosB males were included only if the over-expression was located bilaterally in the IL.
361 Of the five NoT@P-ΔFosB males removed from analysis, three of them had evidence of
362 viral vector expression in other regions of the brain (two in the Cg1/ posterior PrL and
363 one in the secondary motor cortex), while the other two males did not show any
364 evidence of viral vector expression. Thus, a total of 10 males were excluded from
365 behavioral analysis yielding a total of 24 males, with T@P-GFP n = 7, NoT@P-GFP n =
366 10, and NoT@P- ΔFosB n = 7. 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. Final sample sizes for each behavior analyzed are provided in the respective figures or figure legends.

Sexual behavior statistical analysis:

Statistical analysis for sexual behavior in Experiment 2 was the same as previously described for Experiment 1. In addition, the nature of the main effect of experimental group (T@P-GFP, NoT@P-GFP, NoT@P- ΔFosB) 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.

Tissue processing

Twenty-four hours after the final behavior test, all hamsters were deeply anesthetized with an overdose of sodium pentobarbital (150 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. To verify the correct placement of the injection using GFP as a marker, sections were cut into 40μm coronal sections using a cryostat and stored in cryoprotectant for 30-60 minutes until mounting. The sections were then washed in TBS, mounted, and coverslipped while still wet with Vectashield hard set mounting medium (Vector Laboratories, Burlingame, CA). 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 using an Olympus BX51 microscope under fluorescence illumination.

Radioimmunoassay

Plasma from blood collection was used to determine testosterone concentrations by radioimmunoassay as described above. The intra-assay coefficient of variance was 5.4% and the minimum limit of detectability was 0.11 ng/ml. All males had adult physiological concentrations of circulating testosterone, with no significant differences among the groups (T@P-GFP 4.07 ± 1.16 ng/ml, NoT@P-GFP 4.76 ± 0.85 ng/ml, NoT@P-ΔFosB 3.84 ± 0.73 ng/ml).

Experiment 3: Determine the effects of sexual experience and over-expression of ΔFosB in IL on dendritic spine number and morphology

Experiments 1 and 2 provided evidence that pubertal testosterone programs sexual proficiency through the regulation of ΔFosB in the IL. To explore a possible mechanism through which ΔFosB in the IL mediates this experience-induced plasticity, Experiment 3 investigated whether sexual experience and over-expression of ΔFosB in IL have similar effects on IL dendritic spine number and morphology, as has been reported for sexual experience and ΔFosB in NAc (Pitchers et al., 2013).

Animals

A total of 10 gonad-intact adult male hamsters were used for this experiment. Stereotaxic surgery and sexual behavior testing were performed as described in Experiment 2. Hamsters were randomly assigned to one of three groups to investigate the effects of sexual experience and ΔFosB overexpression on spine density in the IL: sexually naïve plus GFP (Naïve-GFP; n = 4), sexually experienced (Experienced-GFP; n = 3), and sexually naïve plus ΔFosB (Naïve-ΔFosB; n = 3) males. AAV injections were performed as previously described in Experiment 2. Twenty-one days later, males in the

Experienced-GFP group underwent 5 weeks of sexual behavior experience as described above; males in the Naïve-GFP and Naïve-ΔFosB groups were placed in clean empty glass aquaria for 5 min in lieu of a sexual behavior experience.

Tissue collection and processing

Twenty-four hours after the final behavior test, all hamsters were deeply anesthetized with an overdose of sodium pentobarbital (150 mg/kg, ip). Animals were then transcardially perfused with 100ml ice-cold phosphate buffered saline (PBS) followed by 150 ml 4% paraformaldehyde. Brains were postfixed 24 h in 4% paraformaldehyde and cryopreserved in 20% sucrose. Brains were cut into 100 µm sections on a Vibratome 3000 EP (Leica Microsystems) and rinsed in PBS. Free-floating immunofluorescent staining was performed using a goat anti-GFP primary antibody (ab5450, 1:1000, Abcam) followed by an Alexa Fluor 488 secondary antibody (705-545-147, 1:200, Jackson Immunoresearch Labs). Sections were mounted on slides using DPX mounting medium (Sigma-Aldrich).

Dendritic spine quantification

GFP/Alexa 488 fluorescence was visualized using an Olympus FluoView 1000 Filter-based Laser Scanning Confocal Microscope with the z-step size of 0.5 micrometers and numerical aperture of 1.40 using a 100x lens. Spines were on the dendritic arbors of cortical pyramidal neurons in IL layers III, V, and VI, and analyzed essentially as previously described (Christoffel et al., 2011). Briefly, dendritic segments 50–150 µm away from the soma were randomly chosen from IL neurons that expressed GFP. Z-stack images were acquired for reconstruction and morphological analysis using NeuronStudio with the rayburst algorithm. NeuronStudio classifies spines as thin,

436 mushroom, or stubby based on the following values: (1) aspect ratio, (2) head to neck
437 ratio, and (3) head diameter. Spines with a neck can be classified as either thin or
438 mushroom, and those without a significant neck are classified as stubby. Spines with a
439 neck are labeled as thin or mushroom based on head diameter.

440 *Dendritic spine statistical analysis*

441 The three groups were analyzed for differences in dendritic spine density by one-
442 way ANOVA followed by Bonferroni *post hoc* test for pairwise differences between
443 individual groups. In this model, the unit of analysis was the number of dendrites within
444 each animal group, yielding sample sizes as: Naïve-GFP = 22, Experienced-GFP = 21,
445 and Naïve-ΔFosB = 22. $p \leq 0.05$ was considered significant. Statistical analysis was
446 performed using IBM SPSS software (version 19).

447

448 **Results**

449 Statistics for all results are reported in respective tables.

450 Sham controls: Age at surgery does not affect sexual behavior or ΔFosB in the mPfC

451 Sham controls were used to confirm that any differences we found between T@P
452 and NoT@P males are due to hormonal manipulation and not age of testing or surgery.
453 The sham-T@P and sham-NoT@P males were tested at the same time and using the
454 same protocol as Experiment 1. There were no significant differences between the two
455 sham groups for any of the behaviors (data not shown). In both groups, latency to
456 mount, latency to intromit, latency to ejaculate, and intromissions to ejaculation all
457 significantly decreased with sexual experience (Table 2). Additionally, the two sham
458 groups did not differ in overall expression of ΔFosB in the mPfC subregions analyzed,

and sexual experience induced ΔFosB in the IL in both sham groups (Table 2). These findings from the sham groups indicate that differences in behavioral or neural measures observed between T@P and NoT@P males are due to the presence or absence of testosterone during puberty, and not to age at the time of surgery or at the time of behavior testing. Results from sham groups will not be further discussed nor represented in the figures.

Experiment 1: Pubertal testosterone programs behavioral adaptations to sexual experience and experience-dependent expression of ΔFosB in the mPfC and NAc (Table 3)

The absence of testosterone during puberty led to significantly higher overall rates of ectopic mounting in NoT@P males compared with T@P males, independent of sexual experience (Figure 2). Although the pubertal testosterone x sexual experience interaction was not statistically significant, only T@P males showed a clear decrease in the rate of ectopic mounting with sexual experience (naïve mean ± SEM rate: 0.492 ± 0.156, experienced mean ± SEM rate: 0.239 ± 0.156) compared to NoT@P males (naïve mean ± SEM rate: 0.748 ± 0.151, experienced mean ± SEM rate: 0.732 ± 0.151). In contrast, the latency to mount, latency to intromit, and number of intromissions to reach ejaculation were all significantly reduced by sexual experience (Figure 3), independent of pubertal testosterone. There was an interaction between sexual experience and pubertal testosterone for ejaculation latency because latency to ejaculate was much higher in naïve NoT@P males compared with naïve T@P males (Figure 3).

The subregions of the mPfC were traced according to the Morin and Wood (2001) hamster atlas (Figure 4A) as shown by a representative microphotograph in Figure 4B. Sexual experience led to a significant increase in ΔFosB expression in the IL of the mPfC only in T@P males (Figure 4C and D). In contrast, sexual experience did not increase ΔFosB expression in the Cg1 or PrL in either T@P or NoT@P males (Figure 4D). Although the interaction between pubertal testosterone and experience was not statistically significant within the PrL (p = 0.122), experience appeared to increase ΔFosB expression in the PrL in T@P, but not NoT@P, males.

The shell and core of the NAc were also traced according to the Morin and Wood (2001) hamster atlas (Figure 5A) with a representative microphotograph shown in Figure 5B. Sexual experience significantly increased ΔFosB expression in the NAc core only in T@P males (Figure 5C). Sexual experience led to a significant increase in ΔFosB expression in NAc shell (Figure 5C); however, pubertal testosterone did not affect ΔFosB expression in the shell nor was there an interaction. Similar to the PrL, although the interaction between pubertal testosterone and experience was not statistically significant within the shell (p = 0.115), experience appeared to increase ΔFosB expression in the shell in T@P, but not NoT@P, males.

Experiment 2: Over-expression of ΔFosB in the IL restores social proficiency in NoT@P males (Table 4)

Placement of the injection site and extent of transduced cells in the IL was verified using fluorescence microscopy (Figure 6). Overexpression of ΔFosB in the IL decreased the overall rate of ectopic mounting (Figure 7), with NoT@P-ΔFosB males displaying less ectopic mounts per minute than NoT@P-GFP males. Sexual experience

504 did not affect the rate of ectopic mounting, nor was there an interaction between
505 experimental group and sexual experience. Interestingly, sexually naïve NoT@P-
506 ΔFosB males had similar rates of ectopic mounting as sexually experienced T@P
507 males, suggesting that they become sexually proficient within a single sexual encounter.
508 Sexual experience significantly reduced the latency to intromit, latency to
509 ejaculate, and number of intromissions to reach ejaculation (Figure 8), independent of
510 overexpression of ΔFosB in the IL, and with no interactions. There was an interaction
511 between sexual experience and over-expression of ΔFosB in the IL for mount latency
512 due to sexual experience decreasing the latency to mount in NoT@P-ΔFosB males, but
513 not in GFP control males (Figure 8). To assess region specificity, behaviors of the three
514 NoT@P-ΔFosB males with misplaced injections were compared with those of NoT@P-
515 ΔFosB males with accurately placed injections. Due to the low sample size and variable
516 brain region hits, these data were not analyzed statistically, but overall NoT@P-ΔFosB
517 males with misplaced injections did not show evidence of social proficiency similar to
518 that seen in NoT@P-ΔFosB males with accurately placed injections (data not shown).
519 Experiment 3: Both sexual experience and overexpression of ΔFosB increase spine
520 density in the IL (Table 5)
521 Overexpression of ΔFosB in the IL increased thin dendritic spine density (Figure
522 9B), with naïve-ΔFosB males having significantly more thin spines compared to both
523 groups of GFP males. There was no effect of sexual experience or overexpression of
524 ΔFosB in the IL on stubby spines (Figure 9B). Sexual experience significantly increased
525 mushroom spine density (Figure 9B;), with experienced-GFP males having more
526 mushroom spines compared to naïve-GFP males. Overexpression of ΔFosB in the IL

increased total dendritic spine density (Figure 9B), with naïve-ΔFosB males having significantly more total spines compared to naïve-GFP control males and experienced-GFP males not differing significantly from either group. Thus, we found that males with overexpressed ΔFosB in the IL had a significant increase in total spine density driven by a significant increase in thin spines and a (nonsignificant) increase in mushroom spines.

Discussion

These studies provide evidence that during puberty, testosterone is necessary for experience-dependent induction of ΔFosB in the IL, thereby programming sexual proficiency in adulthood, possibly through modulation of synaptic lability. We first replicated our previous finding that pubertal testosterone is required for male hamsters to display behavioral adaptations with sexual experience (i.e., social proficiency). We then showed that the presence of testosterone during puberty is necessary for social experience-dependent increases in ΔFosB in both the ventral mPFC and NAc core, providing a correlation between dysregulation of ΔFosB and impairment in social proficiency. Next, we demonstrated that over-expression of ΔFosB in the IL is sufficient to restore a socially proficient phenotype in males that lacked testosterone during puberty. In addition, both ΔFosB and sexual experience increase the density of dendritic spines on IL neurons, albeit in different patterns, suggesting a potential mechanism by which pubertal testosterone programs experience-dependent neural and behavioral adaptations in adulthood. To our knowledge, this is the first report to implicate ΔFosB as a key player in experience-dependent neural and behavioral plasticity that is hormonally

549 programmed during a sensitive period of social development, providing new mechanistic
550 insight into the development of social cognition during puberty and adolescence.

551 In the present study, we found that induction of ΔFosB in the IL occurred after
552 sexual experience in only T@P males, suggesting a possible site of action in which
553 pubertal testosterone programs the ability to inhibit maladaptive behaviors. Because
554 ΔFosB is induced by persistent neuronal activity (Robison and Nestler, 2011), these data
555 may indicate that the IL is preferentially activated by sexual experience in T@P males,
556 and is not activated to the same extent in NoT@P males. This suggests that pubertal
557 testosterone may rewire the brain to allow increased excitatory input (or less inhibitory
558 input) to the IL, which then regulates behavioral inhibition during social behaviors.
559 Substantial projections from the ventral hippocampal formation (CA1 and subiculum,
560 specifically) and the basolateral amygdala to the IL may contribute to the formation of
561 social memories and social learning through reward-related experience, both of which
562 are required for adaptive behavioral flexibility (Hoover and Vertes, 2007; Wassum and
563 Izquierdo, 2015; Hegde et al., 2016). Indeed, ovarian hormones during puberty organize
564 inhibitory transmission in the cingulate region of the mPfC to program behavioral
565 flexibility in non-social learning tasks in mice (Piekarski et al., 2017). Thus, the notion
566 that pubertal hormones rewire the brain to fine-tune behavioral inhibition is not unique to
567 testicular hormones and social proficiency, and generalizes to ovarian hormones and
568 other prefrontal-dependent forms of behavioral flexibility across species.

569 The ventral mPfC, which includes the IL and PrL, is critical for behavioral inhibition
570 in sexual contexts. Male rats with ventral mPfC lesions continue to mate with females
571 even when sexual behavior is paired with aversive consequences (Davis et al., 2010).

572 Furthermore, ΔFosB is integral in inducing neural plasticity in response to both natural
573 and drug rewards by regulating gene expression (Nestler et al., 1999; McClung et al.,
574 2004; Pitchers et al., 2013). Therefore, NoT@P males may not have the capacity for
575 behavioral inhibition as a result of lacking this crucial ΔFosB-driven plasticity within the
576 IL. Indeed, we found that NoT@P males over-expressing ΔFosB show low rates of
577 ectopic mounting overall compared to control NoT@P males. Although NoT@P-ΔFosB
578 males did not show a decrease in ectopic mounting with sexual experience, this is likely
579 due to floor effect as they had very low rates even when sexually naïve. In fact, sexually
580 naïve NoT@P-ΔFosB males had ectopic mounting rates similar to sexually experienced
581 T@P males, suggesting that ΔFosB over-expression reduces this maladaptive behavior
582 even in the absence of experience.

583 Our group and others have found that over-expression of ΔFosB causes an
584 increase in immature dendritic spine number in medium spiny neurons of the NAc
585 (Maze et al., 2010; Grueter et al., 2013; Robison et al., 2013) as well as in hippocampal
586 pyramidal neurons (Eagle et al., 2015). Therefore, we hypothesized that ΔFosB might
587 exert its effects on social proficiency through alterations in the number and structure of
588 dendritic spines on IL pyramidal neurons. Indeed, we found that both sexual experience
589 and over-expression of ΔFosB significantly increased the density of spines on IL
590 pyramidal neurons, with sexual experience increasing mature mushroom spines and
591 over-expression of ΔFosB increasing thin and total spines. These results are consistent
592 with previous work: sexual experience in female hamsters increases NAc spine density
593 (Staffend et al., 2014); in other rodents, sexual experience in males increases mPfC
594 pyramidal neuron spine density (Glasper et al., 2015), early-life stress reduces both

595 social interaction and mPfC spines (Farrell et al., 2016), and antidepressant treatment
596 increases both dendritic spine number and ΔFosB expression in PfC (Li et al., 2010;
597 Vialou et al., 2014). One intriguing potential explanation for these findings is that sexual
598 experience increases ΔFosB expression driving an initial increase in thin spines, while
599 consolidation of memories encoding learned sexual behavior is accompanied by a
600 return to basal ΔFosB levels and a maturation of spines into a mushroom shape. In
601 experiments in which ΔFosB expression is maintained virally without sexual experience,
602 as we have done here, new immature spines are constantly generated, but no learning
603 occurs to cause them to form into mature mushroom spines. The gene targets of ΔFosB
604 in the mPfC are largely unknown, though in other brain regions, including NAc, it
605 regulates the expression of multiple genes critical for spine dynamics and glutamatergic
606 synapse function, including CaMKIIα (Robison et al., 2013) and GluA2 (Kelz et al.,
607 1999; Vialou et al., 2010). These studies, together with the current findings, suggest that
608 ΔFosB increases the potential for synaptic plasticity via the formation of immature
609 spines, and that behavioral experience is needed to promote conversion of labile thin
610 spines into more stable mushroom spines.

611 Sexual proficiency is gained through learning from experience and making
612 behavioral adaptations in part through the inhibition of behaviors that become
613 maladaptive with experience, such as ectopic mounting. The major behavioral difference
614 found between T@P and NoT@P males was rate of ectopic mounting. NoT@P males
615 showed initial high rates of ectopic mounting and, even after sexual experience,
616 continued to show high rates. Conversely, sexually naïve T@P males had lower rates of
617 ectopic mounting compared to NoT@P males, and also showed a decrease in ectopic

618 mounting with sexual experience. Being able to inhibit behaviors that are maladaptive is
619 imperative to successful social interactions. Therefore, these data indicate that pubertal
620 testosterone programs the ability to inhibit behaviors that are not rewarding or
621 advantageous, and thus, maladaptive.

622 Here, we provide evidence that pubertal testosterone organizes the ability to
623 inhibit inappropriate behavior. However, there are some limitations to the present set of
624 experiments. Although the male Syrian hamster is an excellent model to study both
625 sexual reward and sexual proficiency, hamsters are a solitary species. Thus, the
626 generalizability of our results to more social species is limited. Perhaps pubertal
627 testosterone plays a bigger role in organizing the brain in solitary species compared to
628 social species, as solitary animals do not have as much exposure to social experience to
629 help shape the brain. Experiments that address this issue in a more social species, such
630 as rats, would be useful. Additionally, sexual proficiency is only one example of social
631 cognition, and it remains to be seen whether the principles we have derived from our
632 studies on sexual proficiency generalize to other types of social proficiency and
633 cognition. We have shown similar deficits in social cognition during male-male agonistic
634 encounters of NoT@P males (Schulz et al., 2006; De Lorme and Sisk, 2013). Thus, we
635 hypothesize that the social ineptness of NoT@P males is not unique to a specific social
636 context, but reflects a more global dysfunction in the expression of context-appropriate
637 social behavior. Finally, it is worth noting that we also found an increase in ΔFosB in the
638 NAc core after sexual experience in T@P, but not NoT@P, males, and it is possible that
639 a similar increase in ΔFosB and/or dendritic spine density occurred in other regions that
640 we did not investigate. Therefore, although we have shown that over-expression of

ΔFosB in ventral mPFC is sufficient to induce a socially proficient behavioral phenotype, additional experiments are needed to show that an increase in ΔFosB in the IL is necessary for social proficiency to be acquired.

Given that social contexts are ever changing, social proficiency is a fundamental asset in adulthood because it increases the probability of successful social interactions. We show here that, in males, exposure to testosterone during puberty is critical for the ability to make behavioral adjustments with sexual experience. Without pubertal testosterone, males show neither the neural (increase in ΔFosB expression) nor behavioral (decrease in ectopic mounts) adaptations to sexual experience in adulthood that are observed in typically developing males. We also show that, in males who were exposed to testosterone during puberty, the over-expression of ΔFosB in IL increases the density of immature spines and sexual experience increases the density of mature spines, suggesting a mechanism by which pubertal testosterone programs neural and behavioral plasticity in adulthood. We speculate that in the absence of testosterone during puberty, adult males fail to acquire social proficiency as a result of the lack of ΔFosB-induced formation of immature spines in the IL that could mature into functional glutamatergic synapses with social experience. That is, without the potential for neural plasticity in executive control brain regions, there is less potential for experience-dependent behavioral plasticity. The sites and mechanisms of action for hormonal programming of brain and behavior during puberty are largely unknown, and thus, our findings prompt new avenues of investigation to advance understanding of adolescent maturation of social cognition.

664

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783

784 **Figure Legends**

785 **Figure 1. Experimental design of Experiment 1.** T@P and sham-T@P males arrived 4
786 weeks prior to NoT@P and sham-NoT@P males to control for the age of shipping and
787 environment during puberty. Two to 7 days after the NoT@P and sham-NoT@P arrived,
788 males were either gonadectomized (GDX) or sham-GDX during adulthood (P56; T@P
789 and sham-T@P) or prepubertally (P28; NoT@P and sham-NoT@P). Four weeks later,
790 T@P and NoT@P males received testosterone (T)-filled capsules and sham males
791 received empty (blank) capsules of the same size. Sexual behavior testing began two
792 weeks later.

793 **Figure 2. Rate of ectopic mounting is dependent on pubertal testosterone.** T@P
794 males had significantly fewer ectopic mounts per minute compared to NoT@P males.
795 Bars represent mean (±SEM); numbers on bars indicate sample size. *Main effect of
796 pubertal testosterone, $p \leq 0.05$.

Figure 3: The effects of pubertal testosterone and sexual experience on latency to mount, intromit, and ejaculate and number of intromissions to ejaculation. Mount latency: There was a main effect (ME) of sexual experience on mount latency with sexually experienced males having shorter latencies to mount compared to sexually naïve males. Intromission latency: There was an ME of sexual experience on intromission latency with sexually experienced males having shorter latencies to intromit compared to sexually naïve males. Ejaculation latency: 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. Intromissions to ejaculate: There was an ME of sexual experience for intromissions to ejaculate with sexually experienced males having less intromissions to achieve ejaculation compared to sexually naïve males. Bars represent mean (±SEM); numbers on bars indicate sample size. +Interaction between pubertal testosterone and sexual experience, $p \leq 0.05$.

Figure 4: Number of ΔFosB-ir cells in infralimbic cortex (IL) is dependent on pubertal testosterone and sexual experience. A: Brain atlas (Morin and Wood, 2001) representation of a coronal section containing the mPfC. B: Photomicrographs of drawn contours of the mPfC onto immunohistochemically-treated tissue sections at 4x objective. The mPfC included the anterior cingulate (Cg1), prelimbic (PrL), and infralimbic (IL) cortex; scale bar = 250 μm. C: The 2 x 2 panel of photomicrographs below the bar graph are representative images of ΔFosB-ir in the IL for the specified group of males; scale bars = 25 μm. D: In the CgL and PrL, there were no effects or

interactions of pubertal testosterone and sexual experience on ΔFosB-ir cells, respectively. In the IL, there was an interaction between pubertal testosterone and sexual experience on ΔFosB-ir cells with sexual experienced T@P males having 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. Bars represent mean (±SEM); numbers on bars indicate sample size. +Interaction between pubertal testosterone and sexual experience, $p \leq 0.05$.

Figure 5: Number of ΔFosB-ir cells in the nucleus accumbens (NAc) core and shell is dependent on pubertal testosterone and sexual experience. A: Brain atlas (Morin and Wood, 2001) representation of a coronal section containing the NAc. B: Photomicrographs of drawn contours of the NAc onto immunohistochemically-treated tissue sections. The NAc included the shell and core. LV = lateral ventricle; ac = anterior commissure. Scale bar = 250 μm. C: In the core, there was an interaction between pubertal testosterone and sexual experience on ΔFosB-ir cells with sexual experienced T@P males having 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. In the shell, there was a main effect (ME) of sexual experience on ΔFosB-ir cells with sexually experienced males having more ΔFosB-ir expression compared to sexually naïve males. Bars represent mean (±SEM); numbers on bars indicate sample sizes. +Interaction between pubertal testosterone and sexual experience, $p \leq 0.05$.

Figure 6: Visualization of GFP to verify injection site and extent of infected cells in the infralimbic cortex (IL). A: Boxes of representative injection sites in the IL for

NoT@P-ΔFosB males over coronal atlas diagram (Morin and Wood, 2001). B: Photomicrograph of GFP over-expression in a NoT@P-ΔFosB male; scale bar = 250 μm. C: Photomicrograph of GFP over-expression in a NoT@P-ΔFosB male; scale bar = 100 μm.

Figure 7: 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. Bars represent mean (±SEM). *Main effect of experimental group, $p \leq 0.05$.

Figure 8. The effects of ΔFosB over-expression in the IL and sexual experience on latency to mount, intromit, and ejaculate and number of intromissions to ejaculation. For mount latency, there was a pubertal testosterone x sexual experience interaction 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. For intromission latency and ejaculation latency, there was a main effect of sexual experience with sexually experienced males having shorter latencies to mount and intromit compared to sexually naïve males. For intromissions to ejaculate, there was a main effect (ME) of sexual experience with sexually experienced males having less intromissions to achieve ejaculation compared to sexually naïve males. Bars represent mean (±SEM); numbers on bars indicate sample size. +Interaction between experimental group and sexual experience, $p \leq 0.05$

Figure 9: Both sexual experience and ΔFosB increase dendritic spines in vmPFC. A: AAV-GFP was injected into the IL of naïve (n = 22) or sexually experienced (n = 21) males, and AAV-GFP-ΔFosB was injected into the IL of naïve males (n = 22). Immunofluorescence using a GFP antibody reveals spines of IL pyramidal neurons in all three groups. B: Thin spine density was increased by ΔFosB overexpression. Stubby spine density was not affected by either sexual experience nor ΔFosB overexpression. Mushroom spine density increased with sexual experience and showed a trend to increase by ΔFosB overexpression. Overall, total spine density was increased by ΔFosB overexpression. Bars represent mean (±SEM). *Main effect of experimental group, $p < 0.05$.

Tables

Table 1. Concentrations of plasma testosterone

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 2. Statistics for Sham Controls

Figure	Independent variable(s)	Dependent variable	Statistics (***significant**)
NA	sexual experience	Latency to mount	***F*(1, 24) = 14.65; *p* = 0.001***
		Latency to intromit	***F*(1, 24) = 14.33; *p* = 0.001***
		Latency to ejaculate	***F*(1, 24) = 31.27; *p* = 0.001***
		intromissions to ejaculation	***F*(1, 17) = 12.24; *p* = 0.003***
		ΔFosB in the IL	***F*(1, 23) = 4.45; *p* = 0.046***

880 Table 3. Statistics for Experiment 1

Figure	Independent variable(s)	Dependent variable	Statistics (***significant**)	Post hoc comparison (if appropriate)	Post hoc statistics (***significant**)
2	pubertal testosterone	ectopic mounting	***F*(1, 27) = 6.75; *p* = 0.015***		
	sexual experience		*F*(1, 27) = 0.69; *p* > 0.05		
	pubertal testosterone x sexual experience		*F*(1, 27) = 0.54; *p* > 0.05		
3	pubertal testosterone	Latency to mount	*F*(1, 27) = 0.272; *p* > 0.05		
	sexual experience		***F*(1, 27) = 17.43; *p* < 0.001***		
	pubertal testosterone x sexual experience		*F*(1, 27) = 0.309; *p* > 0.05		
	pubertal testosterone	Latency to intromit	F(1, 27) = 1.01; *p* > 0.05		
	sexual experience		***F*(1, 27) = 15.84; *p* < 0.001***		
	pubertal testosterone x sexual experience		*F*(1, 27) = 2.50; *p* > 0.05		
	pubertal testosterone x sexual experience	Latency to ejaculate	***F*(1, 27) = 12.70; *p* = 0.001***	pubertal testosterone in naïve males	***F*(1, 24) = 10.33; *p* = 0.004***
				pubertal testosterone in experienced males	*F*(1, 27) = 0.641; *p* > 0.05
	pubertal testosterone	intromissions to ejaculation	F(1, 26) = 0.465; *p* > 0.05		
	sexual experience		***F*(1, 26) = 25.00; *p* < 0.001***		
	pubertal testosterone x sexual experience		*F*(1, 26) = 2.73; *p* > 0.05		
4D	pubertal testosterone x sexual experience	ΔFosB in the IL	***F*(1, 23) = 10.86; *p* = 0.003***	sexual experience in T@P males	***F*(1, 12) = 14.06; *p* = 0.003***
				sexual experience in NoT@P males	*F*(1, 11) = 0.721; *p* > 0.05
	pubertal testosterone	ΔFosB in the Cg1	*F*(1, 23) = 0.046; *p* > 0.05		

	sexual experience		$F(1, 23) = 0.082$; $p > 0.05$		
	pubertal testosterone x sexual experience		$F(1, 23) = 0.66$; $p > 0.05$		
	pubertal testosterone	ΔFosB in the PrL	$F(1, 23) = 0.133$; $p > 0.05$		
	sexual experience		$F(1, 23) = 2.04$; $p > 0.05$		
	pubertal testosterone x sexual experience		$F(1, 23) = 2.57$; $p > 0.05$		
5C	pubertal testosterone x sexual experience	ΔFosB in the NAc core	**$F(1, 23) = 5.42$; $p = 0.029$***	sexual experience in T@P males	**$F(1, 12) = 9.66$; $p = 0.009$***
				sexual experience in NoT@P males	$F(1, 11) = 0.042$; $p > 0.05$
	pubertal testosterone	ΔFosB in the NAc shell	$F(1, 23) = 1.60$; $p > 0.05$		
	sexual experience		**$F(1, 23) = 7.041$; $p = 0.014$***		
	pubertal testosterone x sexual experience		$F(1, 23) = 2.68$; $p > 0.05$		

898 Table 4. Statistics for Experiment 2
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Figure	Independent variable(s)	Dependent variable	Statistics (***significant**)	Post hoc comparison (if appropriate)	Post hoc statistics (***significant**)
7	ΔFosB over-expression	ectopic mounting	***F*(2, 22) = 3.94; *p* = 0.035***	NoT@P-ΔFosB vs. NoT@P-GFP males	***p* = 0.043***
	sexual experience		*F*(1, 21) = 1.25; *p* > 0.05		
	ΔFosB over-expression x sexual experience		*F*(2, 21) = 1.20; *p* > 0.05		
8	ΔFosB over-expression x sexual experience	Latency to mount	***F*(2, 19) = 3.61; *p* = 0.047***	sexual experience in NoT@P-ΔFosB males	***F*(1, 6) = 11.00; *p* = 0.015***
				sexual experience in T@P-GFP males	*F*(1, 6) = 1.61; *p* > 0.05
				sexual experience in NoT@P-GFP males	*F*(1, 7) = 0.61; *p* > 0.05
	ΔFosB over-expression	Latency to intromit	*F*(2, 20) = 1.12; *p* > 0.05		
	sexual experience		***F*(1, 20) = 14.09; *p* = 0.001***		
	ΔFosB over-expression x sexual experience		*F*(2, 20) = 3.06; *p* > 0.05		
	ΔFosB over-expression	Latency to ejaculate	*F*(2, 20) = 2.23; *p* > 0.05		
	sexual experience		***F*(1, 21) = 15.20; *p* < 0.001***		
	ΔFosB over-expression x sexual experience		*F*(2, 20) = 1.64; *p* > 0.05		
	ΔFosB over-expression	intromissions to ejaculation	*F*(2, 21) = 0.87; *p* > 0.05		
	sexual experience		***F*(1, 20) = 11.70; *p* = 0.003***		
	ΔFosB over-expression x sexual experience		*F*(2, 20) = 0.06; *p* > 0.05		

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902 Table 5. Statistics for Experiment 3
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Figure	Independent variable(s)	Dependent variable	Statistics (***significant**)	Post hoc comparison (if appropriate)	Post hoc statistics (***significant**)
9B	experimental group (naïve-GFP, experienced-GFP, naïve-ΔFosB)	thin spines	***F*(2, 62) = 1.717; *p* < 0.001***	naïve-ΔFosB vs. naïve-GFP males	***p* < 0.001***
				naïve-ΔFosB vs. experienced-GFP males	***p* = 0.016***
	experimental group (naïve-GFP, experienced-GFP, naïve-ΔFosB)	stubby spines	*F*(2, 62) = 0.723; *p* > 0.05		
	experimental group (naïve-GFP, experienced-GFP, naïve-ΔFosB)	mushroom spines	***F*(2, 61) = 4.080; *p* = 0.022***	naïve-GFP vs. experienced-GFP males	***p* = 0.029***
	experimental group (naïve-GFP, experienced-GFP, naïve-ΔFosB)	total spines	***F*(2, 62) = 9.359; *p* < 0.001***	naïve-ΔFosB vs. naïve-GFP males	***p* < 0.001***

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