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Sexual experience affects reproductive behavior and preoptic androgen receptors in male mice

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Abstract

Reproductive behavior in male rodents is made up of anticipatory and consummatory elements which are regulated in the brain by sensory systems, reward circuits and hormone signaling. Gonadal steroids play a key role in the regulation of male sexual behavior *via* steroid receptors in the hypothalamus and preoptic area. Typical patterns of male reproductive behavior have been characterized, however these are not fixed but are modulated by adult experience. We assessed the effects of repeated sexual experience on male reproductive behavior of C57BL/6 mice; including measures of olfactory investigation of females, mounting, intromission and ejaculation. The effects of sexual experience on the number of cells expressing either androgen receptor (AR) or estrogen receptor alpha (ERα) in the primary brain nuclei regulating male sexual behavior was also measured. Sexually experienced male mice engaged in less sniffing of females before initiating sexual behavior and exhibited shorter latencies to mount and intromit, increased frequency of intromission, and increased duration of intromission relative to mounting. No changes in numbers of ERα-positive cells were observed, however sexually experienced males had increased numbers of AR-positive cells in the medial preoptic area (MPOA); the primary regulatory nucleus for male sexual behavior. These results indicate that sexual experience results in a qualitative change in male reproductive behavior in mice that is associated with increased testosterone sensitivity in the MPOA and that this nucleus may play a key integrative role in mediating the effects of sexual experience on male behavior.

Keywords: reproduction; sexual behavior; sexual experience; olfaction; testosterone; androgen receptor; estrogen receptor; hypothalamus; preoptic area

Introduction

Sexual behavior in male rodents consists of species-typical patterns of anticipatory and consummatory behavior. These involve approach and olfactory investigation of the female (especially anogenital sniffing), followed by bouts of mounting, intromission and then ejaculation, with a subsequent refractory period of reduced interest in females. The variations on this basic behavioral template have been characterized in different species (Hull et al., 2002) and in strains of the same species (McGill, 1962b), but patterns of male reproductive behavior also change over the adult life of the individual. One important modifier of mating behavior is sexual experience, which has long-term effects on both anticipatory and consummatory behaviors. The detection and investigation of female odor cues is a key trigger for the initiation of male sexual behavior in rodents and male behavioral responses to female odors are sensitive to sexual experience. While disruption of either the olfactory or vomeronasal system has severe effects on male sexual behavior in many rodents (Mandiyan et al.,2005; Powers and Winans, 1975; Steel and Keverne, 1985), these effects are less pronounced if subjects are sexually experienced (Meredith, 1986; Pfeiffer and Johnston, 1994). It should be noted, however, that in mice the data are less clear and sexually experienced males receiving lesions to the main olfactory epithelium have been reported both to retain normal copulatory performance (Edwards and Burge, 1973) and to suffer total loss of mating behavior (Keller et al., 2006). While this more recent study suggests that sexual experience may not protect male mice from the behavioral disruption caused by olfactory lesions, sexually experienced male mice respond to a wider range of female chemosignals than virgins (Sipos et al., 1992) and acquire preferences for the odors of receptive females with sexual experience (Hayashi and Kimura, 1974). As well as affecting pre-copulatory behaviors, sexual experience also has effects on the motor components of sexual behavior

47 itself. Studies in different rodent species, including rats (Dewsbury, 1969; Larsson, 1959),
48 guinea pigs (Valenstein et al., 1955) and lemmings (Coopersmith et al., 1986), have shown
49 that behavioral components of copulation including mounting, intromission and ejaculation
50 occur with shorter latencies and higher frequencies in sexually experienced males.

51 Male mating behavior is governed by a complex interaction between different systems
52 in the brain which process sensory inputs, regulate reward and motivation, and integrate
53 hormonal signals (Hull et al., 2002). Gonadal steroids play a key role in this regulatory
54 system, as is evident from the suppression of sexual behavior caused by castration, and its
55 restoration by subsequent testosterone treatment (McGinnis and Dreifuss, 1989). However
56 even the effects of castration are reduced by sexual experience in some male rodents
57 (Costantini et al., 2007; Manning and Thompson, 1976). This suggests that while androgens
58 occupy a primary role in the regulation of sexual behavior, the brain systems that integrate
59 steroid hormone signals into behavioral output are modified by sexual experience. The
60 secretion of testosterone itself is also affected by sexual experience. During a sexual
61 encounter, levels of circulating androgens increase after initial exposure to female cues and
62 again in response to copulation (Batty, 1978; Gleason et al., 2009). Sexual experience
63 enhances both these female-elicited reflexive releases of testosterone (Bonilla-Jaime et al.,
64 2006; Kamel et al., 1975) and increases baseline levels of circulating testosterone (Edinger
65 and Frye, 2007; Wu and Gore, 2009).

66 Effects of testosterone on sexual behavior are mediated in the brain directly *via* the
67 androgen receptor (AR) and indirectly (after local aromatization to estradiol) *via* estrogen
68 receptors, primarily estrogen receptor alpha (ERα) (Wersinger et al., 1997). The network of
69 brain areas involved in the regulation of male sexual behavior includes the main olfactory and
70 vomeronasal systems (Keverne, 2004), the mesocorticolimbic system that governs reward

and motivation, (Balfour et al., 2004), and regions in the hypothalamus and preoptic area (Hull et al., 2002). These are sites of high expression of AR and ERα such as the bed nucleus of stria terminalis (BNST), medial amygdala (MeAmg), ventromedial hypothalamus (VMH) and medial preoptic area (MPOA) (Simerly et al., 1990). The MPOA is the critical integrative nucleus in male sexual behavior (Hull and Dominguez, 2006) and lesions of the MPOA disrupt mounting, intromission and ejaculation in rats (de Jonge et al., 1989), mice (Bean et al., 1981) and hamsters (Powers et al., 1987). The importance of testosterone sensitivity in the MPOA for male sexual behavior is illustrated by studies in which AR antagonists are injected into the MPOA, resulting in sexual behavior deficits resembling those seen after MPOA lesions (Harding and McGinnis, 2004; McGinnis et al., 1996). Moreover, conditional deletion of AR in the brain increases latencies to perform sexual behaviors and reduces incidences of copulation (Raskin et al, 2009), despite elevated circulating testosterone and intact MPOA ERα levels in these knockout males. Copulation-induced c-Fos has been shown to colocalize with AR in the MPOA, BNST and MeAmg of male hamsters (Wood and Newman, 1993), indicating that testosterone-sensitive neurons are activated in these brain areas during mating. Sexual experience induces a series of changes across this brain network that regulates reproductive behavior. After sexual experience, female odor-elicited c-Fos expression is greater in both the main olfactory and vomeronasal systems and their downstream androgen-sensitive targets, with particularly strong responses seen in the MeAmg, BNST and MPOA (Fewell and Meredith, 2002; Hosokawa and Chiba, 2005; Swaney et al., 2007). Copulation itself increases c-Fos immunoreactivity in the MPOA (Robertson et al., 1991) and this increase is greater in the brains of sexually experienced male rats than virgins (Lumley and Hull, 1999).

Research into the effects of sexual satiety in rats offers further evidence of neuronal plasticity in the circuits regulating male sexual behavior and of changes in testosterone sensitivity. Male rats that are allowed to mate *ad libitum* over a short period of time reach a state of sexual satiety in which they lose interest in females and do not fully recover sexual drive for up to 15 days (Phillips-Farfan and Fernandez-Guasti, 2009). This state of sexual satiety and lack of interest in females is associated with AR density reductions in the nucleus accumbens (NAc), VMH, lateral septum, MeAmg and especially in the MPOA (Fernandez-Guasti et al., 2003; Phillips-Farfan and Fernandez-Guasti, 2009). Conversely, ERα is elevated in the VMH, lateral septum and MPOA of sexually satiated males (Phillips-Farfan et al., 2007, Fernandez-Guasti, 2010). AR and ERα densities recover to previous levels as libido returns and this dynamic variation in gonadal steroid sensitivity indicates that receptor expression in the sexual brain network varies with sexual function in males.

In the current study, we explore the long term effects of sexual experience on reproductive behavior and whether these effects involve changes in regulatory brain areas that are sensitive to testosterone. Our experimental design involved measurement of the effects of repeated sexual experience during cohabitation with females on reproductive behavior in male mice. We predicted that sexual experience would result in changes in interest in females as well as shorter latencies and higher frequencies of sexual behaviors. While such effects have been characterized in other rodent species, such as rats and hamsters, these effects have not been specifically studied in mice despite their increasing use in behavioral neuroscience research. Given previously reported increases in circulating testosterone after sexual experience and the dynamic changes in AR and ERα after sexual activity, we also examined whether behavioral effects of sexual experience are associated with long-term changes in numbers of AR and ERα-positive neurons in the primary nuclei

that regulate sexual behavior in male mice. The MPOA, posterior MeAmg (pMeAmg), and BNST were selected for investigation based on their involvement in reproductive behavior, previously reported activity changes after sexual experience, and high levels of expression of AR and ERα.

Materials and Methods

To characterize the effects of sexual experience on reproductive behaviors, age-matched virgin and sexually experienced C57BL/6 (B6) male mice were given mating tests with receptive virgin B6 female mice. To investigate effects of sexual experience on AR and ERα, age-matched virgin and sexually experienced B6 male mice were sacrificed and their brains processed for AR and ERα immunohistochemistry. All procedures described were approved by the Institutional Animal Care and Use Committee of Columbia University.

Animals

Adult male and female B6 mice were purchased from Charles River Laboratories (Kingston, NY) at approximately 2 months of age. Males and females were separately housed in groups of 4 or 5 at the Department of Psychology at Columbia University for 1 month of habituation to the animal facility. Mice were housed in 13.5" x 8.1" x 5.5" Plexiglas cages under a 12 hour reversed dark-light cycle (8am: lights off, 8pm: lights on) with wood shaving bedding, *ad libitum* water and chow. At the start of the experimental phase (see **Figure 1** for summary of experimental design), male mice were split into two experimental groups: virgin and sexually experienced males. Each sexually experienced male was housed in a novel clean cage with two gonadally intact B6 female mice which each received repeated hormonal priming to induce regular sexual receptivity. This priming involved subcutaneous injections of 50 µg of estradiol benzoate (Fisher Scientific, Pittsburgh PA) dissolved in 50µl of peanut oil, followed 72 hours later by 400µg progesterone (Fisher Scientific, Pittsburgh PA)

142 dissolved in 50µl of peanut oil. This injection schedule was repeated every 6 days so that
143 males had repeated access to females that were sexually receptive. The injection schedules
144 for the two females in each cage were staggered by three days so that males would have the
145 opportunity to mate with a receptive female every three days. Males were housed under these
146 conditions for three weeks, and then for a further week the females housed with the sexually
147 experienced males received a different injection schedule. During this final week females
148 received no estradiol injections and were given subcutaneous injections of 400µg
149 progesterone in 50µl peanut oil every two days to prevent estrus (Morin, 1977) and keep
150 them sexually unreceptive. This ensured that their male cage-mates did not have mating
151 opportunities during the final week of mixed-sex housing and that any behavioral and brain
152 effects measured were likely to be a longer-term consequence of sexual experience rather
153 than recent copulation. Daily observations confirmed that no sexual activity occurred
154 amongst the sexually-experienced males during this period. Virgin males were re-housed in
155 new groups of four into a novel clean cage at the start of the experimental phase of the study
156 (i.e. at the same time that the sexually experienced group males were re-housed with females)
157 so that they experienced social change, albeit with unfamiliar males rather than females. This
158 re-housing occurred at the start of the experimental phase and each co-housed male was
159 included in the analysis. Previous literature has indicated that male-male aggression may
160 induce changes within the behavioral and neuroendocrine outcomes being examined in the
161 present study (Fuxjager et al., 2010). To ensure that aggressive encounters within the virgin
162 male housing condition were not contributing to the long-term effects being assessed in this
163 experimental design, we conducted daily observations of virgin and sexually-experienced
164 housed males. We observed minimal male-directed aggression in either condition within the
165 two hours following re-housing and during the subsequent four week housing period, no
166 aggression was observed. Following the virgin housing/sexual experience phase, half of the

male mice of each experimental group were given mating tests, the other half were sacrificed for analysis of brain immunohistochemistry.

Tests of male reproductive behavior

The mating behaviors of virgin and sexually experienced B6 males were measured in one-hour tests with unfamiliar, hormonally-primed, virgin B6 females. These stimulus females were hormonally primed with subcutaneous injections of 50 µg of estradiol benzoate dissolved in 50µl of peanut oil 72 hours before testing, followed by 400µg progesterone dissolved in 50µl of peanut oil 3 hours before testing. Each male was tested during the first 6 hours after lights out in a clean 13.5" x 8.1" x 5.5" Plexiglas cage without food or water. A male and a stimulus female were placed in the test cage for one hour and behavior was video recorded from above for later blind scoring. The male reproductive behaviors scored were anogenital sniffing of the female, mounting, intromission and ejaculation. The measures scored were latency and frequency of mounting, intromission and ejaculation, duration of anogenital sniffing before first mount, and the difference between duration of mounting and intromission (calculated by subtracting mounting duration from intromission duration). If a subject did not perform a behavior during the hour-long test, a maximum latency value of 3600 seconds was assigned for that behavior.

AR and ERα immunohistochemistry

Virgin and sexually experienced males were euthanized with an overdose of ketamine-xylazine anesthetic, before being transcardially perfused with 20ml of 0.1M phosphate-buffered saline (PBS) followed by 20ml of freshly prepared 4% paraformaldehyde in PBS. Brains were then removed and post-fixed for 4 hours at 4°C in 4% paraformaldehyde in PBS, before being cryoprotected in 30% sucrose in PBS overnight, then frozen and stored at -80°C. Brains were sectioned coronally at 40µm on a cryostat at -20°C into two alternate series, and

191 processed for AR and ERα expression. Sections encompassing the areas of interest were
192 selected and washed twice in PBS, then incubated overnight at 4°C in PBS with 0.3% Triton
193 x-100 (PBST), 1.5% normal goat serum (Vector Laboratories, Burlingame CA) and either
194 1:5000 polyclonal anti-AR (rabbit) primary antibody or 1:5000 polyclonal anti-ERα (rabbit)
195 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After two washes in PBS,
196 sections were incubated for 15 minutes at room temperature in 4% hydrogen peroxide and
197 10% methanol in PBST, washed twice in PBS, then incubated for 30 minutes at room
198 temperature in 1:2000 biotinylated anti-rabbit (goat) secondary antibody (ABC elite kit:
199 Vector Laboratories) in PBST. After two further washes in PBS, sections were incubated for
200 30 minutes in avidin-biotin peroxidase solution (ABC elite kit: Vector Laboratories) in
201 PBST, washed twice in PBS, then stained in Vector SG peroxidase substrate solution (Vector
202 Laboratories) for 3 minutes. The stained sections were washed twice in PBS before being
203 mounted on gelatin-coated glass slides, dehydrated and cleared in a series of alcohol and
204 xylene washes, then coverslipped with DePeX (Fisher Scientific).

205 *Image analysis*

206 For both the AR- and ERα-stained series, the mouse brain atlas of Paxinos and Franklin
207 (2001) was used to select matched, sequential sections for each animal that encompassed the
208 BNST (four sections centered around -0.22mm from Bregma), pMeAmg (six sections
209 centered around -1.46mm from Bregma) and MPOA (six sections centered around 0.02mm
210 from Bregma). Images of these areas were captured at 20x magnification. The sections were
211 analyzed using MCID imaging software (Interfocus Imaging, Linton, UK) to normalize
212 background levels and apply a minimum staining threshold to all images. Stained cells were
213 counted bilaterally within rectangular boxes overlaid on the areas of interest (BNST:

500x350μm; pMeAmg: 500x375μm; MPOA: 600x400μm). In each region the mean number of stained cells per mm^2 per section was calculated for each animal.

Statistical analysis

Latency data from the mating tests were not normally distributed and so were $\log_{10}(y+1)$ transformed before analysis. All measures of male reproductive behavior were analyzed using student's independent two-sample *t*-tests. A Bonferroni correction was applied to account for multiple comparisons: 8 tests were run, resulting in a corrected alpha value of $P=0.00625$. All immunohistochemical data were normally distributed. Counts of stained AR and ERα cells were analyzed using Student's independent two-sample *t*-tests, or Welch's *t*-test when the variances were unequal.

Results

Tests of male reproductive behavior

Significant effects of sexual experience were seen across multiple measures of male reproductive behavior (**Figure 2**). Sexually experienced males had shorter latencies to mount females than virgin males (n=6/group; $t_{10}=-5.78$, $P<0.001$), and shorter latencies to intromit than virgins ($t_{10}=-5.41$, $P<0.001$). However there was no significant difference between the groups in the latency to ejaculate during testing ($t_{10}=-1.86$, $P=0.093$). Comparison of the frequency of behaviors indicated that intromission frequency was significantly higher among sexually experienced males than virgins ($t_{10}=3.95$, $P=0.003$), however differences between sexually experienced and virgin males in frequency of mounting ($t_{10}=2.19$, $P=0.054$) or frequency of ejaculation ($t_{10}=1.95$, $P=0.080$) did not reach statistical significance. Initial olfactory investigation of females was markedly reduced in sexually experienced males, who engaged in significantly less anogenital sniffing of the female before their first mount ($t_{6.03}=-4.51$, $P=0.004$). The difference in duration of intromission relative to mounting was also

238 significantly affected by sexual experience, with longer duration of intromission relative to
239 mounting among sexually experienced males than virgin males (t_{10}=4.24, P=0.002).

240 *AR and ERα cell counts*

241 Both AR and ERα staining was clearly visible in the regions selected for their importance in
242 male reproductive behavior. The effects of sexual experience on AR and ERα expression
243 (n=6 males/group) were not uniform but area and receptor type specific (**Figure 3**). There
244 was no effect of sexual experience on the number of stained AR cells in either the BNST
245 (t_{10}=1.22, P=0.272) or the pMeAmg (t_{10}=0.225, P=0.827). However, sexually experienced
246 males had significantly more stained AR cells than virgins in the MPOA (t_{10}=2.57, P=0.028).
247 There was no effect of sexual experience on the number of ERα cells counted in any of the
248 areas of interest, with no significant difference between the sexually experienced and virgin
249 males in either the BNST (t_{10}=-0.516, P=0.617), the pMeAmg (t_{10}=1.762, P=0.108) or the
250 MPOA (t_{10}=0.755, P=0.468).

251 **Discussion**

252 Sexual experience resulted in robust changes in reproductive behavior in male mice
253 and increased numbers of AR-positive neurons in the MPOA, the primary regulatory nucleus
254 in male reproductive behavior. Mirroring findings in other rodents (Dewsbury, 1969;
255 Larsson, 1959), sexually experienced male mice had shorter latencies to mount and intromit,
256 had higher frequencies of intromission, and spent significantly longer engaged in
257 intromission behavior than mounting. These are interesting results as intromission and
258 mounting have been shown to have qualitatively different properties from the perspective of
259 both the mating male and female. Mating-mediated conditioning studies have shown that
260 while ejaculation is the most effective sexual stimulus for conditioning (Pfaus et al., 2001),
261 intromission is also intrinsically rewarding and is a sufficiently strong stimulus to produce

spatial preferences in B6 male mice (Kudwa et al., 2005). Intromission without ejaculation results in increased motivation to investigate females, while mounting alone has little effect (Whalen, 1961), suggesting that intromission is also more rewarding than mounting alone. Intromission may have been reinforced during previous matings in the sexually experienced males, leading to longer durations of intromission relative to unrewarding mounting behavior. Alternatively, sexually experienced males may be more sexually "competent" and so able to achieve intromission more easily, an idea that is supported by research showing that inappropriate mounting behavior is reduced in sexually experienced male mice (McGill, 1962a).

In addition to the proposed mechanistic explanations for the change in intromissive behavior, there is also evidence that such a change might have adaptive consequences for mating males. Intromission, but not mounting, has been shown to result in an increase in NAc dopamine in mated female hamsters (Kohlert and Meisel, 1999), and extensive mounting without intromission results in reduced lordosis in female rats (Hardy and Debold, 1971). This suggests that intromission is a rewarding component of mating for females and that such stimulation by the male may be important for maintenance of appropriate female behavior during copulation. Moreover, intromission has also been shown to positively affect female reproductive physiology. Female rats are both more likely to conceive and less likely to continue estrous cyclicity after either intromission (Adler, 1969) or analogous artificial cervical stimulation (Terkel et al., 1990). Copulation with male rats that have high frequencies of intromission is also more likely to result in pregnancy (Wilson et al., 1965). In mice, it has been suggested that the pattern of intromission has a direct bearing on the likelihood of induction of pregnancy or pseudopregnancy (Diamond, 1970). Sexually experienced males that exhibit higher intromission frequencies and longer total duration of

intromission may thus be more likely to successfully impregnate females and so produce offspring. Sexually experienced male mice have been reported to have higher fecundity than virgin males (Rastogi et al., 1981), indicating that sexual experience may indeed have such adaptive consequences for male mice.

In contrast to the changes in mounting and intromission, we saw no effect of sexual experience on either the latency or frequency of ejaculation. This absence of an effect on ejaculation behavior appears surprising given the fact that both latency to ejaculate and frequency of ejaculation are changed by sexual experience in rats (Sura et al., 2001). However the species differences in sexual behavior patterns are important in this regard. While rats ejaculate repeatedly during a single mating bout, mice lose sexual drive for at least 24 hours after a single ejaculation (McGill, 1962b). None of the B6 mice we tested ejaculated more than once and some males failed to ejaculate during the hour-long tests despite repeated mounting and intromission with females. Tests of longer duration, such as the 10 hour tests employed by Raskin et al (2009), might have improved our data on ejaculation latency and shown differences between sexually experienced and virgin males. The ejaculation frequency data for each individual was thus limited to a maximum value of 1, resulting in a narrower range of values than would be obtained in analogous tests with rats, where at test an individual is likely to ejaculate repeatedly and variation in ejaculation frequency can be more easily measured.

We also found that sexually experienced males spent significantly less time engaged in olfactory investigation of stimulus females before initiating sexual behavior. Sexually experienced males have been shown to spend longer investigating female odor cues (Hosokawa and Chiba, 2005; Swaney et al., 2007), however these studies involved exposing males to the urine or volatile odors of females, rather than direct interaction with a stimulus

female. Our data suggest that the sexually experienced males required less olfactory and/or vomeronasal stimulation to initiate sexual behavior. This may be due to more rapid arousal of neuroendocrine responses to female odors or to more rapid recognition of females as potential mates.

Behavioral changes as a function of sexual experience were associated with increased numbers of AR-positive cells in the MPOA of sexually experienced males, however we found no effect of sexual experience on numbers of AR-positive cells in the BNST or pMeAmg, nor did we see an effect on numbers of ERα-positive cells. This suggests that sexual experience produces a long-term increase in testosterone sensitivity in the MPOA, the key nucleus in the regulation of sexual behavior. While both AR and ERα are involved in reproductive behaviors in male rodents, previous studies indicate that actions of testosterone *via* AR have a more important role in male sexual behavior. Sexual behavior is restored after castration by administration of testosterone, but not by estradiol or dihydrotestosterone, (McGinnis and Dreifuss, 1989). These actions of testosterone after castration are blocked by AR-antagonists, and while anti-estrogens prevent the testosterone-induced restoration of social behaviors, they not block the restoration of sexual behavior by testosterone (Vagell and McGinnis, 1998). The effects of testosterone on sexual behavior appear thus to be primarily mediated through AR, and our data suggest that the behavioral effects of sexual experience may be due to some degree to an increase in AR levels within the MPOA. Variation in AR expression in the male sexual brain has been extensively studied in the context of sexual satiety, and decreased AR in the MPOA is associated with a lack of sexual drive in sexually satiated males (Romano-Torres et al., 2007). These studies have shown that AR levels in the male sexual brain are dynamic and vary with sexual interest. Our data suggest that while

recent sexual activity reduces AR in the MPOA, the acquisition of sexual experience produces a sustained increase in MPOA AR.

One caveat to our results is that although we found an increase in MPOA AR in sexually experienced male mice, a recent study with sexually experienced and virgin male rats found no difference in AR or ERα levels (Wu and Gore, 2009). This may be due to a species difference in the mechanisms by which sexual experience affects reproductive behavior, however it may also be a function of subtle differences in experimental design. Sexual satiety results in reduced AR in the MPOA, however even a single ejaculation is sufficient to reduce MPOA AR expression in male rats 24 hours later (Fernandez-Guasti et al., 2010). Male rat subjects in the Wu and Gore (2009) study were mated every other day for a month before being sacrificed for immunohistochemistry 24 hours after the final mating test. It may be that the temporal proximity of ejaculation to the time of AR detection affected levels of AR, potentially masking any long-term effect of sexual experience on MPOA AR levels in these male rats.

Mating is associated with increases in circulating testosterone, and testosterone has been shown to upregulate expression of AR (Meek et al., 1997; Wu and Gore, 2010). During the acquisition of sexual experience, the males in our study mated repeatedly over a three week period and would thus have experienced repeated increases in levels of testosterone which may be a mechanism by which levels of AR in the MPOA could be modulated. An increase in AR could increase the capacity of the MPOA to respond to increased testosterone signaling after sexual experience, as both basal and mating-associated testosterone levels are elevated in sexually experienced males (Bonilla-Jaime et al., 2006; Edinger and Frye, 2007). Increased sensitivity to testosterone in the MPOA also has potential ramifications for mating-associated dopaminergic activity in the MPOA. Testosterone mediates female-elicited release

of dopamine in the MPOA, which is necessary for successful copulation (Hull et al., 2003). This dopamine release is regulated by nitric oxide synthase, which is itself regulated by testosterone levels (Du and Hull, 1999; Sato et al., 2005). Increased sensitivity to testosterone *via* MPOA AR might play a role in nitric oxide synthase-mediated local dopamine release. Indeed, levels of nitric oxide synthase in the MPOA have been shown to be elevated in sexually experienced male rats (Dominguez et al., 2006), indicating that this circuit is also sensitive to sexual experience and potentially pointing to an interaction between gonadal steroids and dopamine within the MPOA. Elucidating these interactions would provide insight into the mechanisms of plasticity in the male brain that are recruited during reproductive experience.

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547

548 **Figure Captions**

549 Figure 1. Summary of experimental design indicating the treatment of virgin *vs.* sexually
550 experienced males

551 Figure 2. Measures of sexual behaviour of virgin (n=6) and sexually experienced (n=6) male
552 B6 mice in hour long mating tests with sexually receptive B6 female virgins. All values are
553 means ± S.E.M. Asterisks signify $P<0.05$. A) mean latency in minutes of mounting,
554 intromission and ejaculation; B) mean frequency per minute of mounting, intromission and
555 ejaculation; C) comparison of mean duration of mounting and intromission; D) mean duration
556 in seconds of sniffing of the female prior to the male's first mounting attempt.

557 Figure 3. Counts of A) AR- and B) ERα-positive cells in the BNST, MPOA and pMeAmg in
558 virgin (n=6) and sexually experienced (n=6) male B6 mice. C) Representative
559 photomicrographs indicating density of AR- and ERα-positive cells in the MPOA of virgin
560 and sexually-experienced males. All values are means ± S.E.M. Asterisks signify $P<0.05$.

Figure 1

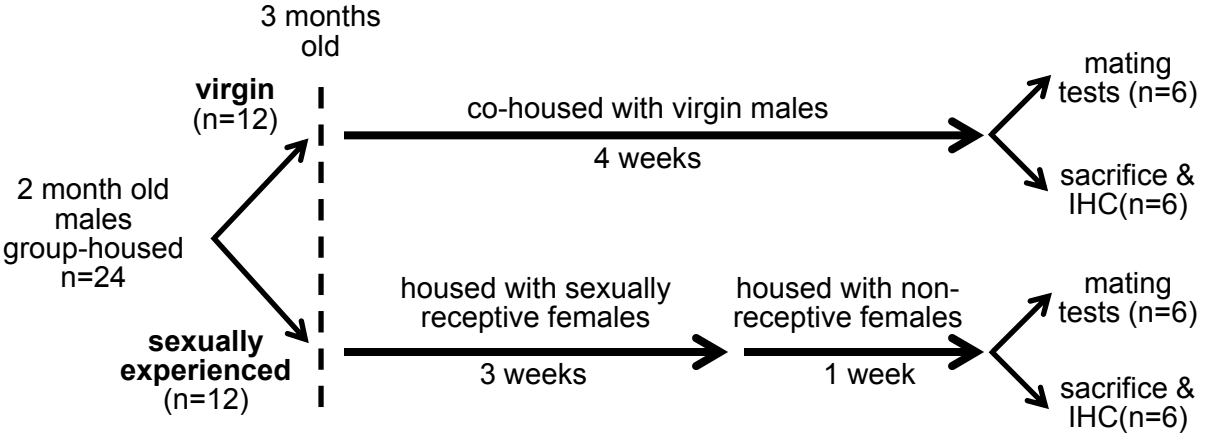

3 months old
virgin (n=12)
2 month old males group-housed n=24
sexually experienced (n=12)
co-housed with virgin males
4 weeks
housed with sexually receptive females
3 weeks
housed with non-receptive females
1 week
mating tests (n=6)
sacrifice & IHC(n=6)
mating tests (n=6)
sacrifice & IHC(n=6)

Figure 2

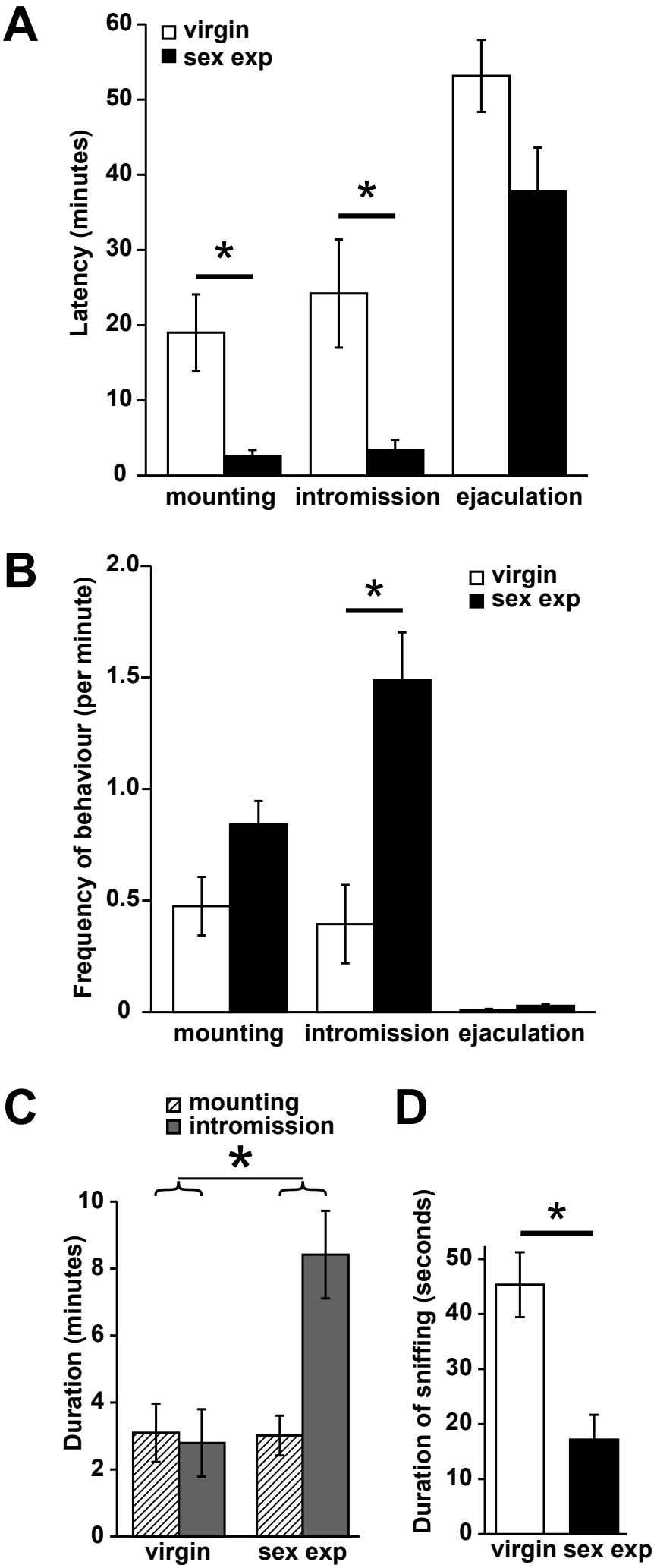
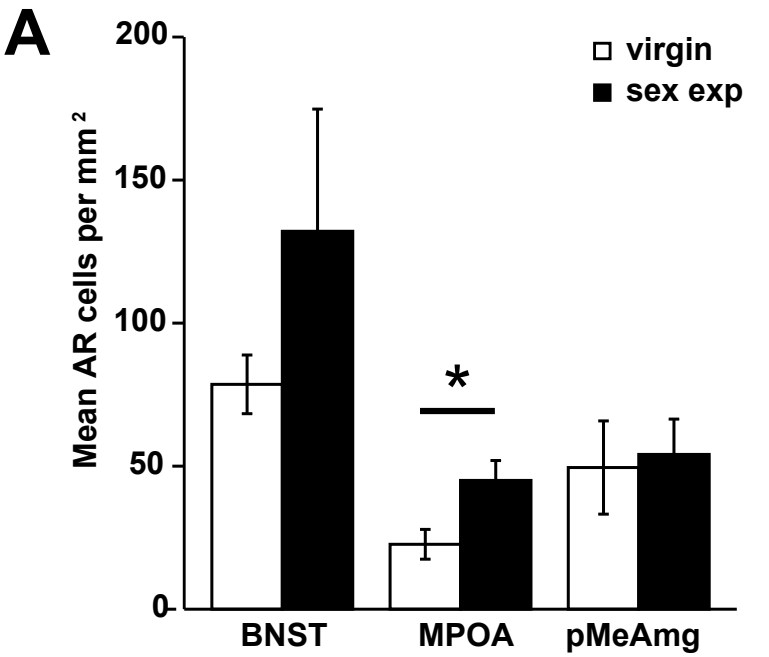
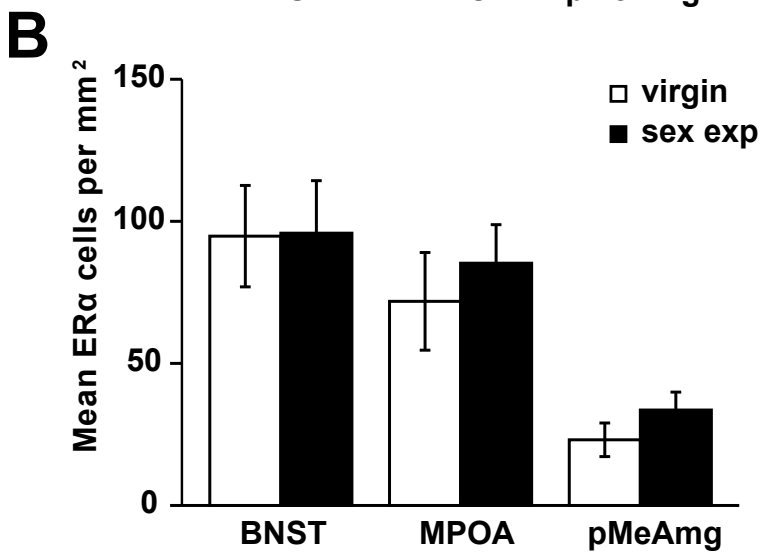
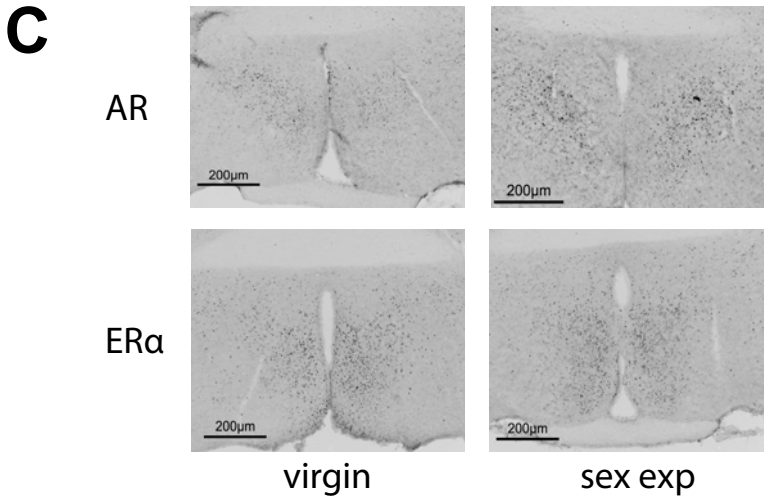

A
virgin
sex exp
Latency (minutes)
60
50
40
30
20
10
0
*
*
mounting
intromission
ejaculation
B
virgin
sex exp
Frequency of behaviour (per minute)
2.0
1.5
1.0
0.5
0
*
mounting
intromission
ejaculation
C
mounting
intromission
*
Duration (minutes)
10
8
6
4
2
0
virgin
sex exp
D
*
Duration of sniffing (seconds)
50
40
30
20
10
0
virgin
sex exp

Figure 3


A
200
150
100
50
0
Mean AR cells per mm²
virgin
sex exp
*
BNST
MPOA
pMeAmg


B
150
100
50
0
Mean ERα cells per mm²
virgin
sex exp
BNST
MPOA
pMeAmg


C
AR
ERα
200µm
virgin
sex exp