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

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2    **Abstract**

3    Reproductive behavior in male rodents is made up of anticipatory and consummatory

4    elements which are regulated in the brain by sensory systems, reward circuits and hormone

5    signaling. Gonadal steroids play a key role in the regulation of male sexual behavior *via*

6    steroid receptors in the hypothalamus and preoptic area. Typical patterns of male

7    reproductive behavior have been characterized, however these are not fixed but are

8    modulated by adult experience. We assessed the effects of repeated sexual experience on

9    male reproductive behavior of C57BL/6 mice; including measures of olfactory investigation

10   of females, mounting, intromission and ejaculation. The effects of sexual experience on the

11   number of cells expressing either androgen receptor (AR) or estrogen receptor alpha (ERα) in

12   the primary brain nuclei regulating male sexual behavior was also measured. Sexually

13   experienced male mice engaged in less sniffing of females before initiating sexual behavior

14   and exhibited shorter latencies to mount and intromit, increased frequency of intromission,

15   and increased duration of intromission relative to mounting. No changes in numbers of ERα-

16   positive cells were observed, however sexually experienced males had increased numbers of

17   AR-positive cells in the medial preoptic area (MPOA); the primary regulatory nucleus for

18   male sexual behavior. These results indicate that sexual experience results in a qualitative

19   change in male reproductive behavior in mice that is associated with increased testosterone

20   sensitivity in the MPOA and that this nucleus may play a key integrative role in mediating the

21   effects of sexual experience on male behavior.

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## 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

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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

71 and motivation, (Balfour et al., 2004), and regions in the hypothalamus and preoptic area

72 (Hull et al., 2002). These are sites of high expression of AR and ERα such as the bed nucleus

73 of stria terminalis (BNST), medial amygdala (MeAmg), ventromedial hypothalamus (VMH)

74 and medial preoptic area (MPOA) (Simerly et al., 1990). The MPOA is the critical integrative

75 nucleus in male sexual behavior (Hull and Dominguez, 2006) and lesions of the MPOA

76 disrupt mounting, intromission and ejaculation in rats (de Jonge et al., 1989), mice (Bean et

77 al., 1981) and hamsters (Powers et al., 1987). The importance of testosterone sensitivity in

78 the MPOA for male sexual behavior is illustrated by studies in which AR antagonists are

79 injected into the MPOA, resulting in sexual behavior deficits resembling those seen after

80 MPOA lesions (Harding and McGinnis, 2004; McGinnis et al., 1996). Moreover, conditional

81 deletion of AR in the brain increases latencies to perform sexual behaviors and reduces

82 incidences of copulation (Raskin et al, 2009), despite elevated circulating testosterone and

83 intact MPOA ERα levels in these knockout males. Copulation-induced c-Fos has been shown

84 to colocalize with AR in the MPOA, BNST and MeAmg of male hamsters (Wood and

85 Newman, 1993), indicating that testosterone-sensitive neurons are activated in these brain

86 areas during mating. Sexual experience induces a series of changes across this brain network

87 that regulates reproductive behavior. After sexual experience, female odor-elicited c-Fos

88 expression is greater in both the main olfactory and vomeronasal systems and their

89 downstream androgen-sensitive targets, with particularly strong responses seen in the

90 MeAmg, BNST and MPOA (Fewell and Meredith, 2002; Hosokawa and Chiba, 2005;

91 Swaney et al., 2007). Copulation itself increases c-Fos immunoreactivity in the MPOA

92 (Robertson et al., 1991) and this increase is greater in the brains of sexually experienced male

93 rats than virgins (Lumley and Hull, 1999).

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94      Research into the effects of sexual satiety in rats offers further evidence of neuronal

95      plasticity in the circuits regulating male sexual behavior and of changes in testosterone

96      sensitivity. Male rats that are allowed to mate *ad libitum* over a short period of time reach a

97      state of sexual satiety in which they lose interest in females and do not fully recover sexual

98      drive for up to 15 days (Phillips-Farfan and Fernandez-Guasti, 2009). This state of sexual

99      satiety and lack of interest in females is associated with AR density reductions in the nucleus

100     accumbens (NAc), VMH, lateral septum, MeAmg and especially in the MPOA (Fernandez-

101     Guasti et al., 2003; Phillips-Farfan and Fernandez-Guasti, 2009). Conversely, ERα is elevated

102     in the VMH, lateral septum and MPOA of sexually satiated males (Phillips-Farfan et al.,

103     2007, Fernandez-Guasti, 2010). AR and ERα densities recover to previous levels as libido

104     returns and this dynamic variation in gonadal steroid sensitivity indicates that receptor

105     expression in the sexual brain network varies with sexual function in males.

106     In the current study, we explore the long term effects of sexual experience on

107     reproductive behavior and whether these effects involve changes in regulatory brain areas

108     that are sensitive to testosterone. Our experimental design involved measurement of the

109     effects of repeated sexual experience during cohabitation with females on reproductive

110     behavior in male mice. We predicted that sexual experience would result in changes in

111     interest in females as well as shorter latencies and higher frequencies of sexual behaviors.

112     While such effects have been characterized in other rodent species, such as rats and hamsters,

113     these effects have not been specifically studied in mice despite their increasing use in

114     behavioral neuroscience research. Given previously reported increases in circulating

115     testosterone after sexual experience and the dynamic changes in AR and ERα after sexual

116     activity, we also examined whether behavioral effects of sexual experience are associated

117     with long-term changes in numbers of AR and ERα-positive neurons in the primary nuclei

118    that regulate sexual behavior in male mice. The MPOA, posterior MeAmg (pMeAmg), and

119    BNST were selected for investigation based on their involvement in reproductive behavior,

120    previously reported activity changes after sexual experience, and high levels of expression of

121    AR and ERα.

122    **Materials and Methods**

123    To characterize the effects of sexual experience on reproductive behaviors, age-matched

124    virgin and sexually experienced C57BL/6 (B6) male mice were given mating tests with

125    receptive virgin B6 female mice. To investigate effects of sexual experience on AR and ERα,

126    age-matched virgin and sexually experienced B6 male mice were sacrificed and their brains

127    processed for AR and ERα immunohistochemistry. All procedures described were approved

128    by the Institutional Animal Care and Use Committee of Columbia University.

129    *Animals*

130    Adult male and female B6 mice were purchased from Charles River Laboratories (Kingston,

131    NY) at approximately 2 months of age. Males and females were separately housed in groups

132    of 4 or 5 at the Department of Psychology at Columbia University for 1 month of habituation

133    to the animal facility. Mice were housed in 13.5" x 8.1" x 5.5" Plexiglas cages under a 12

134    hour reversed dark-light cycle (8am: lights off, 8pm: lights on) with wood shaving bedding,

135    *ad libitum* water and chow. At the start of the experimental phase (see **Figure 1** for summary

136    of experimental design), male mice were split into two experimental groups: virgin and

137    sexually experienced males. Each sexually experienced male was housed in a novel clean

138    cage with two gonadally intact B6 female mice which each received repeated hormonal

139    priming to induce regular sexual receptivity. This priming involved subcutaneous injections

140    of 50 μg of estradiol benzoate (Fisher Scientific, Pittsburgh PA) dissolved in 50μl of peanut

141    oil, followed 72 hours later by 400μg progesterone (Fisher Scientific, Pittsburgh PA)

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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

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167    male mice of each experimental group were given mating tests, the other half were sacrificed

168    for analysis of brain immunohistochemistry.

169    *Tests of male reproductive behavior*

170    The mating behaviors of virgin and sexually experienced B6 males were measured in one-

171    hour tests with unfamiliar, hormonally-primed, virgin B6 females. These stimulus females

172    were hormonally primed with subcutaneous injections of 50 μg of estradiol benzoate

173    dissolved in 50μl of peanut oil 72 hours before testing, followed by 400μg progesterone

174    dissolved in 50μl of peanut oil 3 hours before testing. Each male was tested during the first 6

175    hours after lights out in a clean 13.5" x 8.1" x 5.5" Plexiglas cage without food or water. A

176    male and a stimulus female were placed in the test cage for one hour and behavior was video

177    recorded from above for later blind scoring. The male reproductive behaviors scored were

178    anogenital sniffing of the female, mounting, intromission and ejaculation. The measures

179    scored were latency and frequency of mounting, intromission and ejaculation, duration of

180    anogenital sniffing before first mount, and the difference between duration of mounting and

181    intromission (calculated by subtracting mounting duration from intromission duration). If a

182    subject did not perform a behavior during the hour-long test, a maximum latency value of

183    3600 seconds was assigned for that behavior.

184    *AR and ERα immunohistochemistry*

185    Virgin and sexually experienced males were euthanized with an overdose of ketamine-

186    xylazine anesthetic, before being transcardially perfused with 20ml of 0.1M phosphate-

187    buffered saline (PBS) followed by 20ml of freshly prepared 4% paraformaldehyde in PBS.

188    Brains were then removed and post-fixed for 4 hours at 4°C in 4% paraformaldehyde in PBS,

189    before being cryoprotected in 30% sucrose in PBS overnight, then frozen and stored at -80°C.

190    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:

214    500x350μm; pMeAmg: 500x375μm; MPOA: 600x400μm). In each region the mean number

215    of stained cells per mm$^2$ per section was calculated for each animal.

216    *Statistical analysis*

217    Latency data from the mating tests were not normally distributed and so were $\log_{10}(y+1)$

218    transformed before analysis. All measures of male reproductive behavior were analyzed using

219    student's independent two-sample *t*-tests. A Bonferroni correction was applied to account for

220    multiple comparisons: 8 tests were run, resulting in a corrected alpha value of *P*=0.00625. All

221    immunohistochemical data were normally distributed. Counts of stained AR and ERα cells

222    were analyzed using Student's independent two-sample *t*-tests, or Welch's *t*-test when the

223    variances were unequal.

224    **Results**

225    *Tests of male reproductive behavior*

226    Significant effects of sexual experience were seen across multiple measures of male

227    reproductive behavior (**Figure 2**). Sexually experienced males had shorter latencies to mount

228    females than virgin males (n=6/group; $t_{10}$=-5.78, *P*<0.001), and shorter latencies to intromit

229    than virgins ($t_{10}$=-5.41, *P*<0.001). However there was no significant difference between the

230    groups in the latency to ejaculate during testing ($t_{10}$=-1.86, *P*=0.093). Comparison of the

231    frequency of behaviors indicated that intromission frequency was significantly higher among

232    sexually experienced males than virgins ($t_{10}$=3.95, *P*=0.003), however differences between

233    sexually experienced and virgin males in frequency of mounting ($t_{10}$=2.19, *P*=0.054) or

234    frequency of ejaculation ($t_{10}$=1.95, *P*=0.080) did not reach statistical significance. Initial

235    olfactory investigation of females was markedly reduced in sexually experienced males, who

236    engaged in significantly less anogenital sniffing of the female before their first mount ($t_{6.03}$=-

237    4.51, *P*=0.004). The difference in duration of intromission relative to mounting was also

[11]

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

[12]

262  spatial preferences in B6 male mice (Kudwa et al., 2005). Intromission without ejaculation

263  results in increased motivation to investigate females, while mounting alone has little effect

264  (Whalen, 1961), suggesting that intromission is also more rewarding than mounting alone.

265  Intromission may have been reinforced during previous matings in the sexually experienced

266  males, leading to longer durations of intromission relative to unrewarding mounting behavior.

267  Alternatively, sexually experienced males may be more sexually "competent" and so able to

268  achieve intromission more easily, an idea that is supported by research showing that

269  inappropriate mounting behavior is reduced in sexually experienced male mice (McGill,

270  1962a).

271       In addition to the proposed mechanistic explanations for the change in intromissive

272  behavior, there is also evidence that such a change might have adaptive consequences for

273  mating males. Intromission, but not mounting, has been shown to result in an increase in NAc

274  dopamine in mated female hamsters (Kohlert and Meisel, 1999), and extensive mounting

275  without intromission results in reduced lordosis in female rats (Hardy and Debold, 1971).

276  This suggests that intromission is a rewarding component of mating for females and that such

277  stimulation by the male may be important for maintenance of appropriate female behavior

278  during copulation. Moreover, intromission has also been shown to positively affect female

279  reproductive physiology. Female rats are both more likely to conceive and less likely to

280  continue estrous cyclicity after either intromission (Adler, 1969) or analogous artificial

281  cervical stimulation (Terkel et al., 1990). Copulation with male rats that have high

282  frequencies of intromission is also more likely to result in pregnancy (Wilson et al., 1965). In

283  mice, it has been suggested that the pattern of intromission has a direct bearing on the

284  likelihood of induction of pregnancy or pseudopregnancy (Diamond, 1970). Sexually

285  experienced males that exhibit higher intromission frequencies and longer total duration of

286    intromission may thus be more likely to successfully impregnate females and so produce

287    offspring. Sexually experienced male mice have been reported to have higher fecundity than

288    virgin males (Rastogi et al., 1981), indicating that sexual experience may indeed have such

289    adaptive consequences for male mice.

290        In contrast to the changes in mounting and intromission, we saw no effect of sexual

291    experience on either the latency or frequency of ejaculation. This absence of an effect on

292    ejaculation behavior appears surprising given the fact that both latency to ejaculate and

293    frequency of ejaculation are changed by sexual experience in rats (Sura et al., 2001).

294    However the species differences in sexual behavior patterns are important in this regard.

295    While rats ejaculate repeatedly during a single mating bout, mice lose sexual drive for at least

296    24 hours after a single ejaculation (McGill, 1962b). None of the B6 mice we tested ejaculated

297    more than once and some males failed to ejaculate during the hour-long tests despite repeated

298    mounting and intromission with females. Tests of longer duration, such as the 10 hour tests

299    employed by Raskin et al (2009), might have improved our data on ejaculation latency and

300    shown differences between sexually experienced and virgin males. The ejaculation frequency

301    data for each individual was thus limited to a maximum value of 1, resulting in a narrower

302    range of values than would be obtained in analogous tests with rats, where at test an

303    individual is likely to ejaculate repeatedly and variation in ejaculation frequency can be more

304    easily measured.

305        We also found that sexually experienced males spent significantly less time engaged

306    in olfactory investigation of stimulus females before initiating sexual behavior. Sexually

307    experienced males have been shown to spend longer investigating female odor cues

308    (Hosokawa and Chiba, 2005; Swaney et al., 2007), however these studies involved exposing

309    males to the urine or volatile odors of females, rather than direct interaction with a stimulus

310    female. Our data suggest that the sexually experienced males required less olfactory and/or

311    vomeronasal stimulation to initiate sexual behavior. This may be due to more rapid arousal of

312    neuroendocrine responses to female odors or to more rapid recognition of females as

313    potential mates.

314         Behavioral changes as a function of sexual experience were associated with increased

315    numbers of AR-positive cells in the MPOA of sexually experienced males, however we

316    found no effect of sexual experience on numbers of AR-positive cells in the BNST or

317    pMeAmg, nor did we see an effect on numbers of ERα-positive cells. This suggests that

318    sexual experience produces a long-term increase in testosterone sensitivity in the MPOA, the

319    key nucleus in the regulation of sexual behavior. While both AR and ERα are involved in

320    reproductive behaviors in male rodents, previous studies indicate that actions of testosterone

321    *via* AR have a more important role in male sexual behavior. Sexual behavior is restored after

322    castration by administration of testosterone, but not by estradiol or dihydrotestosterone,

323    (McGinnis and Dreifuss, 1989). These actions of testosterone after castration are blocked by

324    AR-antagonists, and while anti-estrogens prevent the testosterone-induced restoration of

325    social behaviors, they not block the restoration of sexual behavior by testosterone (Vagell and

326    McGinnis, 1998). The effects of testosterone on sexual behavior appear thus to be primarily

327    mediated through AR, and our data suggest that the behavioral effects of sexual experience

328    may be due to some degree to an increase in AR levels within the MPOA. Variation in AR

329    expression in the male sexual brain has been extensively studied in the context of sexual

330    satiety, and decreased AR in the MPOA is associated with a lack of sexual drive in sexually

331    satiated males (Romano-Torres et al., 2007). These studies have shown that AR levels in the

332    male sexual brain are dynamic and vary with sexual interest. Our data suggest that while

[15]

333    recent sexual activity reduces AR in the MPOA, the acquisition of sexual experience

334    produces a sustained increase in MPOA AR.

335        One caveat to our results is that although we found an increase in MPOA AR in

336    sexually experienced male mice, a recent study with sexually experienced and virgin male

337    rats found no difference in AR or ERα levels (Wu and Gore, 2009). This may be due to a

338    species difference in the mechanisms by which sexual experience affects reproductive

339    behavior, however it may also be a function of subtle differences in experimental design.

340    Sexual satiety results in reduced AR in the MPOA, however even a single ejaculation is

341    sufficient to reduce MPOA AR expression in male rats 24 hours later (Fernandez-Guasti et

342    al., 2010). Male rat subjects in the Wu and Gore (2009) study were mated every other day for

343    a month before being sacrificed for immunohistochemistry 24 hours after the final mating

344    test. It may be that the temporal proximity of ejaculation to the time of AR detection affected

345    levels of AR, potentially masking any long-term effect of sexual experience on MPOA AR

346    levels in these male rats.

347        Mating is associated with increases in circulating testosterone, and testosterone has

348    been shown to upregulate expression of AR (Meek et al., 1997; Wu and Gore, 2010). During

349    the acquisition of sexual experience, the males in our study mated repeatedly over a three

350    week period and would thus have experienced repeated increases in levels of testosterone

351    which may be a mechanism by which levels of AR in the MPOA could be modulated. An

352    increase in AR could increase the capacity of the MPOA to respond to increased testosterone

353    signaling after sexual experience, as both basal and mating-associated testosterone levels are

354    elevated in sexually experienced males (Bonilla-Jaime et al., 2006; Edinger and Frye, 2007).

355    Increased sensitivity to testosterone in the MPOA also has potential ramifications for mating-

356    associated dopaminergic activity in the MPOA. Testosterone mediates female-elicited release

[16]

357 of dopamine in the MPOA, which is necessary for successful copulation (Hull et al., 2003).

358 This dopamine release is regulated by nitric oxide synthase, which is itself regulated by

359 testosterone levels (Du and Hull, 1999; Sato et al., 2005). Increased sensitivity to testosterone

360 *via* MPOA AR might play a role in nitric oxide synthase-mediated local dopamine release.

361 Indeed, levels of nitric oxide synthase in the MPOA have been shown to be elevated in

362 sexually experienced male rats (Dominguez et al., 2006), indicating that this circuit is also

363 sensitive to sexual experience and potentially pointing to an interaction between gonadal

364 steroids and dopamine within the MPOA. Elucidating these interactions would provide

365 insight into the mechanisms of plasticity in the male brain that are recruited during

366 reproductive experience.

367

## Acknowledgements

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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

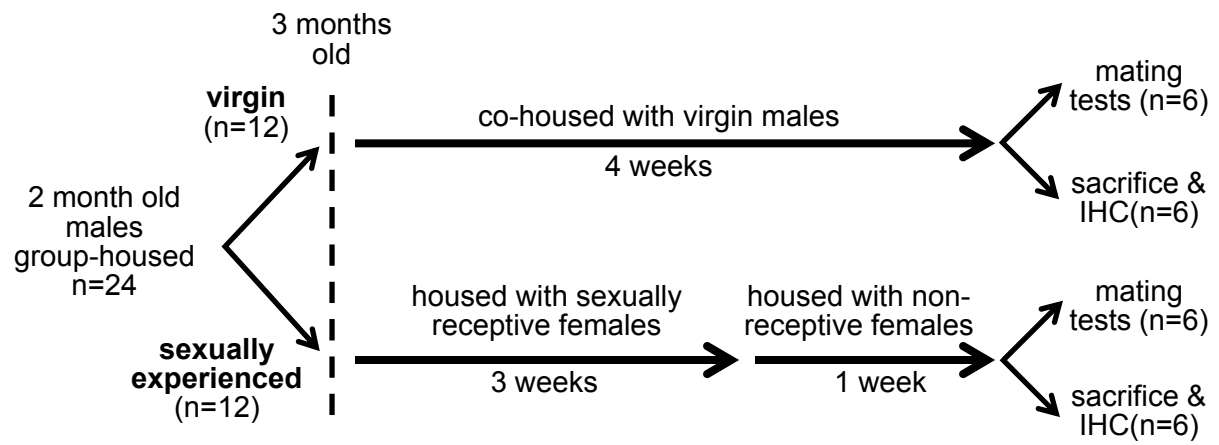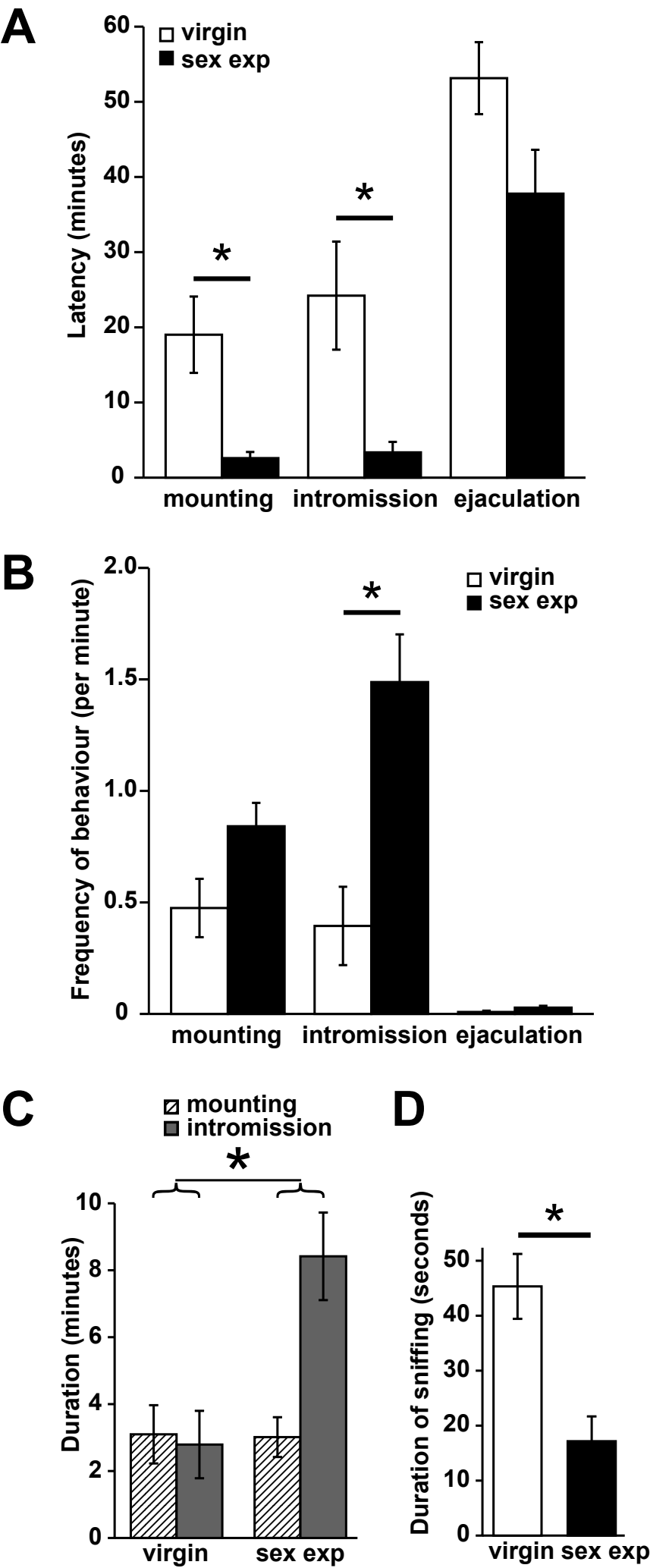560 and sexually-experienced males.  All values are means ± S.E.M. Asterisks signify P<0.05.

**Figure 1**

# Figure 2

# Figure 3