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Phospho-ERK and sex steroids in the mPOA: involvement in male mouse sexual behaviour

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1 **Title:** phospho-ERK and sex steroids in the mPOA : involvement in male mouse sexual behaviour

2

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19

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21

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23

24

25 **Abstract**

26 This paper aimed to investigate the mechanisms triggering ERK phosphorylation and its functional role
27 in male sexual behaviour. ERK1/2 phosphorylated form was detected in the medial preoptic area of
28 the hypothalamus (mPOA) during the sexual stimulation of naive and sexually experienced males who
29 were sacrificed 5 minutes after the first intromission. This mating-induced ERK phosphorylation was
30 increased in sexually experienced males compared to naive mice. The functional role of the ERK1/2
31 pathway activation during sexual behaviour was explored with the administration of a MEK inhibitor,
32 SL-327 (30 mg/kg, i.p.) 45 minutes before the contact with a receptive female. Inhibition of ERK
33 phosphorylation was found to decrease sexual motivation in both naive and experienced males
34 without altering their copulatory ability. The mechanisms potentially involved in this rapid ERK1/2
35 pathway activation were specified *ex vivo* on hypothalamic slices. A thirty-minute incubation with 100
36 nM of Testosterone (T), dihydrotestosterone (DHT) or estradiol (E2) led to ERK phosphorylation. No
37 changes were observed following incubation with testosterone 3-(O-carboxymethyl)oxime-BSA (T-
38 BSA), an impermeable to the plasma membrane form of testosterone. All these results indicate that
39 ERK phosphorylation within the mPOA could be a key player in the motivational signalling pathway and
40 considered as an index of sexual motivation. They also demonstrate the involvement of oestrogen
41 receptor (ER) and androgen receptor (AR) transduction pathways in steroid-dependent ERK activation.

42 Introduction

43 Sexual behaviour in male rodents can be described as a series of behavioural elements
44 cumulating in ejaculation. These involve approaches and olfactory investigations of the female during
45 the motivational phase of sexual behaviour, which is followed by bouts of mounting, intromission and
46 then ejaculation constituting the copulatory phase. The mating behaviour is governed by complex
47 central interactions between different systems which process sensory inputs, regulate rewards and
48 integrate hormonal signals (Hull *et al.* 2002). The medial preoptic area of the hypothalamus (mPOA)
49 represents the critical integrative site for male sexual behaviour regulation because lesions in this area
50 are known to disrupt mounting, intromission and ejaculation in rats (De Jonge *et al.* 1989) and
51 mounting behaviour in mice (Bean *et al.* 1981). Peripheral olfactory signals are received in sensory
52 neurons of the olfactory epithelium and the vomeronasal organ, processed and relayed to the main
53 and accessory olfactory bulbs and then to the medial amygdala and in bed nucleus of the stria
54 terminalis before finally being integrated in the mPOA (Simerly & Swanson 1986; Hull & Dominguez
55 2007). In turn, activation of the mPOA network results in the stimulation of premotor areas responsible
56 for the sexual response (Simerly & Swanson
57 1988)<http://www.sciencedirect.com/science/article/pii/S0091305714000343> - [bb0610](#) and
58 descending systems providing excitatory stimuli controlling the spinal generators for erection and
59 ejaculation (Giuliano *et al.* 1996; Veening & Coolen 2014).

60 The mPOA is activated in the first moments of mating as demonstrated by c-Fos mapping of
61 the neural networks underlying the control of sexual behaviour (Baum & Everitt 1992; Pfau & Heeb
62 1997). This has been confirmed by (Taziaux *et al.* 2011) by detecting the phosphorylated form of the
63 mitogen-activated protein kinase/extracellular signal-regulated kinase (pERK) which was increased 10
64 minutes after exposure of sexually experienced males to female olfactory cues or after the display of
65 coital behaviour. Hence, measuring pERK levels appeared as an accurate alternative tool to detect
66 mPOA short-term activation since the timeline of kinase-mediated protein phosphorylation is much
67 shorter than the induction of genomic transcription and translation of immediate early genes (IEG)

68 (Murphy & Blenis 2006). ERK phosphorylation is the final step of an intracellular signalling cascade
69 which participates in the regulation of various cellular processes such as proliferation and
70 differentiation (Shaul & Seger 2007). Within the central nervous system, it plays a critical role in the
71 modulation of synaptic plasticity, neuronal excitability, memory formation, storage and processing
72 (Kelleher *et al.* 2004; Thomas & Huganir 2004; Shiflett & Balleine 2011a; Girault 2012), as well as in the
73 control of reward, aversive or learning behaviour (Shiflett & Balleine 2011b; Pascoli *et al.* 2014; Goto
74 *et al.* 2015). Among the different signals triggering the ERK pathway activation, the stimulation of
75 hormonal receptors, namely androgen (AR) and oestrogen receptors (ER), is known to be a potent
76 activator_of ERK phosphorylation in different brain areas such as the hippocampus (Zhao & Brinton
77 2007; Pike *et al.* 2008) and the cerebellum (Wong *et al.* 2003) but also in other cell types in skeletal
78 muscle (Estrada *et al.* 2003), the breast (Chia *et al.* 2011) and prostate (Liao *et al.* 2013).

79

80 Although, pERK level has been shown to be increased in the mPOA of sexually experienced
81 copulating males, currently no data are available on the hormonal signals triggering this
82 phosphorylation and its functional relevance.

83 We first focused on the role of sex steroid hormones, since the mPOA highly expresses AR
84 (Raskin *et al.* 2009), which can be activated by testosterone (T) or its metabolite dihydrotestosterone
85 (DHT). mPOA also expresses ER α (Swaney *et al.* 2012) and ER β (Orikasa *et al.* 2002) activated by
86 estradiol (E2) which can be produced by neural aromatization of T. These experiments were conducted
87 *ex vivo* on hypothalamic slices including the mPOA. This model has been found to efficiently decipher
88 signalling pathways since it has the advantage of maintaining the integrity of the cellular
89 microenvironment while using various pharmacological agents (Maaloud *et al.* 2008; St-Louis *et al.*
90 2014). In the second part of our work, we analysed the functional role of ERK phosphorylation *in vivo*.
91 We first established that ERK is phosphorylated at the beginning of the copulatory phase. We then
92 compared the levels of ERK phosphorylation in naive and sexually experienced mice because ERK
93 phosphorylation is involved in memory consolidation and network potentiation (Adams & Sweatt

94 2002; Thomas & Huganir 2004; Davis & Laroche 2006). Indeed, although behavioural components of
95 mating are known to be subjected to learning processes (Hull & Dominguez 2006; Swaney *et al.* 2012),
96 the potential role of ERK phosphorylation in this improvement has never been addressed. Finally, we
97 administrated SL-327, an inhibitor of ERK phosphorylation crossing the blood-brain barrier (Atkins *et*
98 *al.* 1998; Valjent *et al.* 2000), to naive and experienced mice before the interaction with a receptive
99 female to analyse the importance of ERK phosphorylation in conducting the various phases of sexual
100 behaviour.

101 **Material and methods**

102 ***Animals***

103 All the experiments were conducted in accordance with French and European laws (Decrees
104 2013-118, L214 and R214-87/130, 2013/63/ECC) and approved by the “Charles Darwin” ethical
105 committee (project number 01490-01)

106 C57Bl/6J mice (Janvier Breeding Centre, Le Genest, France) were bred in our animal facility and
107 housed under a controlled photoperiod (12h light and 12h darkness cycle - lights on at 2 a.m.) at 20 ±
108 2 °C with food and water *ad libitum*. Ten weeks old-adult males were isolated for two weeks before
109 any behavioural experiment.

110

111 ***Preparation of receptive females***

112 C57BL/6J females were ovariectomized under general anaesthesia (xylazine 10 mg/kg -
113 ketamine 100 mg/kg, i.p.) and implanted with Silastic (Dow Corning) implants filled with 50 µg of
114 estradiol-benzoate (Sigma-Aldrich, Saint-Louis, USA) in 30 µl of sesame oil. Four to five hours before
115 the tests, they were subcutaneously treated with 1 mg of progesterone (Sigma-Aldrich, Saint-Louis,
116 USA) in 100 µl of sesame oil, as previously reported (Raskin *et al.* 2009). Female receptivity was verified
117 before the beginning of experiments with a sexually experienced male.

118

119 ***Male sexual experience***

120 Sexually experienced males were obtained after a 10-hours mating session with a receptive
121 female. The test was conducted under red-light illumination 2h after lights off, videotaped and
122 analysed to confirm that each male exhibited copulatory behaviour and reached the ejaculation. Male
123 sexual behaviour was analysed by scoring the sniffing time before the first intromission, the latency to
124 first intromission, the number of mounts and the mating duration defined as the time from the first
125 intromission to ejaculation.

126

127 ***Effect of sex steroid hormones on ERK phosphorylation within the mPOA***

128 Naive males were decapitated, brains were quickly removed and immersed in cooled artificial
129 cerebrospinal fluid (aCSF): 117 mM NaCl, 4.7 mM KCl, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, 2.5 mM
130 CaCl₂, 1.2 mM MgCl₂ and 10 mM glucose (Maolood et al., 2008; St-Louis et al., 2014). Coronal
131 hypothalamic slices were cut with a Vibroslice (World Precision Instruments, Sarasota, USA). Using the
132 anterior commissure as a landmark, one 400- μ m-thick section including the mPOA was selected for
133 each mouse, transferred to a brain slice chamber system and incubated in aCSF with either
134 Testosterone (T, 100 nM; Sigma-Aldrich, Saint-Louis, USA), dihydrotestosterone (DHT, 100 nM; Sigma-
135 Aldrich, Saint-Louis, USA), estradiol (E2, 100 nM; Sigma-Aldrich, Saint-Louis, USA) or Testosterone 3-
136 (O-carboxymethyl)oxime-BSA (T-BSA, 20-30 testosterone molecules per molecule BSA, 100 nM; Sigma-
137 Aldrich, Saint-Louis, USA) diluted in DMSO (7 males per group). Control slices were incubated with only
138 DMSO. In a pilot experiment, the viability of the slices was verified by Fluoro-Jade staining (Millipore,
139 Guyancourt, France). After 30 minutes of treatment, mPOA was punched for Western blot analysis.

140

141 ***Effect of sexual experience on mating-induced ERK phosphorylation***

142 Twenty-eight males were assigned to four different groups (7 males per group, figure 2A):
143 naive staying in home cage (Naive: N), naive allowed to mate with a receptive female (Naive + sex
144 stimulus: N+S), sexually experienced (one mating 14 days before the experiment) staying in home cage
145 (sexually experienced: E) and sexually experienced allowed to mate with a receptive female (sexually
146 experienced + sex stimulus: E+S). Males were allowed to mate with a receptive female for 30 min and
147 their sexual behaviour analysed. Animals showing no sexual behaviour during the 30-minute test were
148 removed from the study. Five minutes after the first intromission, they were decapitated and the brain
149 rapidly removed. One 400- μ m-thick hypothalamic section was cut with a Vibroslice in cold 0.1M
150 phosphate buffer (PB) and the mPOA was punched for Western blot analysis.

151

152

153 ***Involvement of ERK phosphorylation in the induction of sexual experience***

154 Forty-five minutes before mating, 17 naïve males received an i.p. injection of SL-327 (Sigma-Aldrich,
155 Saint-Louis, USA, 30 mg/kg dissolved in 5% DMSO, 5% Tween 20 and 15% polyethylene glycol 400),
156 and 15 males received an i.p. injection of vehicle (figure 3A). Each male was tested in its home cage for
157 10h after the introduction of a receptive female and male sexual behaviour was analysed (mating 1).
158 To determine the role of pERK in sexual experience, male sexual behaviour of the same animals was
159 also analysed 14 days later without any injection (mating 2).

160

161 ***Involvement of ERK phosphorylation in olfactory preference of sexually experienced males***

162 Twenty-two sexually experienced (one mating 14 days before the experiment) males were
163 tested for olfactory preference (figure 4A). Two hours after lights off, each male was placed into an
164 enclosed Plexiglas Y-maze without any stimuli, for 5 minutes on two consecutive days, allowing them
165 to adapt to the apparatus. On the third day, 45 minutes after i.p. injection of SL-327 (30 mg/kg) or
166 vehicle, animals (11 per group) were given a choice between an anesthetized receptive female or an
167 anesthetized gonadally intact male as previously described (Picot *et al.* 2014). The time spent sniffing
168 at each partition and the number of entrances in the different arms were scored over the nine-minute
169 test. Results were expressed as a preference score [(time spent sniffing the female - time spent sniffing
170 the male)/total time sniffing] and in a percentage of the entries into the female arm. The maze was
171 cleaned with 10% ethanol between trials.

172

173 ***Involvement of ERK phosphorylation in locomotor activity of sexually experienced males***

174 Another group of 22 sexually experienced males were i.p. injected either with SL-327 (30
175 mg/kg) or vehicle (11 per group) 45 minutes before the test (figure 4A). Their locomotor activity was
176 evaluated for 120 minutes using a circular corridor with four infrared beams placed at every 90°
177 (Imetronic, Pessac, France) in a low luminosity environment as previously described (Valjent *et al.*
178 2006). Evolution of locomotor activity during the 120 minutes-test was assessed by counting the

179 number of beam interruptions every 5-minute intervals. The total locomotor activity represents the
180 number of quarters travelled during the 120-minutes test

181

182 ***Involvement of ERK phosphorylation in sexual behaviour of sexually experienced males.***

183 A group of sixty sexually experienced males was either injected with SL-327 (30 mg/kg) or
184 vehicle (8 per group) 45 minutes before a mating test (figure 4A). A control group of 5 sexually
185 experienced males who stayed in their home cage was also analysed. Male sexual behaviour was
186 followed and five minutes after the first intromission, mPOA was punched for Western blot analysis.

187

188 ***Western blot analysis***

189 Punches were homogenized in 50mM Tris (pH 7.2), 150mM NaCl, 0.1% SDS, 0.5% sodium
190 deoxycholate, 10 mM EDTA, 10 mM EGTA, 1% Triton-X100 and 1% protease Inhibitor (Roche, Meylan,
191 France) and sonicated 7 times during 30 seconds (Bioruptor Plus®; Diagenode, Seraing, Belgium). After
192 centrifugation (13 minutes, 13000 rpm at 4°C), protein concentrations were determined in the
193 supernatant with a Coomassie assay (Thermo Fisher Scientific, Waltham, USA). Samples were
194 denatured in Laemmli buffer (5 minutes at 95 °C). 15 µg protein were subjected to electrophoresis (120
195 V) on 4-12% Bis-Tris NuPAGE (Thermo Fisher Scientific, Waltham, USA) and electrotransferred
196 overnight (30 V at 4°C) onto a PVDF membrane (Millipore, Guyancourt, France). Blots were blocked
197 with 5% non-fat milk in Tris-buffered saline (TBS) and 0.2% Tween 20 (2 h) and incubated overnight
198 with primary antibodies against pERK1/2 (1:2000; polyclonal rabbit; Cell Signalling Technology,
199 Danvers, USA), ERK1/2 (1:2000; polyclonal rabbit; Cell Signalling Technology, Danvers, USA), GAPDH
200 (Glyceraldehyde 3-phosphate dehydrogenase; 1:20000; monoclonal mouse; Santa Cruz Biotechnology,
201 Dallas, USA) diluted in the same solution. Antibodies binding to blots were detected by 2h incubation
202 with horseradish peroxidase anti-rabbit or anti-mouse secondary antibodies (1:10000; Jackson
203 Immunoresearch, West Grove, USA). Signals were visualized with a pico-Super Signal detection kit
204 (Thermo Fisher Scientific, Waltham, USA) with an Amersham Imager 600 (GE Healthcare Life Sciences,

205 Velizy-Villacoublay, France) and quantified with ImageJ software (NIH). To determine the level of ERK
206 activation, the phospho-ERK signal was normalized to the total-ERK signal and expressed as a
207 percentage of the control.

208

209 ***Statistical analysis***

210 Statistical analysis were performed using GraphPad Prism software (GraphPad Software, La
211 Jolla, California, USA). Data were expressed as mean \pm SEM and compared with a Student's test. One-
212 way ANOVA followed by Dunnett's multiple comparison test was used to analyse slice treatments.
213 Two-way ANOVA followed by Bonferroni tests were used to analyse the effects of mating and sexual
214 experience on ERK phosphorylation. Two-way repeated measures ANOVA followed by Bonferroni tests
215 were used to analyse the effects of SL-327 treatment and the number of mating on sexual behaviour
216 and also to analyse the effects of SL-327 treatment and time on locomotor activity. *p* values of less
217 than 0.05 were considered to be significant.

218 **Results**

219 ***Activation of ERK by testosterone and its metabolites***

220 Slices containing mPOA were incubated with T or T-BSA, a non-aromatisable form of
221 testosterone which is impermeable to the plasma membrane (Nguyen *et al.* 2005). Incubations were
222 also performed with testosterone metabolites such as DHT or E2. An incubation time of 30 minutes
223 was chosen to be consistent with the rapid time course of ERK phosphorylation observed *in vivo*
224 (Taziaux *et al.* 2011). Slices were incubated with 100 nM of steroid corresponding to the plasmatic level
225 of testosterone found in copulating mice (Coquelin & Desjardins 1982; James & Nyby 2002).

226 The level of ERK phosphorylation in the mPOA was analysed by Western blot (figure 1A). One-way
227 ANOVA showed a significant effect of treatment on ERK phosphorylation ($F=5.623$, $p<0.001$, figure 1B).
228 Post hoc analyses showed a 86% significant increase in ERK phosphorylation induced by T treatment
229 when compared to the control group ($p<0.05$). This increase was also significant for the incubation
230 with DHT (80%, $p<0.05$) or with E2 (86%, $p<0.05$). By contrast, the levels of pERK were unchanged after
231 incubation with T-BSA.

232

233 ***Mating-induced ERK phosphorylation in the mPOA and sexual experience***

234 Sexually naive (N+S group) or experienced (E+S group) male mice were faced to a receptive female
235 for 30 minutes and sacrificed 5 minutes after the first intromission. Sexually naive (N group) or
236 experienced (S group) control mice were kept in their home cage without any contact with a receptive
237 female (figure 2A). The 5-minute delay was adapted to the time-course of ERK phosphorylation which
238 is known to be activated in the first 10 minutes after female exposure (Taziaux *et al.* 2011).

239 Analyses of sexual parameters showed a 53% reduction of the time spent in anogenital sniffing
240 ($p<0.01$), a 48% reduction of latency to the first intromission ($p<0.05$) and a 74% increase in the number
241 of mounts ($p<0.05$) in the E+S group compared to the N+S group, confirming the improvement of
242 sexual behaviour in experienced mice (figure 2B, C, D).

243 Two-way ANOVA demonstrated a significant effect of mating ($F_{(1-24)}=7.28$, $p<0.05$) and sexual
244 experience ($F_{(1-24)}=30.4$, $p<0.001$) on ERK phosphorylation (figure 2E). Post hoc analyses showed
245 that pERK level was increased for N+S males sacrificed 5 minutes after the first intromission (N+S:
246 241 ± 26.9 , $p<0.05$), but was unchanged in the control experienced group (E: 146 ± 33.9) when both were
247 compared to the control naive group (N: 100 ± 27.9). By contrast, ERK phosphorylation was increased
248 in the E+S group when compared to the N+S group (E+S: 378 ± 44 , $p<0.05$). The changes in pERK levels
249 were independent of any variation of total ERK protein normalized to the amount of GAPDH,
250 demonstrating a specific regulation on ERK phosphorylation rather than on its protein level.

251 These results demonstrate that ERK was phosphorylated in the mPOA 5 minutes after the first
252 intromission. The basal levels of pERK were not modified by sexual experience but the mPOA ability to
253 phosphorylate ERK during mating was enhanced by sexual experience.

254

255 ***Involvement of ERK phosphorylation in the regulation of male sexual behaviour***

256 To determine the role of ERK phosphorylation in the regulation of sexual behaviour, SL-327 (30
257 mg/kg, i.p.) was administered to naive males 45 minutes before the introduction of a receptive female
258 (figure 3A) and its effect on sexual behaviour was analysed (mating 1). To determine whether the
259 effects of this unique injection of SL-327 are transient or maintained in the long term, males were
260 subjected to a second sexual behavioural test 14 days later (mating 2, figure 3B, C, D).

261 Two-way repeated measures ANOVA showed a significant effect of SL-327 treatment on the time
262 spent in anogenital sniffing ($F_{(1-30)}=4.74$, $p<0.0001$) and on the latency to first intromission ($F_{(1-30)}=51.68$, $p<0.0001$) but not on mating duration ($F_{(1-30)}=29.05$, $p=0.92$). A significant effect of sexual
263 experience was also observed on the time spent in anogenital sniffing ($F_{(1-30)}=39.87$, $p<0.0001$), the
264 latency to first intromission ($F_{(1-30)}=5.78$, $p<0,05$) and the mating duration ($F_{(1-30)}=29.05$, $p<0.0001$).
265 Post hoc analyses showed that SL-327 treatment induced a significant increase by 34% ($p<0.01$) of the
266 time spent in anogenital sniffing and a 53% increase ($p<0.05$) of the latency to first intromission for SL-
267 327 treated males compared to the vehicle group in the first mating test.

269 These behavioural differences were not observed during the second mating test for the sniffing
270 time (54.39±5.2 vs. 63.8±7.9), the latency to the first intromission (2±0.21 vs. 2.46±0.26) and the
271 mating duration (12.7±1.38 vs. 12.5±1.31). This indicates that the SL-327 treatment transiently altered
272 sexual behaviour but did not interfere with sexual improvement assessed two weeks later.

273

274 ***Involvement of ERK phosphorylation in the control of male sexual behaviour in experienced males***

275 The short-term effect of the SL-327 (30 mg/kg, i.p.) treatment was further analysed on sexually
276 experienced males by comparing the vehicle group and SL-327 injected groups (figure 4A). The results
277 showed a significant increase of 34% ($p < 0.05$) in the time spent in anogenital sniffing and an increase
278 of 37% ($p < 0.05$) in the latency to the first intromission in the SL-327 treated group (Figure 4B, C).

279 To ensure that SL-327 did not affect behaviours which might interfere with the expression of sexual
280 behaviour controlled by the mPOA, olfactory preference towards female and locomotor activity were
281 analysed 45 minutes after SL-327 or vehicle injection.

282 For olfactory preference, tested males were given a choice between an anesthetized receptive
283 female and an anesthetized intact male. The total time spent in chemo-investigation was similar
284 between the groups (221±6.8 sec vs. 242±11.2 sec). The percentage of entries in the female arm
285 (44.1±3.3 vs. 50 ± 2.7) and preference score (14.1±2.9 vs. 16.1 ± 4.2) were not modified by SL-327
286 administration (figure 4D, E).

287 Two-way repeated measures ANOVA demonstrated a significant effect of time ($F_{(23-460)}=84.02$,
288 $p < 0.0001$) but not of treatment ($F_{(1-460)}=0.46$, $p=0.507$) on locomotor activity measured at 5-minute
289 intervals (Figure 4F). The cumulative activity expressed as the total number of quarters crossed during
290 the 2h-test was not different between the two groups (933±77.3 vs. 864±85.1, figure 4G). Taken
291 together, these data indicate that SL-327 does not interfere with olfactory preference and locomotor
292 activity.

293

294

295 ***Phospho-ERK inhibition by SL-327***

296 The level of pERK was measured in the mPOA of experienced male mice kept in their home cage
297 without a female (home cage) or injected either with SL-327 (SL-327) or vehicle (vehicle), put in the
298 presence of a receptive female and sacrificed 5 minutes after the first intromission (figure 4A). One-
299 way ANOVA showed the treatment had a significant effect on the ERK phosphorylation level ($F=8.020$,
300 $p<0.005$). Mated mice injected with the vehicle had significantly higher levels of pERK level than
301 unmated males (220 ± 31 vs. 100 ± 34 , $p<0.05$). This increase was totally abolished by SL-327 treatment
302 (86.4 ± 14 , figure 4H, I).

303

304 Taken together, these results demonstrate that blocking ERK phosphorylation increased the time
305 spent in chemo-investigation and delayed the initiation of sexual behaviour in both naive and
306 experienced mice. These transient effects did not disrupt the sexual improvement of mating and were
307 not due to altered olfactory preference or locomotor activity.

308 **Discussion**

309 The medial preoptic area of the hypothalamus (mPOA) is considered as the main integrative
310 site for male sexual behaviour regulation (Hull & Dominguez 2006), established for a long time by lesion
311 and stimulation experiments. Studies in a variety of rodents demonstrated an increased expression of
312 immediate early gene (IEG), such as c-Fos, in the mPOA with sexual activity (Robertson *et al.* 1991;
313 Heeb & Yahr 1996; Kollack-Walker & Newman 1997; Nutsch *et al.* 2016). Since pERK was induced in
314 the first 10 minutes of sexually experienced male coital behaviour by Taziaux *et al.* 2011, this signalling
315 pathway was suspected to be one of the first events involved in the control of sexual behaviour. This
316 paper investigated the mechanisms triggering ERK phosphorylation and its functional role in male
317 sexual behaviour.

318 We first confirmed the rapid activation of ERK phosphorylation in the early phases of
319 copulation. pERK was increased 5 minutes after the first intromission in both naive and experienced
320 males. Given the ability of steroid hormones to induce its phosphorylation (Wong *et al.* 2003; Abrahám
321 *et al.* 2004; Pike *et al.* 2008; Liao *et al.* 2013) and the increased circulating levels of testosterone (T)
322 during an interaction with a female (Batty 1978; Coquelin & Desjardins 1982), we thus investigated
323 whether T or its metabolites were able to induce ERK phosphorylation within the mPOA. Thirty minutes
324 treatment of hypothalamic slices by T was found to induce ERK activation. By contrast, treatment with
325 the plasma membrane-impermeable T-BSA was inefficient, thereby eliminating a potential membrane-
326 dependent signalling pathway. Furthermore, DHT treatment reproduced T activation of ERK,
327 suggesting the involvement of an intracellular AR-dependent mechanism in agreement with previously
328 described regulation of ERK phosphorylation by the intracellular AR in hippocampal neurons (Nguyen
329 *et al.* 2005). T-induced effects were also mimicked by E2 treatment, evidencing the participation of ER
330 in steroid mediated ERK phosphorylation. The possibility that steroids might use either the AR or the
331 ER pathway to mediate the same effect has already been described in the male rat namely to restore
332 a complete sexual behaviour after castration (Attila *et al.* 2010). Finally, the rapid ERK activation (30
333 min) suggests a rapid non-genomic action of sex steroids in the mPOA. In this way it is now well

334 established that cytoplasmic ER or AR can rapidly activate members of the ERK signalling cascade, via
335 direct interaction with the non tyrosine kinase receptor Src (Migliaccio *et al.* 2000). Of interest, sex
336 steroids can also activate members of the ERK pathway in hippocampal and cerebellar neurons in less
337 than 15 minutes (Wong *et al.* 2003; Nguyen *et al.* 2005).

338 Because the ERK pathway is involved in behavioural learning and reinforcing processes (Adams
339 & Sweatt 2002; Thomas & Haganir 2004), the level of ERK phosphorylation was compared between
340 naive and experienced males. Sexually experienced males showed a typical improved behaviour with
341 decreased anogenital sniffing time and latency to the first intromission and also a higher number of
342 mounts (Hull & Dominguez 2006; Swaney *et al.* 2012) thus demonstrating an enhancement of sexual
343 motivation (Fiorino & Phillips 1999).

344 ERK phosphorylation was found to be increased within the mPOA 5 minutes after the first
345 intromission in both naive and experienced males. To our knowledge, no study has reported an
346 increase of ERK phosphorylation in less than 5 minutes of stimulation. Thus, it can be hypothesized
347 that ERK phosphorylation was triggered by events taking place at least 5 minutes before the sacrifice,
348 thus during the motivational phase of sexual behaviour. In this line of thought, the level of ERK
349 phosphorylation may thus represent an index of sexual motivation and performance.

350 Interestingly, mating-induced ERK phosphorylation was more elevated in sexually experienced
351 males who are supposed to be more sensitive to androgen since they display elevated levels of
352 circulating testosterone and higher AR levels in the mPOA (Swaney *et al.* 2012). They also show an
353 increased number of copulation-induced c-Fos positive neurons in their mPOA (Lumley & Hull 1999).
354 As we found that T and its metabolites could induce a rapid activation of the MAPK pathway, the
355 increased ability of experienced males to phosphorylate ERK could be thus explained by a higher
356 sensitivity of the mPOA to steroids.

357 To assess the functional role of ERK phosphorylation in sexual behaviour, males were treated
358 with SL-327, a brain-penetrating selective inhibitor of MAP-kinase/ERK kinase (MEK1/2), the enzyme
359 which selectively activates ERK (Selcher *et al.* 1999). Naive males treated with SL-327 showed

360 decreased sexual motivation without altering the copulatory phase since mating duration and
361 copulatory patterns remained similar to the vehicle group. These results are consistent with those
362 obtained by Niessen *et al.* (2013) where c-Fos inhibition in the mPOA resulted in the reduction of
363 appetitive male sexual behaviour in the quail. In experienced males treated with SL-327, similar results
364 were observed: anogenital sniffing time and latency to the first intromission were increased compared
365 to experienced males treated with the vehicle. The increase observed in the motivational phase length
366 could be considered specific and not linked to a side effect of the SL-327 treatment since locomotion
367 was not altered. The rapid non-genomic ERK activation mediated by steroids that we exhibited *ex vivo*
368 could be one of the mechanisms involved in mating-induced ERK activation since sexual motivation
369 was shown to be partly regulated by rapid non-classical actions of steroids in the quail (Seredynski *et*
370 *al.* 2013).

371 Pre-treatment of naive males with SL-327 before their first sexual behaviour did not alter the
372 improvement of their sexual performance observed during the second mating test. Thus, inhibition of
373 ERK phosphorylation did not disrupt the learning associated with sexual experience even if the
374 motivational phase was prolonged. These results show that ERK phosphorylation is not required for
375 learning processes of sexual experience as it has been previously demonstrated in other brain areas
376 for spatial memory (Blum *et al.* 1999) and fear conditioning (Schafe *et al.* 2008).

377 To summarize, our results show that ERK phosphorylation probably took place during the
378 motivational phase, that experienced males, who are more motivated than naïve males, have an
379 increased level of phosphorylation and that inhibition of ERK phosphorylation decreased sexual
380 motivation. These results strongly suggest that ERK phosphorylation in the mPOA could be considered
381 as a key player in the motivational signalling pathway transduction and as an index of sexual
382 motivation.

383 Our results also revealed new aspects regarding the mechanisms leading to olfactory
384 preference since it was not modified by SL-327 treatment although exposure of a male to an oestrous
385 female was found to activate the ERK pathway in the main and accessory olfactory bulb and in the

386 amygdala (Taziaux *et al.* 2011). These data may be linked to the observation that pheromone
387 stimulation of a male mouse causes ERK activation in the vomeronasal organ, in amygdala and in the
388 hypothalamus but not in the accessory olfactory bulb (Dudley *et al.* 2001). Furthermore, even if
389 olfactory interactions are important for copulatory behaviour (Keller *et al.* 2006), several other
390 neuronal pathways coming from BST, septum and other areas converging on the mPOA (Dhungel *et al.*
391 2011) are activated when a male is placed in front of a receptive female (Pfaus & Heeb 1997). These
392 results are also consistent with data reported by Satoh *et al.* 2011 showing that mice lacking ERK2 in
393 the brain have deficits in social interaction without olfactory defect. Taken together, these
394 observations indicate that ERK phosphorylation is not essential to induce olfactory preference.
395 Furthermore, it suggests that the mechanisms controlling the motivational phase and the mPOA
396 activation are integrated downstream the olfactory bulb.

397

398 In conclusion, this paper has established for the first time that ERK is phosphorylated within
399 the mPOA during the early events of the male copulation in both naive and sexually experienced males.
400 This phosphorylation can be triggered *ex vivo* by rapid action of testosterone or its metabolites such
401 as DHT or E2. Although the ERK pathway was involved in numerous learning processes, ERK
402 phosphorylation in the mPOA is not an underlying factor in sexual improvement induced by sexual
403 experience. Nevertheless, the level of pERK could be considered as an index of sexual motivation since
404 sexually experienced males exhibited increased in mating-induced ERK activation, and its inhibition
405 decreased the motivational phase efficiency.

406 Our results could represent an interesting tool for the investigation of the effects of
407 hormonomimetic molecules able to interfere with the control of sexual behaviour, such as endocrine
408 disruptors. Furthermore, the ability to rapidly activate the ERK pathway on an *ex vivo* model of mPOA
409 slices could also be a very useful tool to help decipher the negative effects of such molecules perturbing
410 the signalling pathways of steroid hormones.

411 **Conflicts of interest :**

412 There is no conflict of interest that could be perceived as prejudicing the impartiality of the research
413 reported.

414

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420

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596

597

598 **Legends**

599 **Figure 1: Activation of ERK by testosterone and its metabolites**

600 Brain slices including mPOA were incubated 30 minutes in aCSF+DMSO (control), Testosterone
601 (T, 100 nM), Dihydrotestosterone (DHT, 100 nM), Estradiol (E2, 100 nM) or Testosterone 3-(O-
602 carboxymethyl)oxime-BSA (T-BSA, 100 nM). A: representative blot detecting pERK, ERK and GAPDH in
603 the mPOA after 30 minutes of incubation B: densitometric evaluation of the Western blots. Results
604 (pERK/ERK) are presented as mean percentage of the control group \pm SEM, n = 7 in each group analysed
605 by one-way ANOVA followed by Dunnett's multiple comparison test, * $p < 0.05$ when compared to
606 control group.

607

608 **Figure 2: Mating-induced ERK phosphorylation in the mPOA and sexual experience**

609 A: Experimental design used to analyse the effect of mating and sexual experience on ERK
610 phosphorylation in the mPOA. N: naive male staying in home cage, N+S: naive male with sex stimulus
611 sacrificed 5 minutes after the first intromission, E: experienced male staying in home cage, E+S:
612 experienced male with sex stimulus sacrificed 5 minutes after the first intromission.

613 Sexual behaviour expressed as B: total anogenital sniffing time before the first intromission, C: latency
614 to the first intromission and D: number of mounts for N+S and E+S males. Results are presented as
615 mean \pm SEM and analysed by Student's test, * $p < 0.05$, ** $p < 0.01$ compared to N+S group.

616 E: representative blot detecting pERK, ERK and GAPDH in the mPOA for N, N+S, E and E+S males and
617 densitometric evaluation. Results (pERK/ERK) are presented as mean percentage of the N group \pm SEM
618 (n=7 in each group) and analysed by two-way ANOVA followed by Bonferroni post-hoc tests, * $p < 0.05$,
619 *** $p < 0,001$, # $p < 0.05$ when compared to N+S group.

620

621 **Figure 3: Involvement of ERK phosphorylation in the induction of male sexual experience**

622 A: Experimental design used to analyse the effect of a unique administration of the ERK
623 phosphorylation inhibitor SL-327 administration (30 mg/kg, i.p.) on sexual behaviour improvement. SL-

624 327 and vehicle injected groups were compared during the first and the second mating (separated by
625 14 days) for B: total anogenital sniffing time before the first intromission, C: latency to the first
626 intromission and D: mating duration. Results are presented as mean \pm SEM (n=15 for vehicle group and
627 n=17 for SL-327 group) and analysed by two-way repeated measures ANOVA followed by Bonferroni
628 post-hoc tests #p<0.05, ###p<0,001 for sexual experience effect and *p<0.05, **p<0.01 when
629 compared to mating 1 of vehicle group.

630

631 **Figure 4: Involvement of ERK phosphorylation in sexual behaviour, locomotor activity and olfactory**
632 **preference of sexually experienced males**

633 A: Experimental design used to analyse the effect of SL-327 administration (30 mg/kg, i.p.) on sexual
634 behaviour, locomotor activity, olfactory preference and ERK phosphorylation in the mPOA of sexually
635 experienced males.

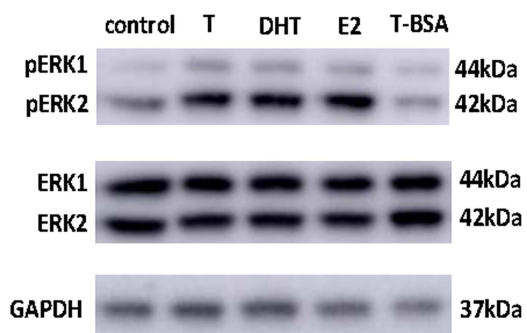
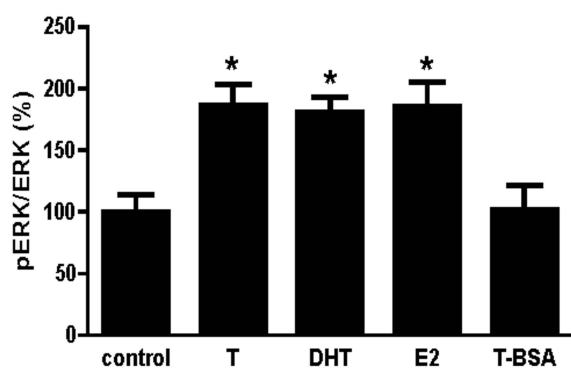
636 Sexual behaviour expressed as B: total anogenital sniffing time before the first intromission and C:
637 latency to the first intromission (n=8 in each group) presented as mean \pm SEM and analysed by
638 Student's test, *p<0.05 when compared to vehicle group.

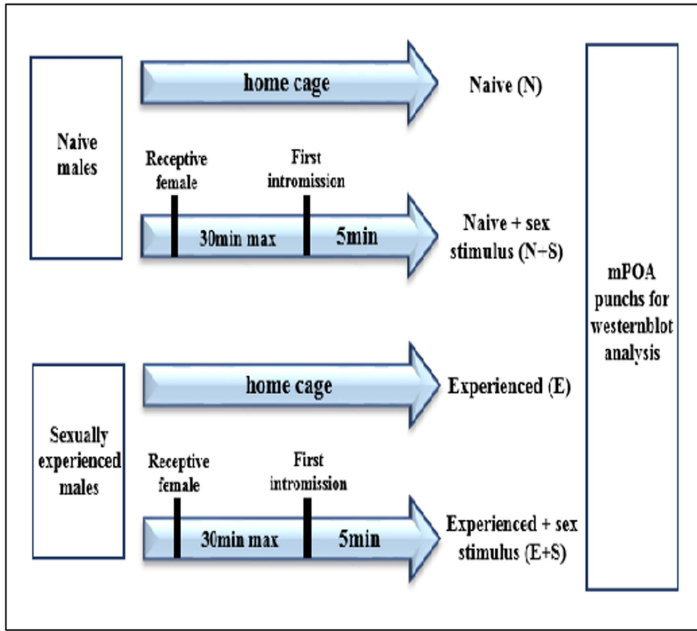
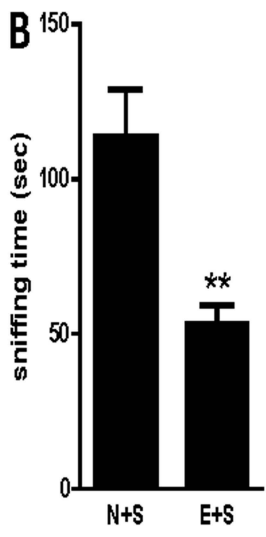
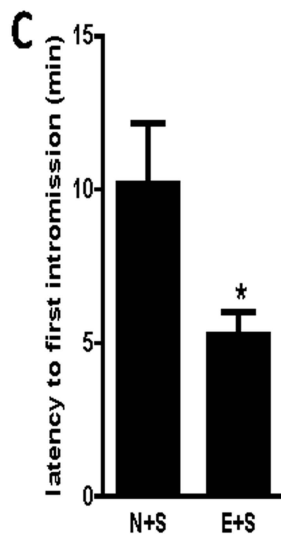
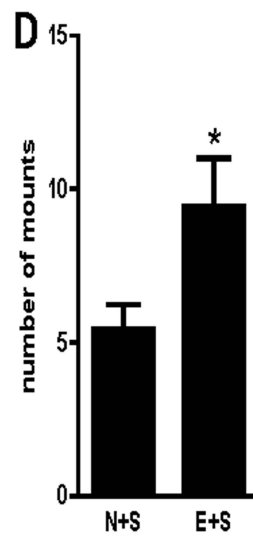
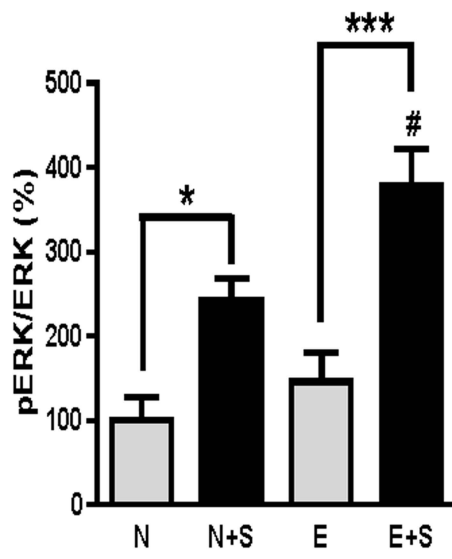
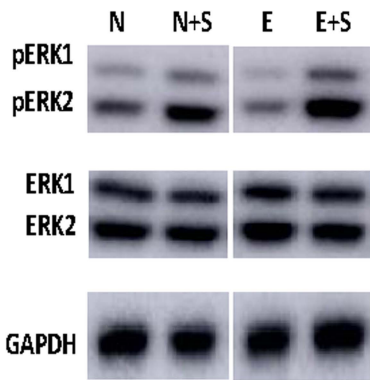
639 Olfactory preference in D and E. D: olfactory preference score defined as the [(time spent sniffing the
640 female - time spent sniffing the male)/total time sniffing], E: percentage of entries in female arm
641 analysed by Student's test (n=11 in each group).

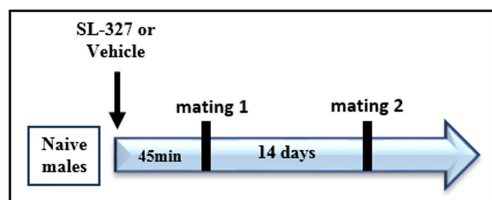
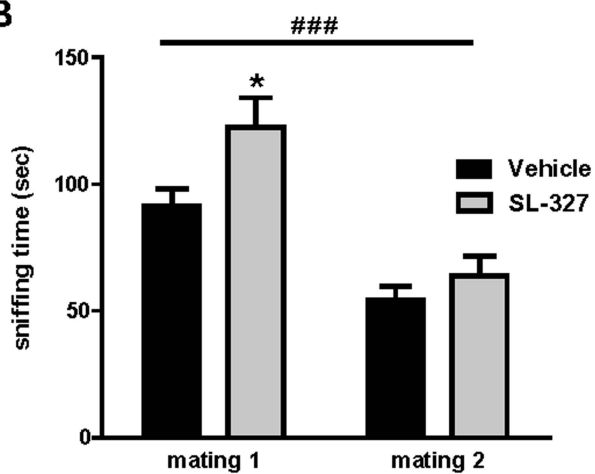
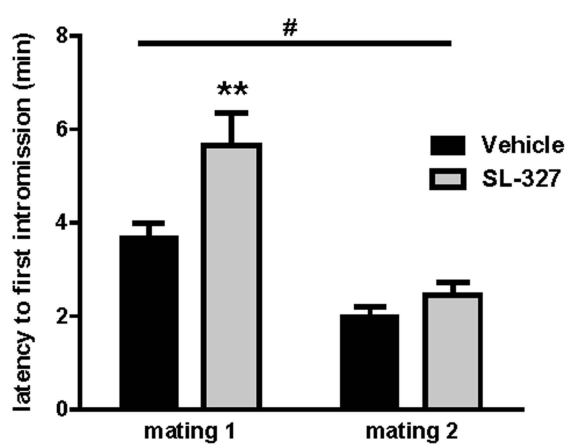
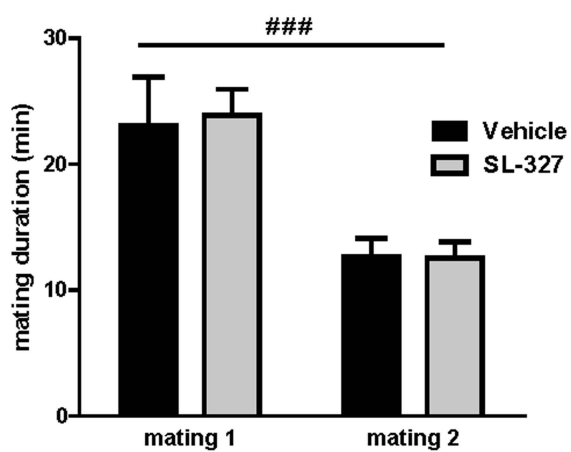
642 Locomotor activity in F and G. F: Evolution of locomotor activity during the 120-minutes test, assessed
643 by counting the number of beam interruptions every 5-minute intervals, analysed by two-way
644 repeated-measures ANOVA followed by Bonferroni post-hoc test, G: total locomotor activity, defined
645 as the total number of beam interruptions during the 120-minute test analysed by Student's test (n=11
646 in each group).

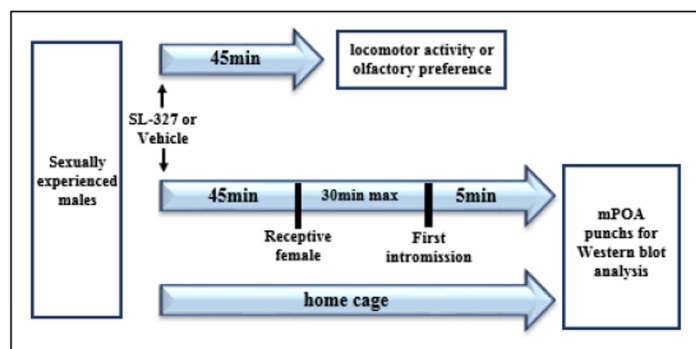
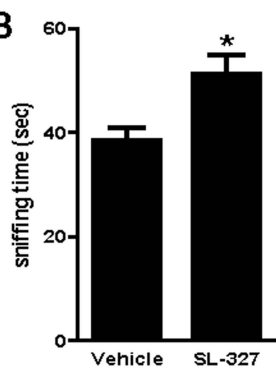
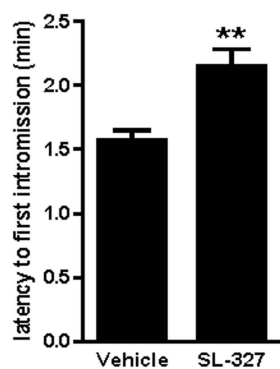
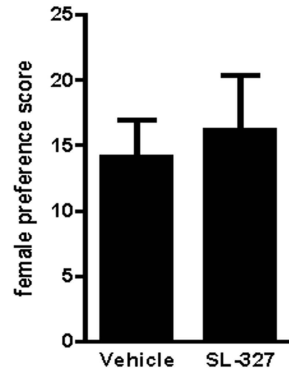
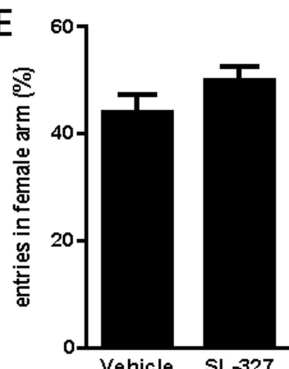
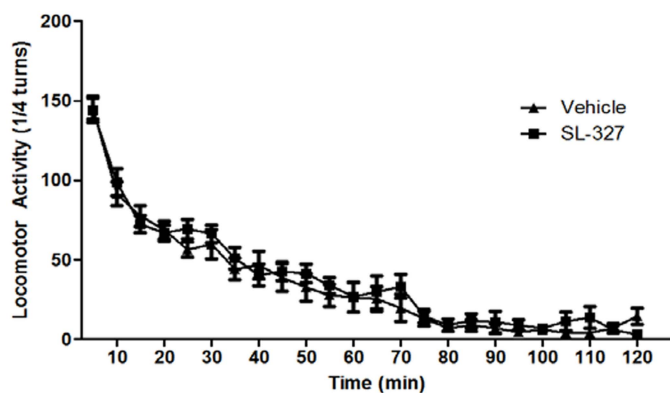
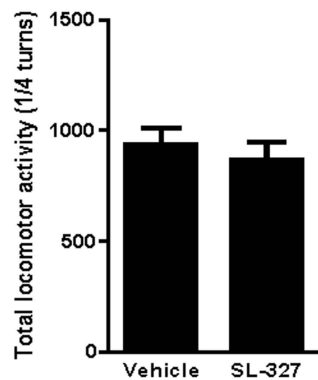
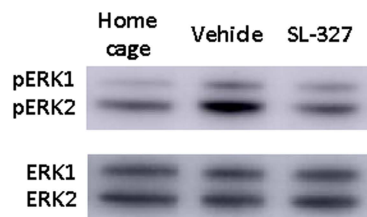
647 H: representative blot detecting pERK and ERK in the mPOA of experienced male mice kept in their
648 home cage without a female (home cage) or injected either with SL-327 (SL-327) or vehicle (vehicle),
649 put in the presence of a receptive female and sacrificed 5 minutes after the first intromission.

650 I: densitometric evaluation of the Western blot. Results (pERK/ERK) are expressed as mean percentage
651 of the home cage group \pm SEM and analysed by one-way ANOVA followed by Tukey post-hoc test (n=8
652 for vehicle and SL-327 groups, n=5 for home cage group). *p<0.05, **p<0.01 compared to home cage
653 group.

A**B**

A**B****C****D****E**

A**B****C****D**

A**B****C****D****E****F****G****H****I**