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

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Abstract

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

Key Words

- ▶ phospho-ERK
- ▶ sexual behaviour
- ▶ SL-327
- ▶ testosterone
- ▶ hypothalamic preoptic area

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Introduction

Sexual behaviour in male rodents can be described as a series of behavioural elements cumulating in ejaculation. These involve approaches and olfactory investigations of the female during the motivational phase of sexual behaviour, which is followed by bouts of mounting, intromission and then ejaculation constituting the copulatory phase. The mating behaviour is governed by complex central interactions between different systems,

which process sensory inputs, regulate rewards and integrate hormonal signals (Hull *et al.* 2002). The medial preoptic area of the hypothalamus (mPOA) represents the critical integrative site for male sexual behaviour regulation because lesions in this area are known to disrupt mounting, intromission and ejaculation in rats (De Jonge *et al.* 1989) and mounting behaviour in mice (Bean *et al.* 1981). Peripheral olfactory signals are received

in sensory neurons of the olfactory epithelium and the vomeronasal organ, processed and relayed to the main and accessory olfactory bulbs and then to the medial amygdala and in bed nucleus of the stria terminalis before finally being integrated in the mPOA (Simerly & Swanson 1986, Hull & Dominguez 2007). In turn, activation of the mPOA network results in the stimulation of premotor areas responsible for the sexual response (Simerly & Swanson 1988) and descending systems providing excitatory stimuli controlling the spinal generators for erection and ejaculation (Giuliano *et al.* 1996, Veening & Coolen 2014).

The mPOA is activated in the first moments of mating as demonstrated by c-Fos mapping of the neural networks underlying the control of sexual behaviour (Baum & Everitt 1992, Pfau & Heeb 1997). This has been confirmed by Taziaux and coworkers (Taziaux *et al.* 2011) by detecting the phosphorylated form of the mitogen-activated protein kinase/extracellular signal-regulated kinase (pERK), which was increased 10 min after exposure of sexually experienced males to female olfactory cues or after the display of coital behaviour. Hence, measuring pERK levels appeared as an accurate alternative tool to detect mPOA short-term activation as the timeline of kinase-mediated protein phosphorylation is much shorter than the induction of genomic transcription and translation of immediate early genes (IEG) (Murphy & Blenis 2006). ERK phosphorylation is the final step of an intracellular signalling cascade that participates in the regulation of various cellular processes such as proliferation and differentiation (Shaul & Seger 2007). Within the central nervous system, it plays a critical role in the modulation of synaptic plasticity, neuronal excitability, memory formation, storage and processing (Kelleher *et al.* 2004, Thomas & Huganir 2004, Shiflett & Balleine 2011a, Girault 2012), as well as in the control of reward, aversive or learning behaviour (Shiflett & Balleine 2011b, Pascoli *et al.* 2014, Goto *et al.* 2015). Among the different signals triggering the ERK pathway activation, the stimulation of hormonal receptors, namely androgen (AR) and oestrogen receptors (ER), is known to be a potent activator of ERK phosphorylation in different brain areas such as the hippocampus (Zhao & Brinton 2007, Pike *et al.* 2008) and the cerebellum (Wong *et al.* 2003) and also in other cell types in skeletal muscle (Estrada *et al.* 2003), the breast (Chia *et al.* 2011) and prostate (Liao *et al.* 2013).

Although pERK level has been shown to be increased in the mPOA of sexually experienced copulating males, currently no data are available on the hormonal

signals triggering this phosphorylation and its functional relevance.

We first focused on the role of sex steroid hormones as the mPOA highly expresses AR (Raskin *et al.* 2009), which can be activated by testosterone (T) or its metabolite dihydrotestosterone (DHT). mPOA also expresses ER α (Swaney *et al.* 2012) and ER β (Orikasa *et al.* 2002) activated by oestradiol (E2), which can be produced by neural aromatisation of T. These experiments were conducted *ex vivo* on hypothalamic slices including the mPOA. This model has been found to efficiently decipher signalling pathways as it has the advantage of maintaining the integrity of the cellular microenvironment while using various pharmacological agents (Maolood *et al.* 2008, St-Louis *et al.* 2014). In the second part of our work, we analysed the functional role of ERK phosphorylation *in vivo*. We first established that ERK is phosphorylated at the beginning of the copulatory phase. We then compared the levels of ERK phosphorylation in naive and sexually experienced mice because ERK phosphorylation is involved in memory consolidation and network potentiation (Adams & Sweatt 2002, Thomas & Huganir 2004, Davis & Laroche 2006). Indeed, although behavioural components of mating are known to be subjected to learning processes (Hull & Dominguez 2006, Swaney *et al.* 2012), the potential role of ERK phosphorylation in this improvement has never been addressed. Finally, we administrated SL-327, an inhibitor of ERK phosphorylation crossing the blood-brain barrier (Atkins *et al.* 1998, Valjent *et al.* 2000), to naive and experienced mice before the interaction with a receptive female to analyse the importance of ERK phosphorylation in conducting the various phases of sexual behaviour.

Material and methods

Animals

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

C57Bl/6J mice (Janvier Breeding Centre, Le Genest, France) were bred in our animal facility and housed under a controlled photoperiod (12-h light and 12-h darkness cycle – lights on at 02:00h) at 20 $\pm$ 2°C with food and water *ad libitum*. Ten-week-old adult males were isolated for two weeks before any behavioural experiment.

Preparation of receptive females

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

Male sexual experience

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

Effect of sex steroid hormones on ERK phosphorylation within the mPOA

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

Figure 1

Activation of ERK by testosterone and its metabolites. Brain slices including mPOA were incubated 30min in aCSF + DMSO (control), testosterone (T, 100nM), dihydrotestosterone (DHT, 100nM), oestradiol (E2, 100nM) or testosterone 3-(O-carboxymethyl)oxime-BSA (T-BSA, 100nM). (A) Representative blot detecting pERK, ERK and GAPDH in the mPOA after 30min of incubation. (B) Densitometric evaluation of the Western blots. Results (pERK/ERK) are presented as mean percentage of the control group $\pm$ s.e.m., $n=7$ in each group analysed by one-way ANOVA followed by Dunnett's multiple comparison test, * $P<0.05$ when compared to control group.

Effect of sexual experience on mating-induced ERK phosphorylation

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

Figure 2

Mating-induced ERK phosphorylation in the mPOA and sexual experience. (A) Experimental design used to analyse the effect of mating and sexual experience on ERK phosphorylation in the mPOA. N: naive male staying in home cage, N+S: naive male with sex stimulus killed 5 min after the first intromission, E: experienced male staying in home cage, E+S: experienced male with sex stimulus killed 5 min after the first intromission. Sexual behaviour expressed as (B) total anogenital sniffing time before the first intromission, (C) latency to the first intromission and (D) number of mounts for N+S and E+S males. Results are presented as mean $\pm$ S.E.M. and analysed by Student's *t* test, **P* < 0.05, ***P* < 0.01 compared to N+S group. (E) Representative blot detecting pERK, ERK and GAPDH in the mPOA for N, N+S, E and E+S males and densitometric evaluation. Results (pERK/ERK) are presented as mean percentage of the N group $\pm$ S.E.M. (*n* = 7 in each group) and analysed by two-way ANOVA followed by Bonferroni *post hoc* tests, **P* < 0.05, ****P* < 0.001, #*P* < 0.05 when compared to N+S group.

Involvement of ERK phosphorylation in the induction of sexual experience

Forty-five minutes before mating, 17 naive males received an i.p. injection of SL-327 (Sigma-Aldrich, 30 mg/kg dissolved in 5% DMSO, 5% Tween 20 and 15% polyethylene glycol 400), and 15 males received an i.p.

injection of vehicle (Fig. 3A). Each male was tested in its home cage for 10 h after the introduction of a receptive female, and male sexual behaviour was analysed (mating 1). To determine the role of pERK in sexual experience, male sexual behaviour of the same animals was also analysed 14 days later without any injection (mating 2).

Figure 3

Involvement of ERK phosphorylation in the induction of male sexual experience. (A) Experimental design used to analyse the effect of a unique administration of the ERK phosphorylation inhibitor SL-327 administration (30 mg/kg, i.p.) on sexual behaviour improvement. SL-327- and vehicle-injected groups were compared during the first and the second mating (separated by 14 days) for (B) total anogenital sniffing time before the first intromission, (C) latency to the first intromission and (D) mating duration. Results are presented as mean $\pm$ S.E.M. (*n* = 15 for vehicle group and *n* = 17 for SL-327 group) and analysed by two-way repeated measures ANOVA followed by Bonferroni *post hoc* tests. #*P* < 0.05, ****P* < 0.001 for sexual experience effect and **P* < 0.05, ***P* < 0.01 when compared to mating 1 of vehicle group.

Figure 4

Involvement of ERK phosphorylation in sexual behaviour, locomotor activity and olfactory preference of sexually experienced males. (A) Experimental design used to analyse the effect of SL-327 administration (30 mg/kg, i.p.) on sexual behaviour, locomotor activity, olfactory preference and ERK phosphorylation in the mPOA of sexually experienced males. Sexual behaviour expressed as (B) total anogenital sniffing time before the first intromission and (C) latency to the first intromission ($n=8$ in each group) presented as mean $\pm$ S.E.M. and analysed by Student's *t* test, $*P<0.05$ when compared to vehicle group. Olfactory preference in D and E. (D) Olfactory preference score defined as the time spent sniffing the female – time spent sniffing the male/total time sniffing, (E) percentage of entries in female arm analysed by Student's *t* test ($n=11$ in each group). Locomotor activity in F and G. (F) Locomotor activity during the 120-min test, assessed by counting the number of beam interruptions every 5-min intervals, analysed by two-way repeated-measures ANOVA followed by Bonferroni *post hoc* test, (G) total locomotor activity, defined as the total number of beam interruptions during the 120-min test analysed by Student's *t* test ($n=11$ in each group). (H) Representative blot detecting pERK and ERK in the mPOA of experienced male mice kept in their home cage without a female (home cage) or injected either with SL-327 (SL-327) or vehicle (vehicle), put in the presence of a receptive female and killed 5 min after the first intromission. (I) Densitometric evaluation of the Western blot. Results (pERK/ERK) are expressed as mean percentage of the home cage group $\pm$ S.E.M. and analysed by one-way ANOVA followed by Tukey *post hoc* test ($n=8$ for vehicle and SL-327 groups, $n=5$ for home cage group). $*P<0.05$, $**P<0.01$ compared to home cage group.

Involvement of ERK phosphorylation in olfactory preference of sexually experienced males

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

female arm. The maze was cleaned with 10% ethanol between trials.

Involvement of ERK phosphorylation in locomotor activity of sexually experienced males

Another group of 22 sexually experienced males were i.p. injected either with SL-327 (30 mg/kg) or vehicle (11 per group) 45 min before the test (Fig. 4A). Their locomotor activity was evaluated for 120 min using a circular corridor with four infrared beams placed at every 90° (Imetronic, Pessac, France) in a low luminosity environment as previously described (Valjent *et al.* 2006). Locomotor activity during the 120-min test was assessed by counting the number of beam interruptions every 5-min intervals. The total locomotor activity represents the number of quarters travelled during the 120-min test.

Involvement of ERK phosphorylation in the sexual behaviour of sexually experienced males

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

Western blot analysis

Punches were homogenised in 50 mM Tris (pH 7.2), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 10 mM EDTA, 10 mM EGTA, 1% Triton-X100 and 1% protease inhibitor (Roche) and sonicated 7 times during 30 s (Bioruptor Plus; Diagenode, Seraing, Belgium). After centrifugation (13 min, 15,495 g, at 4°C), protein concentrations were determined in the supernatant with a Coomassie assay (Thermo Fisher Scientific). Samples were denatured in Laemmli buffer (5 min at 95°C). 15 µg protein were subjected to electrophoresis (120 V) on 4–12% Bis-Tris NuPAGE (Thermo Fisher Scientific) and electrotransferred overnight (30 V at 4°C) onto a PVDF membrane (Millipore). Blots were blocked with 5% non-fat milk in Tris-buffered saline (TBS) and 0.2% Tween20 (2 h) and incubated overnight with primary antibodies against pERK1/2 (1:2000; polyclonal rabbit; Cell Signalling Technology), ERK1/2 (1:2000; polyclonal rabbit; Cell Signalling Technology) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase; 1:20,000; monoclonal mouse; Santa Cruz Biotechnology) diluted in the same solution. Antibodies binding to blots were detected by 2-h incubation with horseradish peroxidase anti-rabbit or anti-mouse secondary antibodies (1:10,000; Jackson ImmunoResearch). Signals were visualised with a pico-Super Signal detection kit (Thermo Fisher Scientific) with an Amersham Imager 600 (GE Healthcare Life Sciences) and quantified with ImageJ software (NIH). To determine the level of ERK activation, the phospho-ERK signal was normalised to the total ERK signal and expressed as a percentage of the control.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software). Data were expressed as mean ± S.E.M. and compared with a Student's test.

One-way ANOVA followed by Dunnett's multiple comparison test was used to analyse slice treatments. Two-way ANOVA followed by Bonferroni tests were used to analyse the effects of mating and sexual experience on ERK phosphorylation. Two-way repeated-measures ANOVA followed by Bonferroni tests were used to analyse the effects of SL-327 treatment and the number of mating on sexual behaviour and also to analyse the effects of SL-327 treatment and time on locomotor activity. *P* values of less than 0.05 were considered to be significant.

Results

Activation of ERK by testosterone and its metabolites

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

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

Mating-induced ERK phosphorylation in the mPOA is increased by sexual experience

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

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

Two-way ANOVA demonstrated a significant effect of mating ($F_{(1-24)}=30.4$, $P<0.001$) and sexual experience ($F_{(1-24)}=7.28$, $P<0.05$) but no interaction between these main effects ($F_{(1-24)}=1.78$, $P>0.05$) on ERK phosphorylation (Fig. 2E). *Post hoc* analyses showed that pERK level was increased for N+S males killed 5 min after the first intromission (N+S: 241 ± 26.9 , $P<0.05$), but was unchanged in the control experienced group (E: 146 ± 33.9) when both were compared to the control naive group (N: 100 ± 27.9). By contrast, ERK phosphorylation was increased in the E+S group when compared to the N+S group (E+S: 378 ± 44 , $P<0.05$). The changes in pERK levels were independent of any variation of total ERK protein normalised to the amount of GAPDH, demonstrating a specific regulation on ERK phosphorylation rather than on its protein level.

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

Involvement of ERK phosphorylation in the regulation of male sexual behaviour

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

Two-way repeated measures ANOVA showed a significant effect of SL-327 treatment on the time 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 experience was also observed on the time spent in anogenital sniffing ($F_{(1-30)}=39.87$, $P<0.0001$), the latency to first intromission

($F_{(1-30)}=5.78$, $P<0.05$) and the mating duration ($F_{(1-30)}=29.05$, $P<0.0001$). SL-327 treatment induced a significant increase by 34% ($P<0.01$) of the time spent in anogenital sniffing and a 53% increase ($P<0.05$) of the latency to first intromission for SL-327 treated males compared to the vehicle group in the first mating test.

These behavioural differences were not observed during the second mating test for the sniffing 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 mating duration (12.7 ± 1.38 vs 12.5 ± 1.31). This indicates that the SL-327 treatment transiently altered sexual behaviour but did not interfere with sexual improvement assessed two weeks later.

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

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

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

For olfactory preference, tested males were given a choice between an anaesthetised receptive female and an anaesthetised intact male. The total time spent in chemo-investigation was similar between the groups (221 ± 6.8 s vs 242 ± 11.2 s). The percentage of entries in the female arm (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 administration (Fig. 4D and E).

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

Phospho-ERK inhibition by SL-327

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

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

Discussion

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

We first confirmed the rapid activation of ERK phosphorylation in the early phases of copulation. pERK was increased 5 min after the first intromission in both naive and experienced males. Given the ability of steroid hormones to induce its phosphorylation (Wong *et al.* 2003, Abrahám *et al.* 2004, Pike *et al.* 2008, Liao *et al.* 2013) and the increased circulating levels of testosterone (T) during an interaction with a female (Batty 1978, Coquelin & Desjardins 1982), we thus investigated whether T or its metabolites were able to

induce ERK phosphorylation within the mPOA. Thirty-minute treatment of hypothalamic slices by T was found to induce ERK activation. By contrast, treatment with the plasma membrane-impermeable T-BSA was inefficient, thereby eliminating a potential membrane-dependent signalling pathway. Furthermore, DHT treatment reproduced T activation of ERK, suggesting the involvement of an intracellular AR-dependent mechanism in agreement with previously described regulation of ERK phosphorylation by the intracellular AR in hippocampal neurons (Nguyen *et al.* 2005). T-induced effects were also mimicked by E2 treatment, evidencing the participation of ER in steroid-mediated ERK phosphorylation. The possibility that steroids might use either the AR or the ER pathway to mediate the same effect has already been described in the male rat namely to restore a complete sexual behaviour after castration (Attila *et al.* 2010). Finally, the rapid ERK activation (30 min) suggests a rapid non-genomic action of sex steroids in the mPOA. In this way, it is now well established that cytoplasmic ER or AR can rapidly activate members of the ERK signalling cascade, via direct interaction with the non-tyrosine kinase receptor Src (Migliaccio *et al.* 2000). Of interest, sex steroids can also activate members of the ERK pathway in hippocampal and cerebellar neurons in less than 15 min (Wong *et al.* 2003, Nguyen *et al.* 2005).

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

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

Interestingly, mating-induced ERK phosphorylation was more elevated in sexually experienced males who are supposed to be more sensitive to androgen as they display

elevated levels of circulating testosterone and higher AR levels in the mPOA (Swaney *et al.* 2012). They also show an increased number of copulation-induced c-Fos-positive neurons in their mPOA (Lumley & Hull 1999). As we found that T and its metabolites could induce a rapid activation of the MAPK pathway, the increased ability of experienced males to phosphorylate ERK could thus be explained by a higher sensitivity of the mPOA to steroids.

To assess the functional role of ERK phosphorylation in sexual behaviour, males were treated with SL-327, a brain-penetrating selective inhibitor of MAP kinase/ERK kinase (MEK1/2), the enzyme which selectively activates ERK (Selcher *et al.* 1999). Naive males treated with SL-327 showed decreased sexual motivation without altering the copulatory phase as mating duration and copulatory patterns remained similar to the vehicle group. These results are consistent with those obtained by Niessen and coworkers (Niessen *et al.* 2013) in which c-Fos inhibition in the mPOA resulted in the reduction of appetitive male sexual behaviour in the quail. In experienced males treated with SL-327, similar results were observed: anogenital sniffing time and latency to the first intromission were increased compared to experienced males treated with the vehicle. The increase observed in the motivational phase length could be considered specific and not linked to a side effect of the SL-327 treatment as locomotion was not altered. The rapid non-genomic ERK activation mediated by steroids that we exhibited *ex vivo* could be one of the mechanisms involved in mating-induced ERK activation as sexual motivation was shown to be partly regulated by rapid non-classical actions of steroids in the quail (Seredynski *et al.* 2013).

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

To summarise, our results show that ERK phosphorylation probably took place during the motivational phase, that experienced males, who are more motivated than naive males, have an increased level of phosphorylation and that inhibition of ERK

phosphorylation decreased sexual motivation. These results strongly suggest that ERK phosphorylation in the mPOA could be considered as a key player in the motivational signalling pathway transduction and as an index of sexual motivation.

Our results also revealed new aspects regarding the mechanisms leading to olfactory preference as it was not modified by SL-327 treatment although exposure of a male to an oestrous female was found to activate the ERK pathway in the main and accessory olfactory bulb and in the amygdala (Taziaux *et al.* 2011). These data may be linked to the observation that pheromone stimulation of a male mouse causes ERK activation in the vomeronasal organ, in amygdala and in the hypothalamus but not in the accessory olfactory bulb (Dudley *et al.* 2001). Furthermore, even if olfactory interactions are important for the copulatory behaviour (Keller *et al.* 2006), several other neuronal pathways coming from BST, septum and other areas converging on the mPOA (Dhungel *et al.* 2011) are activated when a male is placed in front of a receptive female (Pfaus & Heeb 1997). These results are also consistent with data reported by Satoh and coworkers (Satoh *et al.* 2011) showing that mice lacking ERK2 in the brain have deficits in social interaction without olfactory defect. Taken together, these observations indicate that ERK phosphorylation is not essential to induce olfactory preference. Furthermore, it suggests that the mechanisms controlling the motivational phase and the mPOA activation are integrated downstream the olfactory bulb.

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

Our results could represent an interesting tool for the investigation of the effects of hormonomimetic molecules able to interfere with the control of sexual behaviour, such as endocrine disruptors. Furthermore, the ability to rapidly activate the ERK pathway on an *ex vivo* model

of mPOA slices could also be a very useful tool to help decipher the negative effects of such molecules perturbing the signalling pathways of steroid hormones.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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