Rapid activation of phosphorylated mitogen-activated protein kinase after sexual stimulation in male mice

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We mapped cells immunoreactive for the phosphorylated form (p44/p42) of the mitogen-activated protein kinase (pMAPK – also known as ERK1/2) in the brain of male mice after exposure to female olfactory cues or after the display of male copulatory behaviors. Exposure to soiled bedding from estrous females or the display of coital behaviors rapidly (within 10 min) induced MAPK phosphorylation in most of the brain regions known to be involved in the processing of olfactory cues (main and accessory olfactory bulbs, amygdala, and medial preoptic area) and in the control of copulatory behavior (amygdala and medial preoptic area). MAPK phosphorylation thus seems to be a useful marker to study short-term neural activation associated with the expression of specific behaviors. ***NeuroReport***

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Introduction

The mapping of neural networks underlying the control of sexual behavior has almost exclusively been achieved by analyzing the induction of immediate early genes (IEG) after the display of male or female sexual behaviors [1]. Although the protein products of IEG like *c-fos* or *zif268* have become common tools for mapping functional activity in the brain, it is still difficult to link neuronal induction with precise behavioral parameters (e.g. frequencies of specific behaviors) mostly because it takes at least 1 h [2,3] to obtain sufficient IEG protein induction for immunohistochemical detection. In contrast with IEG, the timeline of kinase-dependent protein phosphorylations is much shorter as this process only involves one enzymatic activity as opposed to the induction of genomic transcription and translation [4,5]. Thus, the phosphorylation state of a signaling enzyme or inducible transcription factors might be a better tool for mapping neuronal activity as it allows much shorter stimulation periods than what is possible with IEG. Therefore, in this study, we used the phosphorylated form of the mitogen-activated protein kinase (pMAPK) as a marker of neuronal activity after reproductively related olfactory stimulation and coital behavior in male mice. These results indicate that MAPK phosphorylation is affected in an anatomically specific manner during the processing of olfactory stimuli and the display of coital behavior, suggesting that pMAPK represents an excellent marker to map functional pathways associated with brain activation in a reproductive context.

Materials and methods

Mice and general procedure

Gonadally intact male mice from the *C57Bl/6J* strain were used. All experimental mice were housed alone under a reversed light/dark (12 h LD) cycle. Food and water were available *ad libitum*. All experiments were conducted in accordance with the guidelines set forth by the National Institutes of Health Guiding Principles for the Care and Use of Research Animals and were approved by the Ethics Committee for Animal Use of the University of Liege. Stimulus females were ovariectomized in adulthood and implanted subcutaneously with a Silastic capsule (inner diameter, 1.57 mm; outer diameter, 2.41 mm; length, 5 mm) containing crystalline estradiol-17β (E_2) diluted 1:1 with cholesterol. Three hours before testing, stimulus females were given a subcutaneous injection of 500 µg progesterone (P). This treatment induces high levels of sexual receptivity in female mice [6].

Preparation of odor stimuli – soiled bedding

A group of female mice ($n = 18$) that were ovariectomized and treated with E_2 and P to induce behavioral estrous were placed in clean cages containing fresh sawdust. Bedding was collected 10 h later. All bedding was stored in plastic freezer bags at −80°C until used in the experiment.

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Experimental procedures

MAPK phosphorylation after exposure to female olfactory cues

Thirteen sexually experienced male mice were used in this experiment. Twenty-four hours before odor exposure, all mice were placed on clean sawdust and were moved to a different animal room containing no female mice. On the day of odor exposure, male mice were exposed to estrous female bedding ($n = 7$) or clean bedding ($n = 6$) and brains were collected 10 min later.

MAPK phosphorylation after male copulatory behavior

Twenty-one sexually experienced male mice were exposed to a sexually receptive female ($n = 18$) or left in their homecage ($n = 3$). Brains were collected 10 min later.

Immunohistochemistry

The mice were decapitated and the brain was dissected out of the skull. The brains were placed in acrolein (5% in Tris buffer 0.05 M-saline 0.9%, pH 7.6, TBS) for 2.5 h, washed twice in TBS (30 min) and cryoprotected in 30% sucrose for 24 h at 4°C. The brains were then frozen on dry ice and stored at −80°C until used. Olfactory bulbs and forebrains were cut in the coronal plane at −20°C in three series of 30 µm thick sections. One series of sections was stained by immunohistochemistry for pMAPK. All incubations were carried out at room temperature, unless otherwise mentioned, and all washings were carried out using TBS. In brief, sections were incubated sequentially in $NaBH_4$ to inactivate the aldehydes (15 min), in 5% normal goat serum to decrease nonspecific binding (1 h), and overnight at 4°C in the primary antibody (rabbit polyclonal anti-p44/42 MAPK antibody; Cell Signaling Technology, #9101; 1 : 500 in TBST/2% NGS). They were then incubated for 1 hour in a goat antirabbit biotinylated antibody (Dako Cytomation, Denmark; 1 : 400 in TBST/2% NGS), and in H_2O_2 (0.6%) to eliminate endogenous peroxidase (20 min). The antibody–antigen complex was localized by the avidin–biotin complex method (Kit ABC Vectastain Elite PK-6100, Vector Laboratories PLC, Cambridge, UK). Finally, the peroxidase was visualized with diaminobenzidine. Sections were mounted on microscope slides in a gelatin-based medium and coverslipped.

Data analysis

Number of pMAPK-immunoreactive (pMAPK-ir) cells were counted in several brain areas implicated in olfactory processing, both the accessory and the main olfactory pathways and in brain areas known to be implicated in the control of male sexual behavior. These areas in which p-MAPK expression was quantified are schematically shown in Fig. 1. Number of pMAPK-ir cells were quantified on both sides of the section and summed by an experimenter (MT) who was blind to the experimental treatments using computer-assisted image analysis. Sections were digitized through a video camera attached to the microscope (× 40 objective for olfactory bulb areas, × 20 objective for all other brain areas) and pMAPK-ir cells were counted with a Macintosh-based image analysis system using the particle counting protocol of the NIH Image program (1.62). Digital images were made binary and manual threshold was used for discriminating the labeled material from the background. At × 20 objective, exclusion thresholds were set at 20 (low threshold) and 200 (high threshold) pixels to remove from the counts dark objects that did not have the size of a cell nucleus (thresholds at 50 and 500, respectively, for the × 40 objective used for analyzing sections through the olfactory bulbs). Brain structures were identified based on the mouse brain atlas [7] and pMAPK-ir cells were measured in one entire field (computer screen; length: 0.536 mm; height: 0.360 mm = 0.193 mm^2; field placed in landscape position in each case) that was placed in a standardized manner based on predefined anatomical landmarks in the sections (e.g. edge of ventricles or prominent fiber tracts).

Statistical analysis

Student *t*-tests were used to analyze differences in the number of pMAPK-ir cells after exposure to clean or estrous bedding. Owing to a rather large number of males ($n = 9$) exposed to a receptive female displayed only anogenital investigations (AGI) and performed no mounts and no intromissions, we analyzed these data based on the type of sexual stimulation received and behavior shown by the mice [males which stayed in their home cage (HC) vs. males which displayed only AGI vs. males which copulated with the female (SEX)]. One-way ANOVAs with the type of sexual stimulation (HC vs. AGI vs. SEX) as independent factors and number of pMAPK-ir cells as dependent factor were used to analyze the overall differences in pMAPK immunoreactivity in each area analyzed. All ANOVAs were followed when appropriate by *post hoc* tests using Fisher's protected least significant difference tests. Differences were considered significant for P value of less than 0.05.

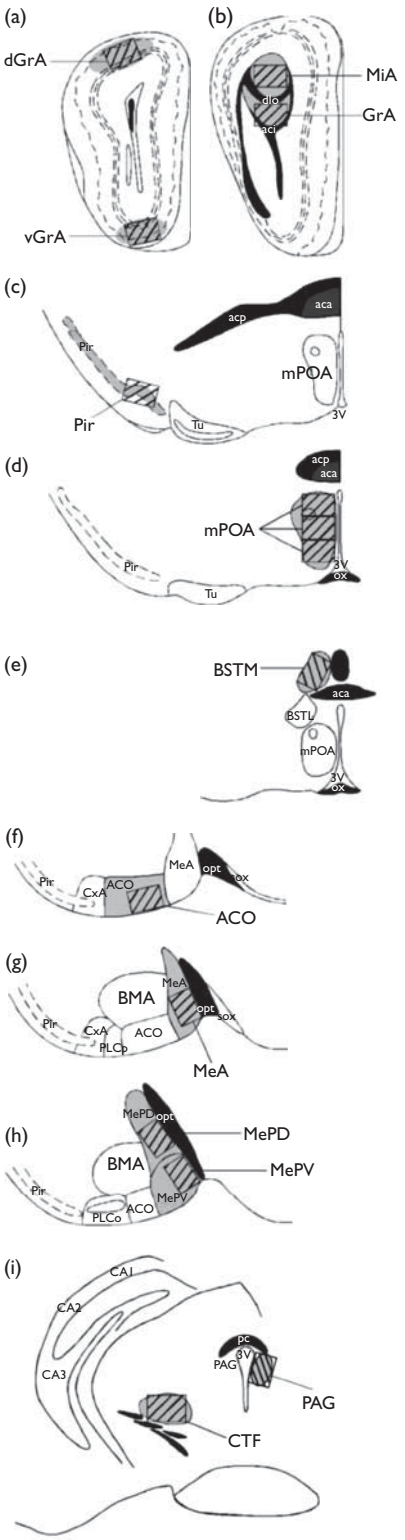
Results

MAPK phosphorylation after exposure to female olfactory cues

Overall, exposure to estrous female bedding induced a significant pMAPK expression in brain regions previously known to be involved in olfactory processing compared with exposure to clean bedding (Fig. 2a). This was confirmed by *t*-tests showing that exposure to estrous female bedding induced significantly more pMAPK expression than clean bedding in both the granular and the mitral cell layer of the accessory olfactory bulb (AOB) [granular cell layer: $t_{(11)} = 4.818$, $P = 0.0005$; mitral cell layer: $t_{(11)} = 3.042$, $P = 0.0112$], both the dorsal and the ventral part of the granular layer of the main olfactory bulb (MOB) [dorsal part: $t_{(11)} = 4.233$, $P = 0.0014$; ventral part: $t_{(11)} = 2.707$, $P = 0.0204$], postero-dorsal part of the medial amygdala (MePD) [$t_{(11)} = 3.788$, $P = 0.0030$], and postero-ventral

part of the medial amygdala (MePV) [$t_{(11)} = 4.525$, $P = 0.0009$]. In the anterior part of the medial amygdala (MeA) and medial preoptic area (mPOA), males exposed to estrous female bedding displayed a higher number of pMAPK-ir cells compared with males exposed to clean bedding but this difference did not reach statistical significance [MeA: $t_{(11)} = 1.450$, $P = 0.1750$; mPOA: $t_{(11)} = 1.929$, $P = 0.0799$]. Representative photomicrographs showing these effects in the AOB and MePV are shown in Fig. 2b.

No significant effects of estrous female bedding exposure were observed in medial part of the bed nucleus of the stria terminalis (BSTM), piriform cortex, and anterior cortical amygdaloid nucleus ($P > 0.5$; data not shown).



MAPK phosphorylation after expression of male copulatory behavior

Differential pMAPK expression after exposure to a sexually receptive female was observed in four specific brain areas, including the mPOA, MeA, MePV, and MePD. Furthermore, the pattern of pMAPK expression in each of these fourth areas was related to the amount of sexual stimulation received (Fig. 3a). One-way ANOVAs showed a significant effect of the type of sexual stimulation in the mPOA ($F_{2,18} = 6.702$, $P = 0.0067$), MeA ($F_{2,18} = 6.838$, $P = 0.0062$), MePV ($F_{2,18} = 3.972$, $P = 0.0373$), and MePD ($F_{2,18} = 7.141$, $P = 0.0052$). In the mPOA and MeA, *post hoc* tests showed that males expressing copulatory behavior showed an enhanced pMAPK expression compared with males that only displayed AGI ($P < 0.05$), and with males that stayed in their homecage ($P < 0.01$). In the MePV, *post hoc* testing showed that only mice expressing copulatory behavior showed a level of pMAPK induction significantly higher compared with the levels found in HC ($P = 0.0147$) but not in AGI ($P = 0.1030$). Males in the AGI condition did not significantly differ from HC mice ($P = 0.1554$). Finally, in the MePD, *post hoc* testing showed that mice expressing copulatory behavior or AGI showed a level of pMAPK induction significantly higher compared with the levels found in HC mice ($P < 0.05$). SEX males displayed a greater number of pMAPK-ir cells in the MePD than AGI mice, but this difference fell short of significance

Fig. 1

Schematic drawings of coronal sections through the mouse brain showing the areas in which pMAPK expression was quantified (hatched areas). Sections (a) through (i) are shown in a rostral to caudal order. aca, anterior commissure, anterior part; aci, anterior commissure, intrabulbar part; acp, anterior commissure, posterior nerve; ACo, anterior cortical amygdaloid nucleus; BMA, anterior part of the basomedial amygdala; BSTL, bed nucleus of the stria terminalis, lateral part; BSTM, bed nucleus of the stria terminalis, medial part; CXA, cortex-amygdala transition; CA1, field CA1 hippocampus; CA2, field CA2 hippocampus; CA3, field CA3 hippocampus; CTF, central tegmental field; dGrA, granular layer of the main olfactory bulb, dorsal part; dlo, dorso-lateral olfactory tract; GrA, granular layer of the accessory olfactory bulb; MeA, anterior part of the medial amygdala; MePD, postero-dorsal part of the medial amygdala; mPOA, medial preoptic area; MePV, postero-ventral part of the medial amygdala; MiA, mitral layer of the accessory olfactory bulb; opt, optic tract; ox, optic chiasm; PAG, periaqueductal grey; pc, posterior commissure; Pir, piriform cortex; PLCo, posterolateral cortical amygdala; sox, supraoptic decussation; Tu, olfactory tubercle; vGrA, granular part of the main olfactory bulb, ventral part; 3V, third ventricle.

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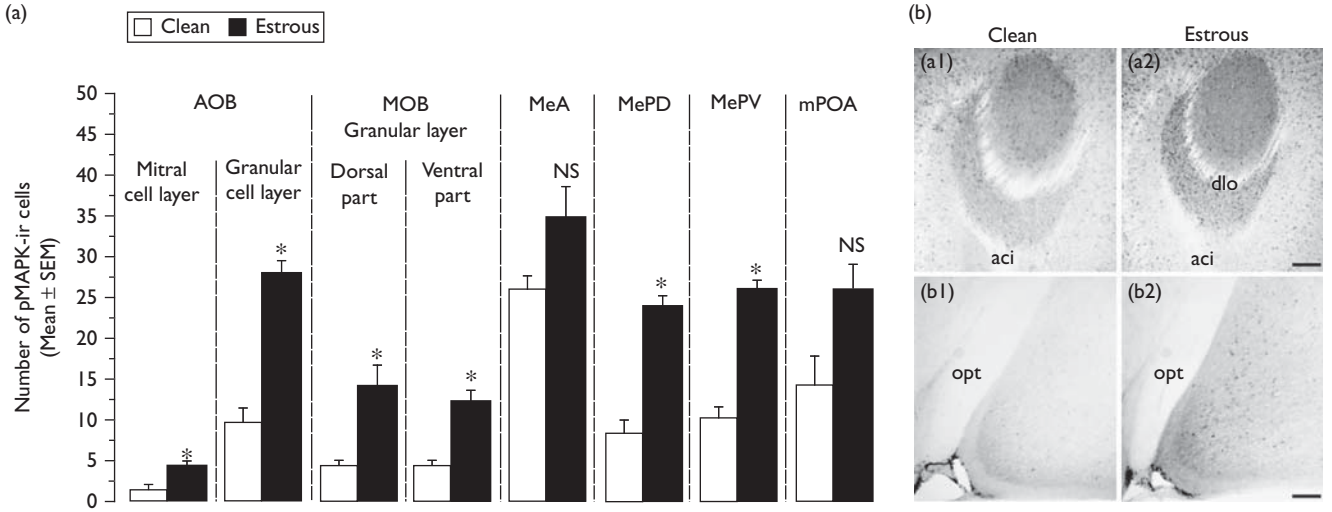
Fig. 2



(a) Number of phosphorylated mitogen-activated protein kinase-immunoreactive (pMAPK-ir) cells in male mice that had been exposed either to a clean bedding ($n=6$) or to estrous female bedding ($n=7$) in the mitral and granular cell layer of the AOB, dorsal, and the ventral part of the granular layer of the MOB, anterior part of the medial amygdala (MeA), postero-dorsal part of the medial amygdala (MePD), postero-ventral part of the medial amygdala (MePV), and medial preoptic area (mPOA). *$P<0.05$; NS, not significant. (b) Photomicrographs (× 10 objective) showing pMAPK expression in the AOB (a1–2) and MePV (b1–2). Scale bars: 100 μm.

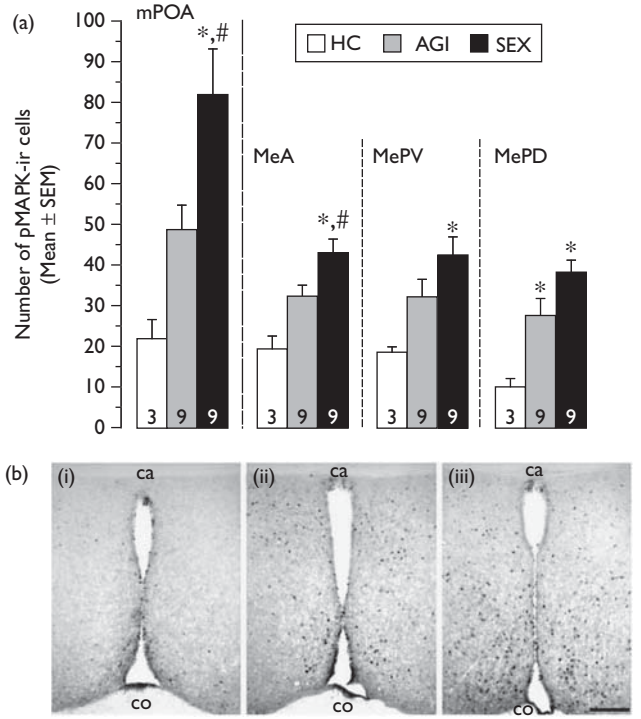
($P = 0.0562$). Representative photomicrographs showing these effects in the mPOA are presented in Fig. 3b. No significant effects of male coital behavior were observed in the BSTM, piriform cortex, central tegmental field, and periaqueductal grey ($P > 0.2$; data not shown). We also analyzed whether there was a positive correlation between the number of mounts and intromissions and the mean number of pMAPK-ir cells counted per section in each of the four brain areas (mPOA, MeA, MePV and, MePD) in which significant effects of the test situation had been detected. These multiple regression analyses identified a significant positive linear correlation between the number of intromissions and the number of pMAPK-ir cells in the mPOA ($r = 0.812$, $P = 0.0185$, $n = 18$).

Discussion

Protein kinase-mediated signaling cascades play a fundamental role in translating extracellular signals into neuronal responses in the central nervous system [8]. MAPK is rapidly (within minutes or even seconds) phosphorylated in neurons that are activated and that use this signaling pathway, making the phosphoryation of this protein potentially very useful for identifying after a short delay neurons that are involved in organizing behavioral responses. In this study we showed a rapid increase (within 10 min) of pMAPK-positive cells in response to sexual stimuli in the male mouse brain. First, exposure to estrous female odors rapidly induced the phosphorylation of MAPK in brain regions known to be involved in the processing of olfactory cues, namely the MOB and the AOB and their target sites including the MePV, and MePD. These results are consistent with previous studies showing that female olfactory cues induce *c-fos* expression in the AOB and MOB, MeA, MePD, MePV, BSTM, and mPOA in male mouse brain [9–11]. A similar pattern of *c-fos* activation in response to sex-related odors was found in male rats [12–15], hamsters [16–18], and gerbils [19]. To our knowledge, only one study has investigated in a qualitative way the phosphorylation of MAPK after exposure to sex-related odors [20] and found a nearly similar pattern of pMAPK activation after exposure to estrous female bedding in male mice.

Copulation increased Fos expression in the mPOA, BSTM, MeA, and the central tegmental field in male rats [13,15,21,22], mice [10], hamsters [16–18,23], and gerbils [19]. These brain areas act in concert to regulate male sexual behavior [24]. Previous studies have also shown differences in neural activation after AGI versus copulation in rats [13,21]. We report here for the first time pMAPK induction after the expression of male copulatory behavior in brain regions previously associated with the control of male sexual behavior, that is, the MeA, MePD, MePV, and mPOA. The observed neuronal activation after expression of copulatory behavior seems to relate specifically to the exposure to the female and/or to the performance of copulatory behavior. Support for this interpretation comes from the following two observations: (i) the number of pMAPK-ir cells in the mPOA is significantly correlated with the number of intromissions they carried out during the final behavioral test; and (ii) the increase in the number of pMAPK-ir cells is directly proportional to the amount of sexual behavior displayed by males.

Fig. 3



(a) Number of phosphorylated mitogen-activated protein kinase-immunoreactive (pMAPK-ir) cells in the medial preoptic area (mPOA), anterior part of the medial amygdala (MeA), postero-ventral part of the medial amygdala (MePV), and postero-dorsal part of the medial amygdala (MePD) of male mice that copulated with a female (SEX group), displayed only anogenital investigation (AGI group) or stayed in their home cage (HC group). $^{*}P<0.05$ vs. HC group; $^{\#}P<0.05$ vs. AGI group. (b) Photomicrographs (× 10 objective) showing pMAPK expression in the mPOA of HC (i), AGI (ii) or SEX (iii) male mice. Scale bar: 100 μm.

Here we also show that the phosphorylation of MAPK is detectable and statistically significant only 10 min after the beginning of sexual stimulation whereas a detectable increase in the protein product of IEG like *c-fos* or *zif268* only culminates 90 min after exposure to a given stimulus [2]. The choice of MAPK phosphorylation for tracking changes of neural activation thus allows a better temporal resolution than IEGs linking the expression of a certain behavior more closely to neural activation.

Conclusion

This study indicates that the assessment by immunohistochemistry of the phosphorylation state of individual components of signal transduction cascades, such as MAPK, provides a powerful approach to identify early consequences of sexual stimulation with a high degree of anatomical resolution.

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