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Rapid activation of phosphorylated MAPK after sexual stimulation in male mice

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Abstract

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 following 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 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 appears thus to be a useful marker to study short-term neural activation associated with the expression of specific behaviors.

Keywords

Neuronal activation; MAPK phosphorylation; Olfaction; Copulatory behavior; Amygdala

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) following 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 one hour [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 since this process only involves one enzymatic activity as opposed to the induction of genomic transcription

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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 since it allows much shorter stimulation periods than what is possible with IEG. Therefore, in the present study, we used the phosphorylated form of the mitogen-activated protein kinase (pMAPK) as a marker of neuronal activity following reproductively-related olfactory stimulation and coital behavior in male mice. Our 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.

Methods

Subjects 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 Ethical Committee for Animal Use of the University of Liège. Stimulus females were ovariectomized (OVX) in adulthood and implanted subcutaneously with a Silastic^{TM} capsule (inner diameter, 1.57 mm; outer diameter, 2.41 mm; length, 5 mm) containing crystalline estradiol-17 β (E₂) diluted 1:1 with cholesterol. Three hours before testing, stimulus females were given a subcutaneous injection with 500 μ g progesterone (P). This treatment induces high levels of sexual receptivity in female mice [6].

Preparation of odor stimuli – soiled bedding

A group of females ($n = 18$) that were OVX and treated with E₂ and P to induce behavioral estrus, 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.

Experimental procedures

MAPK phosphorylation following exposure to female olfactory cues—Thirteen sexually experienced male mice were used in this experiment. Twenty-four hours prior to odor exposure, all mice were placed on clean sawdust and were moved to a different animal room containing no females. 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 following 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

Subjects were decapitated and the brain was dissected out of the skull. The brains were placed into acrolein (5% in Tris buffer 0.05 M-saline 0.9%, pH 7.6, TBS) for 2.5 hours, 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 3 series of 30 μ m 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 washes were performed using TBS. Briefly, sections were incubated sequentially in NaBH₄ to inactivate the aldehydes (15 min), in 5% normal goat serum to decrease non specific binding (1h), 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 one hour in a goat-anti-rabbit 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

Numbers 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 where p-MAPK expression was quantified are schematically illustrated in Fig. 1. Numbers 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 mm²; 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. Because a rather large number of males (n=9) exposed to a receptive female displayed only ano-genital investigations and performed no mounts and no intromissions, we analyzed these data based on the type of sexual stimulation received and behavior performed by the subjects (males which stayed in their home cage (HC) vs. males which displayed only ano-genital investigations (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 (PLSD) tests. Differences were considered significant for $p < 0.05$.

Results

MAPK phosphorylation following 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 to exposure to clean bedding (Fig. 2.1). This was confirmed by t-tests revealing 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 : $t_{(11)} = 2.707$, $p = 0.0204$), MePD ($t_{(11)} = 3.788$, $p = 0.0030$) and MePV ($t_{(11)} = 4.525$, $p = 0.0009$). In the MeA and mPOA, males exposed to estrous female bedding displayed a higher number of pMAPK-ir cells compared to 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 illustrating these effects in the AOB and MePV are presented in Fig. 2.2. No significant effects of estrous female bedding exposure were observed in the BSTM, PIR and ACo ($p > 0.5$; data not shown).

MAPK phosphorylation following 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 four areas was related to the amount of sexual stimulation received (Fig. 3.1). One-way ANOVAs revealed 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 demonstrated that males expressing copulatory behavior showed an enhanced pMAPK expression compared to males that only displayed ano-genital investigations ($p < 0.05$), and to males that stayed in their homecage ($p < 0.01$). In the MePV, *post hoc* testing revealed that only subjects expressing copulatory behavior showed a level of pMAPK induction significantly higher compared to 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 subjects ($p = 0.1554$). Finally, in the MePD, *post hoc* testing revealed that subjects expressing copulatory behavior or ano-genital investigations showed a level of pMAPK induction significantly higher compared to levels found in HC subjects ($p < 0.05$). SEX males displayed a greater number of pMAPK-ir cells in the MePD than AGI subjects, but this difference fell short of significance ($p = 0.0562$). Representative photomicrographs illustrating these effects in the mPOA are presented in Fig. 3, 2. No significant effects of male coital behavior were observed in the BSTM, PIR, CTF and PAG ($p > 0.2$; data not shown). We also analyzed whether there was a positive correlation between the number of mounts and intromissions and the mean numbers of pMAPK-ir cells counted per section in each of the four brain areas (mPOA, MeA, MePV, MePD) where 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 utilize this signaling pathway, making the phosphorylation 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. Our 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 following exposure 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 CTF 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 demonstrated differences in neural activation following anogenital investigation versus copulation in rats [13,21]. We report here for the first time pMAPK induction following the expression of male copulatory behavior in brain regions previously associated with the control of male sexual behavior, i.e. the MeA, MePD, MePV and mPOA. The observed neuronal activation following expression of copulatory behavior appears to relate specifically to the exposure to the female and/or to the performance of copulatory behavior. Support for this interpretation comes from the two following observations: (a) the number of pMAPK-ir cells in the mPOA is significantly correlated with the number of intromissions they performed during the final behavioral test; and (b) the increase in the number of pMAPK-ir cells is directly proportional to the amount of sexual behavior displayed by males.

We also demonstrate here 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

The present 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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Fig. 1.

Schematic drawings of coronal sections through the mouse brain illustrating the areas where pMAPK expression was quantified (hatched areas). Sections A through I are presented 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 accessory olfactory bulb; 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.

Fig. 2.

(1) Number of 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, MeA, MePD, MePV and mPOA. * $p < 0.05$; ns : not significant. (2) Photomicrographs (10x objective) illustrating pMAPK expression in the AOB (A1–2) and MePV (B1–2). Scale bars: 100 µm.

Fig. 3.

(1) Number of pMAPK-ir cells in the mPOA, MeA, MePV, MePD of male mice that copulated with a female (SEX group), displayed only ano-genital investigation (AGI group) or stayed in their home cage (HC group). * $p < 0.05$ vs. HC group; # $p < 0.05$ vs. AGI group. (2) Photomicrographs (10x objective) illustrating pMAPK expression in the mPOA of HC (A), AGI (B) or SEX (C) male mice. Scale bar: 100 μm.