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Repeated Swim-Stress Induces Kappa Opioid-Mediated Activation of ERK1/2 MAPK

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Abstract

Previous studies identified the dynorphin-kappa opioid receptor (KOR) system as a critical mediator of dysphoria-induced aversion following repeated stress exposure, but the molecular signaling mechanisms were not fully characterized. In this study we report that repeated forced swim-stress caused a significant phosphorylation of ERK1/2 MAPK in both the caudate and nucleus accumbens regions of the mouse striatum. Activation was blocked by the KOR antagonist, norbinaltorphimine, and absent in KOR knockout mice. In contrast to p38-MAPK activation by stress-induced dynorphin release, KOR-mediated ERK1/2 phosphorylation was not dependent on G-protein coupled receptor kinase 3 expression. These results indicate stress-induced activation of the dynorphin-KOR systems activates ERK1/2 MAPK signaling, and this may contribute to the behavioral responses to repeated stress exposure.

Keywords

dynorphin; β -arrestin-dependent signaling; forced swim; stress-induced immobility; ERK1/2 MAPK; Kappa opioid receptor; G-protein coupled receptor kinase

Introduction

Repeated stress exposure causes adverse effects by increasing the risk of depression, inducing drug seeking behavior, and stimulating drug craving in drug-dependent humans and rodents [1–4]. Recent studies suggest that the dynorphin-kappa opioid system is one of the principal mediators of this complex behavioral response [1,3–8]. Repeated forced swim, repeated social defeat and prior administration of U50,488 each strongly potentiated the conditioned place preference response to cocaine in a kappa opioid receptor (KOR)-dependent manner [3,4]. The kappa opioid system is comprised of prodynorphin peptide products and KOR [9]. Agonist stimulation of KOR leads to inhibition of adenylate cyclase, activation of potassium channels and inhibition of calcium channels, typical of $G_{i/o}$ coupled receptors. Using *in vitro* approaches, kappa agonists have also been shown to activate all three MAPK family members including the extracellular signal-regulated kinase (ERK1/2), the stress kinase c-Jun N-terminal kinase (JNK), and the stress kinase p38 [5,10–13]. KOR-mediated p38 MAPK activation requires receptor phosphorylation by G-protein coupled receptor kinase 3 and β -arrestin recruitment *in vivo* and *in vitro* [5,11]. KOR-mediated ERK1/2 activation *in vitro* is G_i -mediated and dependent on phosphoinositide 3-kinase/PKC signaling [10,14]; however ERK1/2 activation by the dynorphin/KOR system in brain has not been previously demonstrated. Activation of the ERK1/2 MAPK pathway may be an important mechanism for the long-lasting adverse effects of repeated behavioral stress.

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In this study we used a repeated forced swim-stress paradigm previously demonstrated to induce dynorphin release and subsequent activation of KOR in mice [3–5,8,15]. We found that repeated swim stress caused phosphorylation of ERK1/2 MAPK that was blocked by the KOR-selective antagonist, norBNI, and was absent in mice lacking KOR (KOR^{−/−}). Interestingly, we also found that the stress-induced, KOR-mediated phosphorylation of ERK1/2 was not dependent on GRK3 expression. These data suggest that prolonged stress exposure induces ERK1/2 activation via the kappa opioid system and that this occurs by a mechanism that is different from that responsible for KOR-mediated p38-MAPK activation.

Materials and Methods

Drugs and Chemicals

Nor-binaltorphimine-HCl was obtained from the NIDA Drug Supply Program (Bethesda, MD). NorBNI was dissolved in 0.9% saline, and administered in 0.1ml/10g body weight 1hr prior to behavioral testing.

Animals and Housing

Male C57Bl/6 Mice (Charles River Laboratories, Wilmington, MA) weighing 22–30g were group-housed as previously described [6,18]. Animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee. Homozygous KOR and GRK knockout (−/−) and wildtype (+/+) littermate control mice were prepared by heterozygous crosses and genotyped as previously described [4,16,17].

Forced Swim Stress (FSS)

The modified Porsolt forced swim test paradigm used was a 2-day procedure in which mice swam without the opportunity to escape [4,5,8]. Mice were placed in an opaque 5 liter beaker (40 cm tall x 25 cm in diameter) filled with 3.5 liters of 30 ± 1 °C water. On day 1, animals were placed in water to swim for a single, 15 min trial. On day 2, mice swam for a series of four, 6 min trials; between each trial, mice were towel dried and returned to their home cage for 6 min.

Immunohistochemistry

Mice were anesthetized with isoflurane (Sigma, St. Louis, MO) and intracardially perfused with 4% *para*-formaldehyde in PB (0.1 M sodium phosphate, pH 7.4), and brains were sectioned as described [5,8]. Sections were incubated with phospho-ERK1/2 monoclonal antibody (1:300, Cell Signaling Technologies, Beverly, MA) overnight at 4°C. Immune complexes were then localized using fluorescent secondary antibody FITC anti-mouse IgG (1:250; Jackson ImmunoResearch, West Grove, PA), and sections were viewed with a Nikon Eclipse E600 fluorescence microscope (Tokyo, Japan).

Immunoblotting of Mouse Brain Striatal ERK 1/2 and P-CREB

Immunoblotting was performed as previously described [5,11,13]. Mice were killed at specified time points, and whole striata were dissected. Tissue was homogenized in 500 µl of ice cold lysis buffer containing (50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF, and 10% Glycerol) with protease inhibitor cocktail set 1 (Calbiochem, San Diego, CA), and protein phosphatase inhibitor set 1 (Calbiochem, San Diego, CA) to inhibit proteases and serine/threonine phosphatases. Samples were then centrifuged at 30,000 x g for 15 min. Protein concentrations in the supernatants were determined using the bicinchoninic colorimetric assay according to the manufacturer's protocol (Pierce, Rockford, IL) with bovine serum albumin (BSA) as the standard. Proteins were then resolved by SDS-PAGE using 10% bis-acrylamide non-reducing gels (Invitrogen) and then transferred to

nitrocellulose. Blots were blocked with 5% BSA or 5% Milk TBS for 1hr, then incubated overnight at 4°C with a primary goat antibody recognizing phospho-ERK 1/2 or pCREB (Cell Signaling Technologies, Beverly, MA) at a dilution of 1:1000 (TBST). Following washes, blots were incubated for 1 hr IRDye 800 Conjugated goat anti-rabbit IgG secondary antibody. Protein loading was assessed using goat-anti rabbit β -actin antibody (AbCam, diluted 1:5000) and secondary Alexa-Fluor 680 alloccyanin goat-anti-mouse IgG (Molecular Probes, Eugene, OR). Labeling was dual-visualized in both the 700 (Actin) and 800 (ERK/pCREB) channels using the Odyssey Imaging system (Li-COR, Lincoln, NE), and fluorescence intensity was compared to background as described previously [5,11,13]. Data was normalized to control (no-swim) values for all groups. Student's *t*-test or one way ANOVA was used to determine statistical differences between groups. Data are presented as means $\pm$ S.E.M. of the animal group, with statistical significance set at $p < 0.05$.

Results

Prior studies showed that KOR activation can induce the phosphorylation of ERK 1/2 MAPK in heterologous gene expression systems and primary neuron or astrocyte cultures [10,11]; however whether this pathway can be activated by dynorphin/KOR in brain following behavioral stimulation has not been established. To address this question, we used a modified Porsolt forced swim stress (FSS) [4,5,18] to determine if stress-induced dynorphin release caused KOR-dependent activation (phosphorylation) of ERK1/2 *in vivo*. We observed that FSS caused a robust increase in the phosphorylation of ERK1/2 (P-ERK1/2) MAPK, as measured by P-ERK1/2 immunoreactivity (ir) in both the caudate (Fig. 1A) and the nucleus accumbens (Fig. 1B, C) regions of the mouse striatum 1hr after FSS. Surprisingly, the increase in pERK1/2-ir caused by FSS was completely dependent on KOR activation. KOR knockout mice (KOR $-/-$) did not show an increase in P-ERK 1/2-ir in either the caudate or nucleus accumbens following FSS (Fig. 1A,B). In addition, pretreatment with the selective KOR-antagonist, norbinaltorphimine (norBNI, 10 mg/kg) prevented FSS-induced ERK1/2 activation (Fig. 1C).

These data suggest that stress-induced dynorphin release caused KOR activation leading to ERK1/2 phosphorylation. To quantify these effects and to determine the time course for ERK1/2 activation following FSS, we used immunoblotting techniques. As evident in Fig 2, a single, acute episode of swim-stress exposure was not sufficient to induce P-ERK1/2 (Fig 2A). Prior to stress exposure the p-p42 band of ERK1/2 was more strongly evident than that of p-p44; however, the staining intensities of neither band increased following a single acute swim episode. In contrast, repeated FSS did cause a significant ($n = 3-9$, $P < 0.05$) increase in P-ERK1/2-ir in mouse striatal cell lysates at 30 min and 1 hr after FSS exposure (Fig 2C, E). The effects at 10 min were more variable, and P-ERK-ir was not significantly increased at this time point. The basis for this variability is not known, but interestingly the amplitude and variance of the P-ERK1/2-ir was substantially smaller in mice receiving FSS following norBNI pretreatment or KOR deletion (Fig 2E). The significant increases in P-ERK-ir at 10 and 60 min were completely blocked by pretreatment with norBNI (10mg/kg, i.p) (Fig 2B) and were not evident in KOR ($-/-$) mice. Together, these data suggest that FSS-induced ERK1/2 activation occurs rapidly following repeated stress-exposure in a KOR-dependent manner.

In some instances G-protein coupled receptor activation of MAPK requires receptor phosphorylation and subsequent β -arrestin recruitment [19,20]. Consistent with this, our group recently reported that kappa opioid activation of p38 MAPK both *in vitro* and *in vivo* required phosphorylation of serine-369 in the C-terminal domain of KOR by G-protein receptor kinase 3 (GRK3) and β -arrestin activation [5,11]. To determine if a similar dependence on GRK3 was required for ERK phosphorylation *in vivo*, we measured phospho-ERK1/2-ir in striatal lysates of GRK3($-/-$) and wildtype ($+/+$) mice. In contrast to KOR activation of p38 MAPK, repeated forced swim stress caused a robust increase in P-ERK1/2-ir in mouse striatum in both

wild type and GRK3 knockout mice (Fig. 3A, B). Furthermore, we recently showed that CREB phosphorylation (P-CREB-ir) following stress was p38 MAPK-independent [5]. Consistent with a GRK3-independent ERK1/2 activation following forced swim stress, we found that repeated swim stress induced an increase in P-CREB-ir in both wild type and GRK3 (−/−) mice. The results suggest that the activation of this ERK-CREB pathway is distinct from the stress-induced KOR-dependent p38 MAPK activation that we previously reported. Together, these data suggest a second, GRK3-independent signaling pathway for KOR-mediated ERK1/2 activity *in vivo*.

Discussion

The principal findings of this study were that repeated swim-stress resulted in KOR-mediated activation of ERK1/2 MAPK in the mouse nucleus accumbens (NAc) and mouse caudate (CPu) in a GRK/β-arrestin-independent manner. These results suggest that endogenous dynorphin opioids that are released by chronic swim stress may produce behavioral responses by ERK1/2 MAPK pathway activation. Previous studies showed that the dynorphin release and subsequent KOR activation caused by repeated forced swim effectively increased immobility, induced analgesia, potentiated cocaine conditioned place preference, and produced dysphoria. These effects were blocked by kappa antagonists and are not evident in mice lacking functional KOR or prodynorphin genes [3–5,8]. The role of ERK1/2 in these behavioral responses has not yet been established, but our prior work showed that the stress-induced immobility and aversion could be blocked by p38 MAPK inhibition [5]. Our present findings are also consistent with a previous study that demonstrated initiation of the ERK1/2 MAPK signaling cascade following swim stress [21]. However, this study builds substantially on this initial finding by demonstrating that repeated, prolonged stress causes a significant, robust ERK1/2 activation in both the dorsal and ventral striatum. Because the role of ERK1/2 MAPK activation in synaptic plasticity and other learning events has been established [22], further work on the role of ERK1/2 in mediating the behavioral responses following dynorphin/KOR activation is warranted.

Swim-stress increases immediate early gene activation in the striatum [5,23–25]. In addition phosphorylation of the cyclic AMP response element binding protein (CREB) in the nucleus accumbens has been suggested to alter hedonic state [24]. Consistent with the suggested functional relationship, CREB colocalizes with dynorphin in the nucleus accumbens and can alter the expression of prodynorphin peptides. However the mechanisms linking dynorphin and CREB are not clear. Recently, we found that CREB phosphorylation was not altered by inhibition of p38 MAPK, suggesting that prolonged stress exposure initiates an alternative kinase pathway for CREB phosphorylation [5]. Consistent with this, here we report that repeated swim stress increased phospho-CREB in the striatum; however this increase in P-CREB was GRK3 independent. It has also been demonstrated in a variety of contexts that CREB is a downstream target of phospho-ERK 1/2 and that CREB activation ultimately leads to changes in synaptic plasticity and behavior [22]. The results of the current report reveal an important link between stress, the kappa opioid system, and ERK 1/2 MAPK-P-CREB pathways.

Conclusion

Repeated stress induces behavioral changes that can manifest as depression and can increase the risk of drug abuse. Understanding the signaling pathways mediating these effects provides molecular and cellular insight and suggests novel therapeutic targets. Results from this study build on the relatively new body of work demonstrating the importance of the kappa opioid system in the stress response, and implicate ERK1/2 MAPK signaling as a key downstream component of the stress response.

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Figure 1.

Repeated swim-stress results in KOR-dependent phospho-ERK 1/2 (P-ERK 1/2) staining in mouse caudate (CPu) and nucleus accumbens (NAc). (a) Representative images of P-ERK 1/2 in CPu min after repeated swim-stress exposure (60 min), in stressed or nonstressed KOR wild-type (KOR +/+) and stressed KOR knockout mice (KOR -/-). (b) Representative images of P-ERK1/2-ir in NAc after repeated swim-stress exposure (60 min), in stressed or nonstressed KOR (+/+), stressed KOR (-/-) mice. (c) Higher power representative image of P-ERK1/2 staining in mouse NAc following repeated swim-stress exposure and stressed mice that were pretreated with the selective kappa opioid antagonist, norbinaltorphimine (norBNI, 10mg/kg i.p., 1hr prior to stress) All images representative of 3 independent experiments.

Figure 2.

Repeated swim-stress exposure induces KOR-dependent phospho-ERK1/2-ir in the mouse striatum. (a) Representative western blot of P-ERK1/2 (p44/p42) in wild-type mouse striatum after a single (Acute Swim) forced swim stress (S) in the presence or absence of the kappa-opioid antagonist norBNI (10 mg/kg, given 1hr prior to FSS) (b) Representative western blot of P-ERK1/2 in wild-type mouse striatum after repeated S (Repeated Swim), in the presence (S+norBNI) or absence (S) of norBNI. (c) Representative western blot of P-ERK1/2 time course following repeated S. Blots show P-ERK1/2 levels 10 min, 30 min, and 60 minutes after stress. (d) Representative western blot of P-ERK1/2 time course following repeated S in animals pretreated with the KOR-antagonist, norBNI (10mg/kg, 1hr). Below each blot of the P-ERK1/2 image is the protein loading control showing similar levels of β -actin. (e) Data are the mean $\pm$ S.E.M. of the quantified band intensities taken from repeated swim stress-induced

P-ERK 1/2 activity in WT mice (KOR +/+), mice pretreated with norBNI (10mg/kg), or mice lacking KOR (KOR -/-) compared with the saline-treated no swim-stress comparable group (basal; dashed line). * $p < 0.05$, One-way ANOVA followed by post-hoc t-test, for each WT multiple swim-stress versus saline control. $n = 3-9$, where each n is a separate animal.

Figure 3.

Repeated swim-stress induced KOR-dependent phospho-ERK1/2 is GRK3-independent. (a) Representative western blot of P-ERK1/2 in wild-type mouse striatum (GRK3 +/+) or GRK3 knockout mouse striatum (GRK3 -/-) (60 min) after repeated forced swim stress (S), as compared to non-stressed (NS) wild-type mice. Below the blot of the P-ERK1/2 image is the protein loading control showing similar levels of β -actin. (b) Data are the mean $\pm$ S.E.M. of the quantified band intensities taken from repeated swim stress-induced P-ERK1/2 activity in WT mice (GRK3 +/+) or mice lacking GRK3 (GRK3 -/-) compared with the no swim-stress comparable group (basal; dashed line). * $p < 0.05$, for both swim-stress group versus non-stress control, student's t -test. $n = 4-5$, where each n is a separate animal. (c) Representative western blots of pCREB-ir (43Kda) in wild-type mouse striatum (GRK3 +/+) or GRK3 knockout mouse striatum (GRK3 -/-) (60 min) after repeated forced swim stress (S), as compared to non-stressed (NS) wild-type mice. Below the blot of the P-CREB image is the protein loading control showing similar levels of β -actin. (d) Data are the mean $\pm$ S.E.M. of the quantified band intensities taken from repeated swim stress-induced pCREB activity in WT mice (GRK3 +/+) or mice lacking GRK3 (GRK3 -/-) compared with the no swim-stress group (basal; dashed line). * $p < 0.05$, for both swim-stress group versus non-stress control, student's t -test. $n = 5$, where each n is a separate animal.