Extracellular signal-regulated kinase activation in the amygdala mediates elevated plus maze behavior during opioid withdrawal

Rebecca S. Hofford[a], Stephen R. Hodgson[a], Kris W. Roberts[b], Camron D. Bryant[c], Christopher J. Evans[b] and Shoshana Eitan[a]

This study examined whether activation of extracellular signal-regulated kinase (ERK) contributes to the increased open-arm time observed in the elevated plus maze (EPM) during opioid withdrawal. We applied SL327, a selective ERK kinase (MEK) inhibitor, to specific limbic areas and examined the effect on EPM behaviors of controls and during naloxone-precipitated morphine withdrawal. We next confirmed that ERK activation increased in limbic areas of mice undergoing naloxone-precipitated morphine withdrawal. Direct injection of SL327 into the amygdala blocked the withdrawal-induced increase in open-arm time; however, injecting SL327 into the septum had no effect. Consistent with these results, both 0.2 and 2 mg/kg naloxone increased ERK activation in the central amygdala of morphine-dependent mice. In drug-naive mice, 2 mg/kg naloxone, but not 0.2 mg/kg, increased ERK activation in the central amygdala. During withdrawal, increased ERK activation was also observed in the lateral septum. In the locus coeruleus, a significant increase was observed only in morphine-dependent mice receiving 2 mg/kg, but not 0.2 mg/kg naloxone. In conclusion, ERK activation in limbic areas is likely involved in both the aversive properties of naloxone and in the affective/emotional symptoms of opioid withdrawal, including mediating EPM behaviors. *Behavioural Pharmacology* 20:576–583 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Behavioural Pharmacology 2009, **20**:576–583

Keywords: dependence, mitogen-activated protein kinase, morphine, mouse, naloxone, opioid signaling

[a]Department of Psychology, Behavioral and Cellular Neuroscience, Texas A&M University, College Station, Texas, [b]Department of Psychiatry and Biobehavioral Sciences, UCLA, Neuropsychiatric Institute, Westwood Plaza, Los Angeles, California and [c]Department of Human Genetics, University of Chicago, CLSC, Chicago, Illinois, USA

Correspondence to Shoshana Eitan, PhD, Texas A&M University, Department of Psychology, 4235 TAMU, College Station, TX 77843, USA
E-mail: seitan@psych.tamu.edu

Received 16 December 2008 **Accepted as revised** 7 May 2009

Introduction

Theories of addiction stipulate that the maintenance of drug abuse, despite the severe consequences, is in part driven by the desire to avoid the precipitation of withdrawal or to escape the withdrawal state (Schulteis and Koob, 1996; Koob *et al.*, 1997). Like humans, opioid withdrawal in rodents also elicits numerous somatic and affective signs. We recently showed that during both naloxone-precipitated and spontaneous morphine withdrawal, mice exhibit an increase in the time spent in the open arms of the elevated plus maze (EPM) (Hodgson *et al.*, 2008; Buckman *et al.*, 2009). We hypothesized that this increase in open-arm time might be explained by the different emotionality, motivation, and defensive patterns triggered by withdrawal. Thus, the EPM behaviors of mice undergoing withdrawal may represent a change in their defensive strategies because of an increased motivation to escape. An increase in the motivation to escape might also induce an increase in exploration and/or risk-taking behaviors similar to the behaviors observed in addicts (Hodgson *et al.*, 2008).

Multiple studies point to the importance of the central amygdala, the extended amygdala and the lateral septum in regulating the affective responses during morphine withdrawal (Aston-Jones *et al.*, 1999; Gracy *et al.*, 2001; Frenois *et al.*, 2002; Watanabe *et al.*, 2002, 2003; Veinante *et al.*, 2003; Hamlin *et al.*, 2004; Jin *et al.*, 2005; Nakagawa *et al.*, 2005). Moreover, an increase in ERK activation in the central amygdala was recently shown to be involved in cue-induced drug-seeking during opioid withdrawal (Li *et al.*, 2008). An increase in phospho-ERK (the activated form of ERK) during naloxone-precipitated withdrawal was also observed in the locus coeruleus (LC), solitary tract, hypothalamus (Schulz and Hollt, 1998), cortex, and striatum (Asensio *et al.*, 2006), as well as in the spinal cord (Cao *et al.*, 2005, 2006) and the heart (Almela *et al.*, 2007). In the spinal cord, ERK activation contributes to the precipitation of somatic signs (Cao *et al.*, 2005, 2006). In the heart, ERK was shown to contribute to adaptive processes induced by opioid withdrawal, such as c-Fos expression (Almela *et al.*, 2007).

Given the role of the ERK pathway in withdrawal-induced behaviors, this study examines the involvement of ERK in mediating EPM behavior during withdrawal. Moreover, given the importance of the limbic system in the manifestation of different withdrawal affective signs,

0955-8810 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/FBP.0b013e32832ec57e

we specifically examined the effects of localized ERK inhibition in the amygdala and septum during naloxone-precipitated withdrawal.

Methods

Subjects and drugs

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee. Male C57BL/6 mice (9–10 weeks old; Harlan Lab, Houston, Texas, USA) were housed four to five per cage with food and water *ad libitum*. They were housed in a temperature-controlled vivarium with a 12/12 h light/dark cycle (lights on 07.00 h). Morphine sulfate, naloxone hydrochloride, and SL327 were purchased from Sigma (St. Louis, Missouri, USA). Placebo and 25 mg morphine pellets were supplied by the National Institute on Drug Abuse. Separate mice were used for the immunohistochemical and behavioral analyses.

Stereotaxic manipulation

Cannulae (23 gauge) were inserted bilaterally into the amygdala [position relative to bregma: anteroposterior −1 mm, lateral ±3.2 mm] and unilaterally into the septum [position relative to bregma: anteroposterior +1.5 mm, lateral 0 mm] using Kopf's 1900 Stereotaxic Alignment instrument (Tujunga, California, USA). Mice were given at least 1 week to recover before starting the experiments. To keep the cannulae open and flowing, mice received intracannula saline injections during this week. All intracannula injections were administered using 30-gauge needles inserted 4.2 mm deep for the amygdala and 3 mm deep for the septum. At the end of the experiment, the placement of the cannulae was confirmed by dye injections by an observer blind to the treatments, and any mice with incorrect cannula placement were excluded. We have approximately 90% success rate in the correct placement of bilateral cannulas.

Elevated plus maze

All procedures, except the SL327 and dimethyl sulfoxide (DMSO) injections, were performed in identical manner to our earlier EPM study as described in Hodgson *et al.* (2008). Mice were injected twice daily (09.00 and 17.00 h) for 3 consecutive days with saline or increasing doses of morphine [10–40 mg/kg, subcutaneously (s.c.)] for a total of six injections. Specifically, on day 1, the mice were injected with saline or 10 mg/kg morphine. On day 2, mice were injected with saline or 20 mg/kg morphine. On day 3, they were injected with saline or 40 mg/kg morphine. A final dose of 20 mg/kg (s.c.) was administered on day 4. One hour later, the mice were injected with SL327 or DMSO directly into the amygdala or septum. Intracannula injections of SL327 and DMSO were 0.2 µl per side for the amygdala and 0.4 µl for the septum, and were administered over a 10-min period. Each insert was withdrawn 5 min after completing the injection. One hour later (i.e. 2 h post-morphine), the mice were injected s.c. with saline or 0.2 mg/kg naloxone. A volume of 10 ml/kg was used for the saline, morphine, and naloxone injections. Five minutes after the saline or naloxone injections, mice were examined for EPM behaviors. EPM tests were performed between 11.00 and 13.00 h. Mice were habituated to the room for at least 30 min before testing. The plus-maze apparatus consisted of four arms elevated 63.8 cm above the floor, with each arm (87 mm wide, 155 mm long) positioned at 90° relative to the adjacent arms. Two arms were enclosed on two sides by 16.3 cm high opaque walls, and the other two arms were open. Mice were placed in the center of the maze facing toward a closed arm and recorded for 10 min by an overhead camera. The apparatus was thoroughly cleaned between mice. Behaviors were scored by an observer blind to the treatments. For each mouse, the following behaviors were monitored: (i) length of time (in seconds) spent in the open arms, starting when all four legs had crossed the entrance line to one of the open arms; (ii) total activity, defined as the total number of entries or exits into or out of any arm.

Phospho-extracellular signal-regulated kinase staining

Mice were injected twice daily (09.00 and 17.00 h) for 3 consecutive days with saline or increasing doses of morphine (10–40 mg/kg, s.c.) as described for the EPM study. A final dose was administered on day 4, where mice were injected with saline or 20 mg/kg morphine. Two hours later, the mice were injected s.c. with saline, 0.2 mg/kg naloxone or 2 mg/kg naloxone. Seven minutes after the naloxone injection, they were injected with pentobarbital (100 mg/kg, i.p.) and after another 5 min mice were perfused with 50 ml ice-cold PBS followed by 200 ml of 4% paraformaldehyde. Brains were removed, post-fixed overnight at 4°C, cryoprotected in 30% sucrose at 4°C for 2 days, and frozen embedded in a Tissue-Tek OCT compound (Sakura Finetek Inc., Torrance, California, USA). The brains were then cryosectioned at 40 µm and stored in PBS containing 0.1% thimerosal at 4°C. We performed simultaneous staining on control and treated brain sections using the free-floating method. Briefly, sections were incubated overnight with antibodies against phospho-p42/44 ERK (Cell Signaling Technology, Danvers, Massachusetts, USA) diluted 1:300 at 4°C. The sections were then washed and incubated with biotin-conjugated anti-rabbit antibodies, followed by ABC enhancement (Vector Lab Inc., Burlingame, California, USA). Staining was revealed using a 3,3′-diaminobenzidine kit (Vector Lab Inc., Burlingame, California, USA).

After staining, digital pictures were taken using an Olympus BX51 microscope equipped with a digital camera (Microfire A/R, Optronics, Goleta, California,

USA) and stored using Adobe Photoshop (Adobe Systems Inc., San Jose, California, USA). Particle analysis was performed in regions of interest using National Institutes of Health Image v1.62 as described earlier (Eitan *et al.*, 2003). For each mouse, three brain areas were analyzed – the central amygdala, lateral septum, and the LC. Areas of interest within each of these sections were determined based on the mouse atlas (Paxinos and Franklin, 2001). For the central amygdala, we used coronal sections falling between −0.82 and −1.94 mm anteroposterior relative to bregma. In these sections, we analyzed areas identified in the atlas as central amygdaloid nucleus, capsular part, lateral division, medial division, and medial posteroventral part. For the lateral septum, we used coronal sections falling between 1.18 and 0.14 mm anteroposterior relative to bregma. In these sections, we analyzed areas identified in the atlas as lateral septal nucleus, dorsal part, intermediate part, and ventral part. Finally, the sections between −5.34 and −5.68 mm anteroposterior to the bregma were analyzed for the area identified in the atlas as LC. Examples of the areas of interest for each location are marked in Fig. 3.

In a similar experiment, mice were implanted s.c. with placebo or 25 mg morphine pellets under light isoflurane anesthesia. Three days later, pellets were removed under light anesthesia. An hour later mice were injected with saline or 2 mg/kg naloxone. Brains were then processed for immunohistochemistry and analyzed as described above.

Data analysis

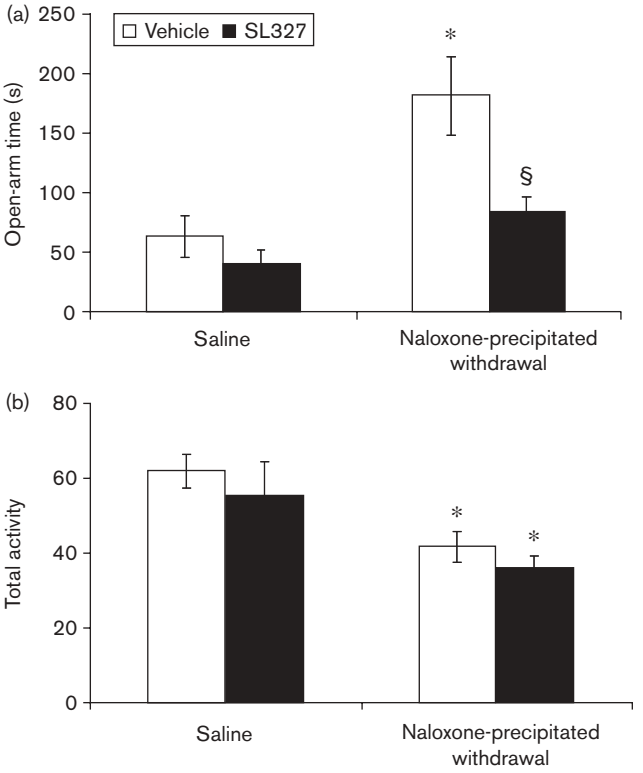
EPM data were analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc comparisons, with condition (control/withdrawal) and treatment (DMSO/SL327) as variables. To analyze the phospho-ERK staining, each brain area of each mouse in the experimental group was normalized to the corresponding staining in the saline control group. The change in staining compared with saline controls was calculated using the following formula: [(experimental value/saline control average) × 100] – 100. Statistical analysis was conducted using two-way ANOVA with pretreatment (placebo/morphine) and treatment (saline/naloxone) as variables, followed by Bonferroni's post-hoc comparisons.

Results

ERK activation in the amygdala, but not the septum, mediates the increase in open-arm time in the EPM during morphine withdrawal

We first examined the effect of administering SL327, an ERK kinase (MEK) inhibitor, into the amygdala on EPM behaviors in saline-injected control mice and the morphine-dependent mice undergoing withdrawal (Fig. 1). Two-way ANOVA revealed significant main effects of both withdrawal [$F(1,26) = 23.59$; $P < 0.005$] and administration of SL327 [$F(1,26) = 13.02$; $P < 0.025$] on open-arm time, but no significant interaction was observed. Bonferroni's post-hoc comparisons revealed a significant increase in open-arm time during naloxone-precipitated morphine withdrawal ($P < 0.01$). Injecting SL327 bilaterally (4 μg per side) into the amygdala of mice undergoing withdrawal completely blocked the increase in open-arm time compared with mice injected with vehicle ($P < 0.05$, Fig. 1a). The effect of SL327 was not simply because of an effect of overall motor activity. Two-way ANOVA revealed a significant main effect of withdrawal [$F(1,26) = 34.48$; $P < 0.001$] on total activity, but no significant effect of SL327 or interaction was observed. Bonferroni's post-hoc comparisons revealed a significant decrease in total activity during naloxone-precipitated morphine withdrawal in both vehicle-treated and SL327-treated mice ($P < 0.05$) compared

Fig. 1



Blockade of extracellular signal-regulated kinase in the amygdala attenuates the withdrawal-induced increase in open-arm time. Mice were injected with increasing doses of morphine for 3 days (10–40 mg/kg, subcutaneously). On day 4, mice were injected SL327 or dimethyl sulfoxide directly into the amygdala. Withdrawal was precipitated using 0.2 mg/kg naloxone. (a) Shows open-arm time and (b) shows total activity. White bars represent vehicle treatment; black bars represent SL327 treatment. Drug-naive mice receiving vehicle or SL327, $n = 6$; morphine-dependent mice undergoing withdrawal and receiving vehicle, $n = 10$; morphine-dependent mice undergoing withdrawal and receiving SL327, $n = 8$. Results are presented as mean ± SEM. (*) indicates a significant difference from control ($P < 0.05$). (§) indicates a significant difference from morphine-dependent mice undergoing withdrawal receiving vehicle ($P < 0.05$).

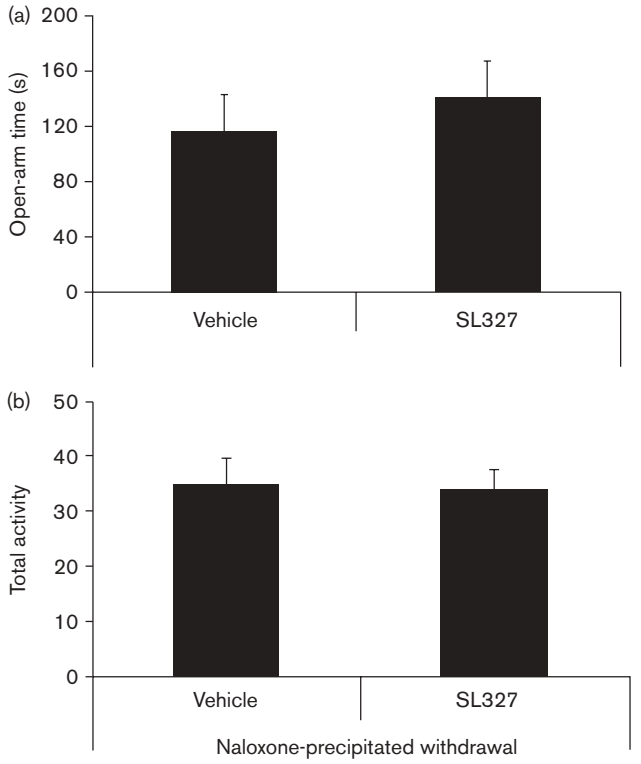
with controls. However, there was no significant difference in the total activity between mice undergoing withdrawal that were injected with vehicle and those injected with SL327 (Fig. 1b). For the drug-naive mice, injecting SL327 into the amygdala had no effect on the time spent in the open arms or total activity (Fig. 1a and b). In mice undergoing morphine withdrawal, SL327 (8 μg) injected into the septum did not change the time spent in the open arms or total activity (Fig. 2a and b).

Naloxone differentially induces an increase in ERK activation in drug-naive and morphine-dependent mice

In rats, increased ERK activation was previously observed in the central, but not basolateral, amygdala during morphine withdrawal (Li *et al.*, 2008). As mice exhibit species-dependent differences in EPM behaviors during withdrawal (Hodgson *et al.*, 2008), we next confirmed that indeed in mice there is a comparable increase in ERK activation in the amygdala during morphine withdrawal. In this experiment, we examined ERK activation in drug-naive and morphine-dependent mice receiving low (0.2 mg/kg) and high (2 mg/kg) naloxone doses. As expected, an increase in ERK activation was observed in the central amygdala. ERK activation was not observed in the basolateral amygdala or any other surrounding areas (data not shown). We also observed increased ERK activation in the lateral septum and LC. An increase in ERK activation was also observed in the bed nucleus of stria terminalis (BNST) in morphine-dependent mice receiving naloxone, but was not quantified (data not shown).

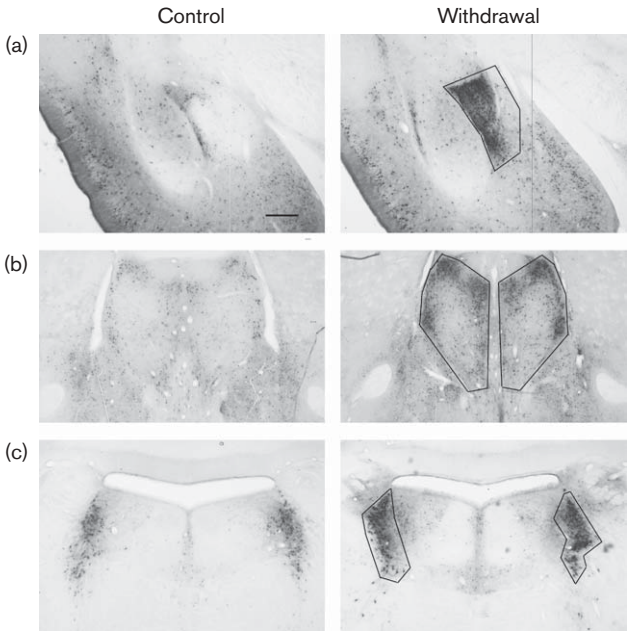
In the central amygdala (Figs 3a and 4a), two-way ANOVA ($n = 10$–14) revealed a significant main effect of naloxone treatment [$F(2,59) = 57.68$; $P < 0.001$] and a significant interaction between naloxone treatment and morphine pretreatment [$F(2,59) = 14.29$; $P < 0.001$]. Bonferroni's post-hoc comparisons revealed that 2 mg/kg naloxone, but not 0.2 mg/kg, significantly increased ERK activation in drug-naive mice ($P < 0.001$). In morphine-dependent mice, a significant decrease was observed in mice receiving saline compared with drug-naive saline controls ($P < 0.01$). For mice undergoing withdrawal, both 0.2 and 2 mg/kg naloxone significantly increased

Fig. 2



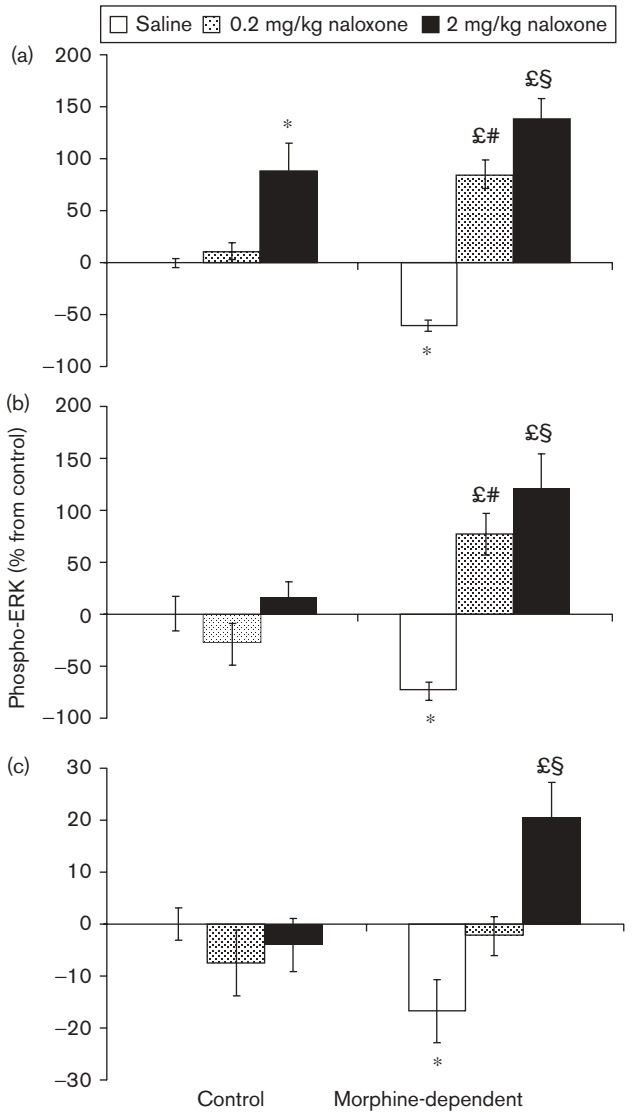
Blockade of extracellular signal-regulated kinase in the septum has no effect on elevated plus maze behaviors during withdrawal. Mice were injected with increasing doses of morphine for 3 days (10–40 mg/kg, subcutaneously.). On day 4, mice were injected SL327 or dimethyl sulfoxide directly into the septum. Withdrawal was precipitated using 0.2 mg/kg naloxone. No significant effect of SL327 was found. (a) Shows open-arm time and (b) shows total activity. Results are presented as mean + SEM, $n = 9$–10.

Fig. 3



Naloxone administration increases extracellular signal-regulated kinase activation in the brain. Mice were injected with increasing doses of morphine for 3 days (10–40 mg/kg, subcutaneously.). On day 4, withdrawal was precipitated using 2 mg/kg naloxone. A naloxone-induced increase in extracellular signal-regulated kinase activation was observed in the central amygdala (a), lateral septum (b), and the LC (c). (a), (b), and (c) are representative diaminobenzidine-stained sections for each brain area. (Control) Drug-naive mice receiving saline; (Withdrawal) morphine-dependent mice receiving 2 mg/kg naloxone. The scale bar is 300 μm. The regions of interests for the semiquantification analysis are outlined on the representative diaminobenzidine-stained sections for withdrawal.

Fig. 4



The differential effect of low and high naloxone doses on extracellular signal-regulated kinase activation in drug-naive and morphine-dependent mice. Mice were injected with increasing doses of morphine for 3 days (10–40 mg/kg, subcutaneously.). On day 4, withdrawal was precipitated using 0.2 or 2 mg/kg naloxone. (a) central amygdala, (b) lateral septum, and (c) the LC. White bars represent saline treatment; gray bars represent 0.2 mg/kg naloxone treatment; black bars represent 2 mg/kg naloxone treatment. Results are presented as mean ± SEM. (*) indicates a significant difference compared to drug-naive mice receiving saline ($P<0.05$); (#) indicates a significant difference compared to drug-naive mice receiving 0.2 mg/kg naloxone ($P<0.05$); (§) indicates a significant difference compared to drug-naive mice receiving 2 mg/kg naloxone ($P<0.05$); (£) indicates a significant difference compared with morphine-dependent mice receiving saline ($P<0.05$).

ERK activation in morphine-dependent mice as compared with their drug-naive counterparts ($P < 0.001$ and $P < 0.05$, respectively). Moreover, 2 mg/kg naloxone significantly increased ERK activation as compared with 0.2 mg/kg naloxone ($P < 0.05$).

In the lateral septum (Figs 3b and 4b), two-way ANOVA ($n = 10$–14) revealed significant main effects of naloxone treatment [$F(2,59) = 14.4$; $P < 0.001$] and morphine pretreatment [$F(1,59) = 7.92$; $P = 0.01$], and a significant interaction between naloxone treatment and morphine pretreatment [$F(2,59) = 13.95$; $P < 0.001$]. Bonferroni's post-hoc comparisons revealed a significant decrease in ERK activation in morphine-dependent mice receiving saline compared with drug-naive saline controls ($P < 0.05$). Both 0.2 and 2 mg/kg naloxone significantly increased ERK in morphine-dependent mice as compared with their drug-naive counterparts ($P < 0.01$). Naloxone did not significantly modulate ERK activation in the lateral septum for drug-naive mice.

In the LC (Figs 3c and 4c), two-way ANOVA ($n = 10$–14) revealed a significant main effect of naloxone treatment [$F(2,59) = 6.193$; $P < 0.005$] and a significant interaction between naloxone treatment and morphine pretreatment [$F(2,59) = 8.672$; $P < 0.001$]. Bonferroni's post-hoc comparisons revealed a significant decrease in ERK activation in morphine-dependent mice receiving saline compared with drug-naive saline controls ($P < 0.05$). In contrast, 2 mg/kg naloxone significantly increased ERK activation in the LC for morphine-dependent mice as compared with their drug-naive counterparts ($P < 0.01$). However, 0.2 mg/kg naloxone did not significantly change ERK activation levels in morphine-dependent mice as compared with drug-naive mice.

Similar results were also observed when morphine dependency was established using 25 mg morphine pellets and withdrawal was precipitated with 2 mg/kg naloxone. Namely, increased ERK activation was observed in the central amygdala but not in the basolateral amygdala or other surrounding brain regions (data not shown). Similarly, increased ERK activation was also observed in the lateral septum, BNST, and LC (data not shown).

Discussion

This study shows the involvement of the ERK pathway in the increased open-arm time observed during naloxone-precipitated morphine withdrawal. Notably, inhibition of ERK in the amygdala blocked the increase in open-arm time, yet inhibiting ERK in the septum had no effect. In addition, increases in phospho-ERK staining (the active form of ERK) were observed in the central amygdala of morphine-dependent mice receiving both 0.2 and 2 mg/kg naloxone. In morphine-dependent mice, the dose required to increase ERK activation in the central amygdala was lower than the dose necessary to precipitate significant jumping behavior (Hodgson *et al.*, 2008) – a behavioral measure commonly taken to determine the severity of somatic signs (Iorio *et al.*, 1975). This low dose, however, is sufficient to precipitate

both the physiological signs of stress from withdrawal, as indicated by significant increases in plasma corticosterone levels, as well as the increase in EPM open-arm time (Hodgson *et al.*, 2008). Note that although total levels of ERK protein were not measured in our experiments, increases in total protein levels are highly unlikely during the 13-min period.

Previously, correlations were made between the motivational components of naloxone-precipitated morphine withdrawal and the activation of c-Fos in the central amygdala, extended amygdala, and lateral septum (Gracy *et al.*, 2001; Veinante *et al.*, 2003; Hamlin *et al.*, 2004; Jin *et al.*, 2005). The lateral septum is implicated in the modulation of anxiety through the mu-opioid receptor (Le Merrer *et al.*, 2006). The central and extended amygdala regulate withdrawal-induced conditioned place aversion (Aston-Jones *et al.*, 1999; Gracy *et al.*, 2001; Jin *et al.*, 2005) and are involved in altered hedonic processing during withdrawal (Harris and Aston-Jones, 2007). In addition, activation of ERK and cAMP response element-binding protein in the central amygdala is involved in mediating drug-seeking and craving during withdrawal (Li *et al.*, 2008). However, these brain regions were demonstrated to have little involvement in the precipitation of somatic signs of withdrawal (Maldonado *et al.*, 1992; Nakagawa *et al.*, 2005).

Consistent with these prior findings, this study implicates ERK activation in the central amygdala in mediating EPM behaviors in mice during withdrawal. It is important to note that in this study, SL327 was injected 1 hour before the precipitation of withdrawal and the EPM test. Although only a very small quantity was administered, we cannot exclude the possibility of some diffusion of SL327 to surroundings areas. Thus, it is possible that the effect on EPM behaviors is because of inhibition of ERK in nearby brain regions. However, consistent with other studies, there were no conditions (i.e. repeated morphine injections, morphine pellets, high and low naloxone doses) in which we observed an increase in phospho-ERK in the areas surrounding the central amygdala. The closest areas in which we observed increased ERK activation were the lateral septum and BNST. Yet, injection of SL327 into the septum had no effect. Thus, it is very likely that the activation of ERK in the central amygdala mediates the effect on EPM behaviors. Nevertheless, we cannot exclude the possibility that changes occurring below the detection threshold in nearby areas are involved in mediating the EPM behaviors during withdrawal.

Naloxone is aversive in both drug-naive (Skoubis *et al.*, 2005) and morphine-dependent mice (Maldonado *et al.*, 2004; Broseta *et al.*, 2005; Shoblock and Maidment, 2006). However, in drug-naive mice, a high naloxone dose is required to establish significant levels of aversion. In contrast, for morphine-dependent mice, withdrawal precipitation even by lower naloxone doses is sufficient to produce a significant emotional reaction. Just as high doses are required to induce aversion in opioid-naive mice, drug-naive mice also require 2 mg/kg naloxone, not just 0.2 mg/kg, to increase ERK activation in the central amygdala. Thus, we hypothesize that the activation of ERK in limbic areas is most likely also involved in the aversive properties of naloxone. The central amygdala is known to have high tonic levels of enkephalin and other endogenous opioid peptides (Mansour *et al.*, 1993; Chieng *et al.*, 2006; Marchant *et al.*, 2007; Poulin *et al.*, 2008). Given that drug-naive mice lacking preproenkephalin do not exhibit increased ERK activation in the central amygdala (Eitan *et al.*, 2002), it supports the hypothesis that naloxone increases ERK activation through inhibition of tonic signaling of endogenous opioid peptides. Similar to ERK, an increase of c-Fos in the central amygdala was observed in drug-naive rats after naloxone administration (Gestreau *et al.*, 2000).

As mentioned above, increased ERK activation was observed in the central amygdala of drug-naive mice administered with 2 mg/kg naloxone, but not 0.2 mg/kg naloxone. However, drug-naive mice did not exhibit a significant increase in open-arm time even when administered 10 mg/kg naloxone (Hodgson *et al.*, 2008). Therefore, it is very unlikely that the differences in EPM behaviors between drug-naive and morphine-dependent mice receiving naloxone can be explained solely by different levels of ERK activation. This leads to the conclusion that the increase in ERK activation is necessary to drive the increased EPM open-arm time during withdrawal. However, the lack of naloxone-induced increases in EPM open-arm time in drug-naive mice suggests that other signaling cascades are required in addition to ERK activation.

This study also showed increased ERK activation in the LC during opioid withdrawal. This is consistent with a previous study that observed an increase in phospho-ERK staining in the LC (Schulz and Hollt, 1998). In contrast to the limbic areas, increased ERK activation in the LC was observed only in morphine-dependent mice receiving the higher naloxone dose – a dose needed to precipitate significant jumping behavior (Hodgson *et al.*, 2008). The LC was suggested to be essential for the development of morphine dependence and the precipitation of withdrawal signs, especially the physical symptoms (Aghajanian, 1978; Crawley *et al.*, 1979; Maldonado *et al.*, 1992). Specifically, inhibition of adrenergic tone, mediated by GIRK2 and GIRK3, was suggested to be required for the development of dependence (Cruz *et al.*, 2008). Likewise, various receptors and signaling molecules in the LC were shown to be involved in the precipitation of withdrawal's

somatic signs. Examples include α-2 adrenoceptors (Aghajanian, 1982; Engberg *et al.*, 1982), γ-aminobutyric acid receptor (Mirzaii-Dizgah *et al.*, 2008), glutamate transporters (Nakagawa and Satoh, 2004; Ozawa *et al.*, 2004), and the cAMP pathway, including adenylyl cyclases 1 and 8 and cAMP response element-binding protein (Lane-Ladd *et al.*, 1997; Valverde *et al.*, 2004; Han *et al.*, 2006; Oh *et al.*, 2007; Zachariou *et al.*, 2008). However, the role of the LC in the precipitation of somatic withdrawal signs remains somewhat debatable, given that lesioning of noradrenergic terminals arising from the LC does not inhibit the precipitation of physical symptoms of opioid withdrawal (Chieng and Christie, 1995).

In this study, we also observed a decreased ERK activation in the central amygdala, lateral septum, and LC of morphine-dependent mice receiving saline (i.e. in which withdrawal was not precipitated with naloxone). We previously observed decreased ERK activation in the central amygdala 30 min after acute or repeated morphine administration (Eitan *et al.*, 2003). This was the only brain region in which we did not observe the development of tolerance for ERK modulation after repeated morphine administration, although antinociceptive tolerance was observed (Eitan *et al.*, 2003). Thus, the decreased ERK activation in the central amygdala is most likely a persistent morphine effect that is already observed at 30 min post-morphine and lasts for at least 2 h. This is unlikely because of spontaneous withdrawal, given that the half-life of morphine (Kalvass *et al.*, 2007) requires a longer duration before withdrawal spontaneously precipitates.

For the LC, we previously observed that acute morphine administration produces an increase in ERK activation (Eitan *et al.*, 2003). However, after repeated morphine administration, tolerance for ERK activation developed; namely, we did not observe an increase or a decrease in ERK activation 30 min post-morphine in mice receiving repeated morphine administration, as compared with controls (Eitan *et al.*, 2003). In this study, morphine-dependent mice showed a decrease in ERK activation at 2 h post-morphine. This decreased ERK activation might represent a delayed adaptive response to a morphine challenge in morphine-dependent mice. Such opposite responses are known to emerge upon cessation of prolonged administration of opioids. These opponent processes in many cases counteract the acute effects of opioids (e.g. hyperalgesia vs. antinociception) and can contribute to the development of tolerance and physical dependence (Bryant *et al.*, 2005). Thus, the decrease in ERK activation may represent one of several cellular opponent processes. Interestingly, this cellular event parallels the opioid-induced hyperalgesia (behavioral rebound) observed at the same time in morphine-tolerant mice (Eitan *et al.*, 2003).

Although opiates are commonly used for pain management, the neuroplastic mechanisms underlying their adaptive processes remain somewhat elusive. The purpose of this study was to examine the involvement of the ERK pathway in EPM behaviors of mice undergoing opioid withdrawal. We focused on the ERK pathway because it plays an important role in synaptic plasticity, and has been implicated in the chronic adaptations to opioids. We observed an increase in ERK activation at the onset of morphine withdrawal. This increase was observed in brain areas previously implicated in mediating the emotional response to withdrawal. Finally, we found that ERK signaling is necessary for mediating the withdrawal-induced increase in EPM open-arm time. Future studies will examine the receptor mechanisms involved, as well as other brain pathways and signaling cascades that are important in driving behavioral adaptations during drug withdrawal.

Acknowledgements

These studies were funded by NIDA (DA022402 and DA05010) and by the Shirley and Stefan Hatos Neuroscience Research Foundation. The authors thank Mr Menachum M. Slodowitz for his editorial assistance. Disclosure/conflicts of interest: no financial interests to disclose.

References

Aghajanian GK (1978). Tolerance of locus coeruleus neurones to morphine and suppression of withdrawal response by clonidine. *Nature* **276**:186–188.

Aghajanian GK (1982). Central noradrenergic neurons: a locus for the functional interplay between alpha-2 adrenoceptors and opiate receptors. *J Clin Psychiatr* **43**:20–24.

Almela P, Milanés MV, Laorden ML (2007). Activation of the ERK signaling pathway contributes to the adaptive changes in rat hearts during naloxone-induced morphine withdrawal. *Br J Pharmacol* **151**:787–797.

Asensio VJ, Miralles A, Garcia-Sevilla JA (2006). Stimulation of mitogen-activated protein kinase kinases (MEK1/2) by mu-, delta- and kappa-opioid receptor agonists in the rat brain: regulation by chronic morphine and opioid withdrawal. *Eur J Pharmacol* **539**:49–56.

Aston-Jones G, Delfs JM, Druhan J, Zhu Y (1999). The bed nucleus of the stria terminalis. A target site for noradrenergic actions in opiate withdrawal. *Ann N Y Acad Sci* **877**:486–498.

Broseta I, Rodriguez-Arias M, Aguilar MA, Minarro J (2005). Isolation decreases physical and motivational aspects of morphine withdrawal. *Behav Pharmacol* **16**:131–138.

Bryant CD, Zaki PA, Carroll FI, Evans CJ (2005). Opioids and addiction: Emerging pharmaceutical strategies for reducing reward and opponent processes. *Clin Neurosci Res* **5**:103–115.

Buckman SG, Hodgson SR, Hofford RS, Eitan S (2009). Increased elevated plus maze open-arm time in mice during spontaneous morphine withdrawal. *Behav Brain Res* **197**:454–456.

Cao JL, He JH, Ding HL, Zeng YM (2005). Activation of the spinal ERK signaling pathway contributes naloxone-precipitated withdrawal in morphine-dependent rats. *Pain* **118**:336–349.

Cao JL, Liu HL, Wang JK, Zeng YM (2006). Cross talk between nitric oxide and ERK1/2 signaling pathway in the spinal cord mediates naloxone-precipitated withdrawal in morphine-dependent rats. *Neuropharmacology* **51**:315–326.

Chieng B, Christie MJ (1995). Lesions to terminals of noradrenergic locus coeruleus neurones do not inhibit opiate withdrawal behaviour in rats. *Neurosci Lett* **186**:37–40.

Chieng BC, Christie MJ, Osborne PB (2006). Characterization of neurons in the rat central nucleus of the amygdala: cellular physiology, morphology, and opioid sensitivity. *J Comp Neurol* **497**:910–927.

Crawley JN, Laverty R, Roth RH (1979). Clonidine reversal of increased norepinephrine metabolite levels during morphine withdrawal. *Eur J Pharmacol* **57**:247–250.

Cruz HG, Berton F, Sollini M, Blanchet C, Pravetoni M, Wickman K, Lüscher C (2008). Absence and rescue of morphine withdrawal in GIRK/Kir3 knock-out mice. *J Neurosci* **28**:4069–4077.

Eitan S, Bryant CD, Saliminejad N, Ansonoff M, Yang YC, Keith D, *et al.* (2002). Modulation of MAPK activation in vivo following acute opioid agonist or antagonist treatment. Society for Neuroscience, Orlando, Florida, poster number 643.2.

Eitan S, Bryant CD, Saliminejad N, Yang YC, Vojdani E, Keith D Jr, *et al.* (2003). Brain region-specific mechanisms for acute morphine-induced mitogen-activated protein kinase modulation and distinct patterns of activation during analgesic tolerance and locomotor sensitization. *J Neurosci* **23**:8360–8369.

Engberg G, Elam M, Svensson TH (1982). Clonidine withdrawal: activation of brain noradrenergic neurons with specifically reduced alpha 2-receptor sensitivity. *Life Sci* **30**:235–243.

Frenois F, Cador M, Caillé S, Stinus L, Le Moine C (2002). Neural correlates of the motivational and somatic components of naloxone-precipitated morphine withdrawal. *Eur J Neurosci* **16**:1377–1389.

Gestreau C, Le Guen S, Besson JM (2000). Is there tonic activity in the endogenous opioid systems? A c-Fos study in the rat central nervous system after intravenous injection of naloxone or naloxone-methiodide. *J Comp Neurol* **427**:285–301.

Gracy KN, Dankiewicz LA, Koob GF (2001). Opiate withdrawal-induced fos immunoreactivity in the rat extended amygdala parallels the development of conditioned place aversion. *Neuropsychopharmacology* **24**:152–160.

Hamlin AS, Buller KM, Day TA, Osborne PB (2004). Effect of naloxone-precipitated morphine withdrawal on c-fos expression in rat corticotropin-releasing hormone neurons in the paraventricular hypothalamus and extended amygdala. *Neurosci Lett* **362**:39–43.

Han MH, Bolaños CA, Green TA, Olson VG, Neve RL, Liu RJ, *et al.* (2006). Role of cAMP response element-binding protein in the rat locus ceruleus: regulation of neuronal activity and opiate withdrawal behaviors. *J Neurosci* **26**:4624–4629.

Harris GC, Aston-Jones G (2007). Activation in extended amygdala corresponds to altered hedonic processing during protracted morphine withdrawal. *Behav Brain Res* **176**:251–258.

Hodgson SR, Hofford RS, Norris CJ, Eitan S (2008). Increased elevated plus maze open-arm time in mice during naloxone-precipitated morphine withdrawal. *Behav Pharmacol* **19**:805–811.

Iorio LC, Deacon MA, Ryan EA (1975). Blockade by narcotic drugs of naloxone precipitated jumping in morphine-dependent mice. *J Pharmacol Exp Ther* **192**:58–63.

Jin C, Araki H, Nagata M, Shimosaka R, Shibata K, Suemaru K, *et al.* (2005). Expression of c-Fos in the rat central amygdala accompanies the acquisition but not expression of conditioned place aversion induced by withdrawal from acute morphine dependence. *Behav Brain Res* **161**:107–112.

Kalvass JC, Olson ER, Cassidy MP, Selley DE, Pollack GM (2007). Pharmacokinetics and pharmacodynamics of seven opioids in P-glycoprotein-competent mice: assessment of unbound brain EC50,u and correlation of *in vitro*, preclinical, and clinical data. *J Pharmacol Exp Ther* **323**:346–355.

Koob GF, Caine SB, Parsons L, Markou A, Weiss F (1997). Opponent process model and psychostimulant addiction. *Pharmacol Biochem Behav* **57**:513–521.

Lane-Ladd SB, Pineda J, Boundy VA, Pfeuffer T, Krupinski J, Aghajanian GK, Nestler EJ (1997). CREB (cAMP response element-binding protein) in the locus coeruleus: biochemical, physiological, and behavioral evidence for a role in opiate dependence. *J Neurosci* **17**:7890–7901.

Le Merrer J, Cagniard B, Cazala P (2006). Modulation of anxiety by mu-opioid receptors of the lateral septal region in mice. *Pharmacol Biochem Behav* **83**:465–479.

Li Y-Q, Li F-Q, Wang X-Y, Wu P, Zhao M, Shaham Y, Lu L (2008). Central amygdala extracellular signal-regulated kinase signaling pathway is critical to incubation of opiate craving. *J Neurosci* **28**:13248–13257.

Maldonado C, Rodríguez-Arias M, Aguilar MA, Miñarro J (2004). GHB ameliorates naloxone-induced conditioned place aversion and physical aspects of morphine withdrawal in mice. *Psychopharmacology (Berl)* **177**:130–140.

Maldonado R, Stinus L, Gold LH, Koob GF (1992). Role of different brain structures in the expression of the physical morphine withdrawal syndrome. *J Pharmacol Exp Ther* **261**:669–677.

Mansour A, Thompson RC, Akil H, Watson SJ (1993). Delta opioid receptor mRNA distribution in the brain: comparison to delta receptor binding and proenkephalin mRNA. *J Chem Neuroanat* **6**:351–362.

Marchant NJ, Densmore VS, Osborne PB (2007). Coexpression of prodynorphin and corticotrophin-releasing hormone in the rat central amygdala: evidence of two distinct endogenous opioid systems in the lateral division. *J Comp Neurol* **504**:702–715.

Mirzaii-Dizgah I, Karimian SM, Hajimashhadi Z, Riahi E, Ghasemi T (2008). Attenuation of morphine withdrawal signs by muscimol in the locus coeruleus of rats. *Behav Pharmacol* **19**:171–175.

Nakagawa T, Satoh M (2004). Involvement of glial glutamate transporters in morphine dependence. *Ann N Y Acad Sci* **1025**:383–388.

Nakagawa T, Yamamoto R, Fujio M, Suzuki Y, Minami M, Satoh M, Kaneko S (2005). Involvement of the bed nucleus of the stria terminalis activated by the central nucleus of the amygdala in the negative affective component of morphine withdrawal in rats. *Neuroscience* **134**:9–19.

Oh KW, Eun JS, Kwon HN, Cho EY, Kim KM (2007). Effects of (–)-epigallocatechin gallate on the development of morphine-induced physical dependence. *Arch Pharm Res* **30**:1111–1115.

Ozawa T, Nakagawa T, Sekiya Y, Minami M, Satoh M (2004). Effect of gene transfer of GLT-1, a glutamate transporter, into the locus coeruleus by recombinant adenoviruses on morphine physical dependence in rats. *Eur J Neurosci* **19**:221–226.

Paxinos G, Franklin KBJ (2001). *The Mouse Brain in Stereotaxic Coordinates, second edition*. San Diego, CA: Academic Press.

Poulin JF, Castonguay-Lebel Z, Laforest S, Drolet G (2008). Enkephalin co-expression with classic neurotransmitters in the amygdaloid complex of the rat. *J Comp Neurol* **506**:943–959.

Schulteis G, Koob GF (1996). Reinforcement processes in opiate addiction: a homeostatic model. *Neurochem Res* **21**:1437–1454.

Schulz S, Hollt V (1998). Opioid withdrawal activates MAP kinase in locus coeruleus neurons in morphine-dependent rats in vivo. *Eur J Neurosci* **10**:1196–1201.

Shoblock JR, Maidment NT (2006). Constitutively active mu opioid receptors mediate the enhanced conditioned aversive effect of naloxone in morphine-dependent mice. *Neuropsychopharmacology* **31**:171–177.

Skoubis PD, Lam HA, Shoblock J, Narayanan S, Maidment NT (2005). Endogenous enkephalins, not endorphins, modulate basal hedonic state in mice. *Eur J Neurosci* **21**:1379–1384.

Valverde O, Mantamadiotis T, Torrecilla M, Ugedo L, Pineda J, Bleckmann S, *et al.* (2004). Modulation of anxiety-like behavior and morphine dependence in CREB-deficient mice. *Neuropsychopharmacology* **29**:1122–1133.

Watanabe T, Nakagawa T, Yamamoto R, Maeda A, Minami M, Satoh M (2002). Involvement of glutamate receptors within the central nucleus of the amygdala in naloxone-precipitated morphine withdrawal-induced conditioned place aversion in rats. *Jpn J Pharmacol* **88**:399–406.

Watanabe T, Nakagawa T, Yamamoto R, Maeda A, Minami M, Satoh M (2003). Involvement of noradrenergic system within the central nucleus of the amygdala in naloxone-precipitated morphine withdrawal-induced conditioned place aversion in rats. *Psychopharmacology (Berl)* **170**:80–88.

Veinante P, Stoeckel ME, Lasbennes F, Freund-Mercier MJ (2003). c-Fos and peptide immunoreactivities in the central extended amygdala of morphine-dependent rats after naloxone-precipitated withdrawal. *Eur J Neurosci* **18**:1295–1305.

Zachariou V, Liu R, LaPlant Q, Xiao G, Renthal W, Chan GC, *et al.* (2008). Distinct roles of adenylyl cyclases 1 and 8 in opiate dependence: behavioral, electrophysiological, and molecular studies. *Biol Psychiatr* **63**:1013–1021.