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Extracellular signal-regulated kinase (ERK) inhibition does not prevent the development or expression of tolerance to and dependence on morphine in the mouse

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Abstract

The clinical use of opioids is limited by the development of tolerance and physical dependence. Opioid tolerance and dependence are believed to result from complex adaptations in the CNS, representing a form of neural plasticity. Extracellular signal-regulated kinases (ERKs) are involved in many forms of neural plasticity, and therefore could also be involved in the development of opioid tolerance and dependence. In this study, we investigated the effect of a systemically bioavailable MEK (ERK kinase) inhibitor, SL327, upon the development and the expression of tolerance to and dependence on morphine in mice. In tolerance and dependence development studies, two strains of mice were treated daily for 8 or 9 days with 5mg/kg morphine s.c. Tolerance development was assessed by tail flick latency. Withdrawal was then precipitated by subcutaneous injection of 2mg/kg naloxone s.c. and signs recorded. Co-administration of 50mg/kg SL327 i.p. prior to morphine administration had no effect on the development of tolerance or withdrawal signs. To study possible effects of ERK inhibition on the expression of tolerance and dependence, mice were implanted with 75mg morphine pellets s.c. Tolerance and dependence were assessed as previously described. An acute i.p. injection of 50mg/kg SL327 after 4 days of morphine exposure had no effect on the expression of either morphine tolerance or physical dependence. To verify that this dose of SL327 inhibited morphine-induced ERK modulation, mice received an acute i.p. injection of 50mg/kg SL327 prior to morphine administration, and sacrificed 30min later. Western blots demonstrated that SL327 did inhibit morphine-induced ERK modulation. Taken together, these data suggest that unlike many other observed forms of neural plasticity, the ERK signaling cascade is not involved in the development or expression of opioid tolerance and dependence.

Keywords: Opioids; Withdrawal; Mitogen-activated protein kinase; Narcotics; Neuronal plasticity

Mu opioid (MOP) receptor agonists such as morphine are among the most effective drugs currently used for pain management. However, their effectiveness is limited by undesirable side effects such as analgesic tolerance and physical dependence. The MOP receptor is coupled to various intracellular effectors such as adenylyl cyclase, voltage-gated calcium channels and G protein-coupled inwardly rectifying potassium channels (Law et al., 2000). Modulation of these effectors by opioids contributes to cellular hyperpolarization. Morphine also blocks presynaptic neurotransmitter release (Vaughan et al., 1997). These phenomena may account for the analgesic effects of morphine (Taylor and Fleming, 2001).

In addition to these well studied pathways, the MOP receptor has been shown to activate ERKs (Extracellular signal-Regulated Kinases) 1 and 2 *in vitro* (Fukuda et al., 1996; Gutstein et al., 1997). These enzymes couple extracellular signals to changes in cellular phenotype by phosphorylation of both cytoplasmic and nuclear proteins, including nuclear transcription factors. ERK activation has been shown to play an important role in many forms of neuronal plasticity (Grewal et al., 1999; Ji and Woolf, 2001; Mazzucchelli and Brambilla, 2000), including learning and memory (Atkins et al., 1998; Selcher et al., 1999), nociceptive sensitization (Ji and Woolf, 2001), and place preference in response to cocaine administration (Valjent et al., 2006; Valjent et al., 2000).

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In a MOP receptor-transfected CHO cell line, ERK activation has been shown to mediate receptor desensitization by phosphorylation (Polakiewicz et al., 1998), a phenomenon that may be related in part to the development of tolerance (Taylor and Fleming, 2001). Moreover, in HEK 293 cells tranfected with the MOP receptor, ERKs 1 and 2 have been shown to play a role in chronic opioid-induced adenylyl cyclase superactivation, a phenomenon that may underlie opioid withdrawal (Tso and Wong, 2001).

Several studies have investigated ERK activation by opioids in vivo. Findings have ranged from no ERK activation seen in vivo after acute morphine administration (Schulz and Hollt, 1998) to the modulation of ERK activation in several brain regions after acute morphine administration (Eitan et al., 2003; Muller and Unterwald, 2004; Valjent et al., 2004). Chronic morphine treatment (Berhow et al., 1996; Eitan et al., 2003; Muller and Unterwald, 2004) as well as naloxone-precipitated withdrawal after chronic treatment (Schulz and Hollt, 1998) have also been shown to alter ERK activation. It has also been shown that chronic morphine administered to induce analgesic tolerance induced tolerance to morphine-induced ERK modulation in most brain regions showing acute activation (Eitan et al., 2003). However, to our knowledge, behavioral studies to determine whether ERK activation played a role in the development and/or the expression of morphine tolerance and dependence behaviors have never been performed.

The goal of these studies was to investigate the effect of ERK inhibition on the development and expression of morphine tolerance and physical dependence. SL327, a systemically bioavailable inhibitor of MEK 1 and 2 (Atkins et al., 1998; Favata et al., 1998), the dual-specificity kinases that phosphorylate ERKs 1 and 2, was used to evaluate the role of ERK in these processes. Co-administration of SL327 with morphine did not prevent the development of tolerance, as assessed using the tail flick test, or physical dependence, assessed by naloxone-precipitated withdrawal behaviors. Acute SL327 injection after chronic morphine treatment did not inhibit the expression of tolerance or physical dependence. Western blotting studies of the cingulate cortex showed that co-administration of SL327 with morphine also blocked acute ERK modulation produced by morphine. The observation that SL327 did not block opioid tolerance or dependence contrasts markedly with other results demonstrating that ERK mediates many other forms of neuronal plasticity. This suggests that the mechanisms underlying the development and expression of opioid tolerance and physical dependence may differ significantly from other forms of neural plasticity.

1. Materials and methods

1.1. Subjects

Male C57BL6 and CD-1 mice (25–30g) (Harlan, Indianapolis, IN) were housed in groups of four in cages on a 12-h light/dark cycle with *ad libitum* access to food and water. Mice were allowed to habituate in the colony room for one week before experimental manipulations were undertaken.

1.2. Drugs

Morphine sulfate powder and 75mg pellets were from NIDA, naloxone was from Sigma (St. Louis, MO). SL327 was from the Du Pont Pharmaceutical Company (Wilmington, DE). Morphine and naloxone were dissolved in 0.9% saline while SL327 was solubilized in 50% DMSO in 0.9% saline.

1.3. Inhibition of ERK modulation by SL-327

Four groups of CD-1 mice ($n = 5$ per group) were injected i.p. with either 50mg/kg SL327 or vehicle (50% DMSO in saline) in a total volume of 10ml/kg. Half an hour after the first injection, either 5mg/kg morphine or saline was injected s.c., creating a 2 by 2 study design. 20min after the second injection, animals were sacrificed under isoflurane anesthesia, and brains quickly frozen in isopentane. Subsequently, the frozen brains were sliced to 500μm thickness and the cingulate cortex dissected using the punch method of Palkovits (Palkovits and Brownstein, 1983). The mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2000) was used to guide the dissections.

1.4. Immunoblot analysis

Proteins in the samples were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% acrylamide gels. Proteins were then transferred to nitrocellulose membranes by electroblotting for 2.5h at 60V. Membranes were blocked with 5% bovine serum albumin in wash solution (0.1% Tween-20, 100mM NaCl, 10mM Tris–pH 7.4) for 1h and incubated with anti-phospho ERK 1 and 2 antibody (New England Biolabs, Ipswich, MA) which detects phosphorylation of threonine 202 and tyrosine 204 of p44 and p42 ERKs at 4°C overnight. Secondary anti-rabbit IgGs antibody conjugated to horseradish peroxidase was then added for 1h at room temperature; the membranes were washed 7 times in wash solution, and developed using ECLplus. Densitometric analysis of ERK 1 and 2 bands was performed using MCID-M5^{+} imaging software (Imaging Research, St Catharines, ON). All samples were run in triplicate.

1.5. Tail-flick assay

Antinociception was assessed using the radiant heat tail-flick test. Animals were loosely restrained in a towel and an 85V lamp was focused on the tail. Withdrawal times were measured using a photocell. Three measurements were obtained at each time point, and the average used in all calculations. A cutoff latency of 10s was used to avoid tissue damage to the tail.

1.6. Induction of tolerance and physical dependence using intermittent morphine injections

In this study, 4 groups of C57BL6 mice were tested ($n = 7$ per group). Baseline tail-flick latency was recorded for each mouse on the first day. Mice then received an i.p. injection of either 50mg/kg SL327 or vehicle (50% DMSO in saline) in a total

volume of 10ml/kg. Half an hour after the first injection, either 5mg/kg morphine or saline was injected s.c., creating a 2 by 2 study design. Tail-flick latency (TFL) was determined 30min after the second injection. This procedure was repeated on day 2 through 8. On day 9, the SL327/vehicle injection was omitted in order to prevent any potential confounding effect of the inhibitor on the expression of tolerance or physical dependence. Preliminary studies using twice daily injection of 50mg/kg SL327/vehicle followed by morphine produced unacceptably high levels of mortality (50%), so a once daily injection protocol was used for these studies.

The same injection protocol was used for two groups of CD-1 mice (vehicle/morphine and SL327/morphine) except that animals were injected for 9days and tested on day 10.

1.7. Induction of tolerance and physical dependence using morphine pellets

This paradigm was employed in order to produce robust signs of tolerance and physical dependence more quickly than the intermittent injection paradigm. Baseline tail-flick latency was recorded for each mouse on the first day. C57BL6 mice were then anesthetized with isoflurane, the base of their necks shaved, and skin sterilized with iodine. A 1cm transverse incision was made between the scapulae and the animals were subcutaneously implanted with either a morphine (75mg) or placebo pellet ($n = 12$ per pellet type). Tail-flick latency was measured 4, 24, 48, and 72h after pellet implantation. 96h after implantation, 6 mice from the placebo group and 6 mice from the morphine group were injected with 50mg/kg SL327 i.p. while the other animals were injected with 50% DMSO in saline vehicle ($n = 6$ per treatment group). Tail-flick latency was determined 30min after the injection.

1.8. Naloxone-precipitated withdrawal

After the final tail-flick test, animals were weighed and withdrawal was precipitated by injection of 2mg/kg naloxone s.c. Animals were then placed in clear Plexiglas withdrawal cages and observed for 15min. The occurrence of withdrawal signs (escape jumps, wet dog shakes, teeth chattering, lacrimation, diarrhea, ptosis, freezing) was recorded. Animals were weighed again 2h after naloxone injection, and weight changes determined.

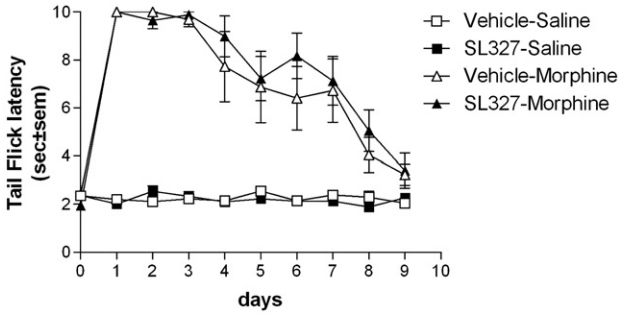

Fig. 1. The effect of chronic SL327 injection on the development of morphine tolerance in C57/BL6 mice: tail flick latency responses of C57BL6 mice ($n = 7$ per treatment group) chronically treated with daily s.c. injections of saline or 5 mg/kg morphine preceded by i.p. injections of 50% DMSO/saline vehicle or 50 mg/kg SL327 30 min earlier. SL327 or vehicle injection was omitted on the final day to control for any possible effect of SL327 on the expression of morphine tolerance. Tail flick latencies were determined 30 min after saline/morphine injection and calculated as mean±sem. There was no statistically significant difference between vehicle/saline (□) and SL327/saline (■) groups, and vehicle/morphine (△) and SL327/morphine (▲) groups at any time point during the study.

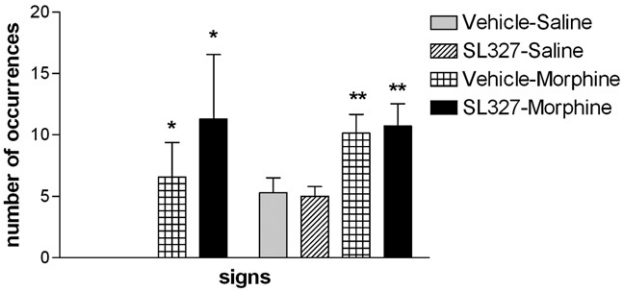

Fig. 2. The effect of chronic SL327 injection on the development of morphine dependence in C57/BL6 mice: withdrawal signs of C57BL6 mice chronically treated with saline or 5 mg/kg morphine along with vehicle or 50 mg/kg SL327, recorded for 15 min after acute injection of 2 mg/kg naloxone ($n = 7$ per group). SL327 or vehicle injection was omitted on the final day to control for any possible effect of SL327 on the expression of morphine physical dependence. Results expressed as mean±SD. Escape jumps (*, $F = 3.40$; $p < 0.05$) and teeth chattering (**, $F = 4.84$; $p < 0.01$) were significantly different between saline and morphine groups. There was no statistically significant difference between vehicle/saline and SL327/saline groups and vehicle/morphine and SL327/morphine groups.

1.9. Statistical analysis

All statistical analyses were performed using the Prism software package (GraphPad Software, San Diego, CA). Tail flick data were analyzed using two-way repeated measures ANOVA followed by Bonferroni post-hoc comparisons at each time point. Withdrawal symptoms that were ordinal variables were analyzed using either one-way ANOVA followed by the Tukey post-hoc test for C57BL6 mice (4 groups) or Student's unpaired t test for CD-1 mice (2 groups). Withdrawal symptoms that were nominal variables (diarrhea, lacrimation, ptosis, freezing) were analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Densitometric data were analyzed using one-way ANOVA followed by Dunnett post-hoc comparisons. $p < 0.05$ was required for significance.

2. Results

To inhibit ERK activation, we chose to use a SL327 dose of 50mg/kg because it was previously shown to block cocaine-induced ERK activation (Valjent et al., 2000) and cocaine-induced changes in gene expression in CD-1 mice (Radwanska et al., 2005). We also used the same route of injection (i.p.) and vehicle (50% DMSO) as these studies.

Our first experiment was designed to determine whether co-administration of SL327 with morphine would inhibit the development of tolerance and physical dependence in C57BL6 mice. Tolerance to daily s.c. injection of 5mg/kg of morphine was evaluated using the tail-flick test. Mice were injected with

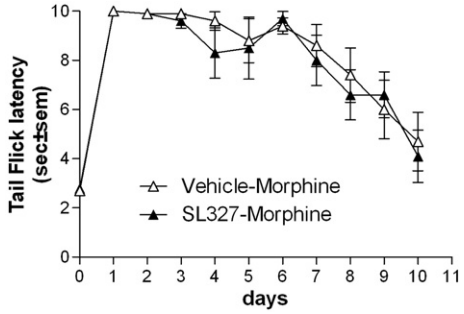

Fig. 3. The effect of chronic SL327 injection on the development of morphine tolerance in CD-1 mice: tail flick latency responses of CD-1 mice chronically treated with 5 mg/kg morphine preceded by vehicle or 50 mg/kg SL327 (n=6 per group). Tail flick latencies were recorded in triplicate 30 min after saline/morphine injections and are expressed as mean±sem. SL327 or vehicle injection was omitted on the final day to control for any possible effect of SL327 on the expression of morphine tolerance. There was no statistically significant difference between vehicle/morphine (△) and SL327/morphine (▲) groups (F=0.66; ns).

either vehicle or SL327 followed by saline or morphine for 8days, and either saline or morphine alone on day 9 (n = 7 per treatment group, 28 total). SL327 co-administered with saline did not cause any change in baseline tail-flick latency compared to 50% DMSO vehicle followed by saline (Fig. 1). Fig. 1 also shows that morphine analgesic tolerance developed over the course of the study. There was no significant difference in tolerance development between the vehicle-morphine and SL327-morphine groups (Fig. 1), suggesting that SL327 did not inhibit the development of morphine tolerance.

After tail-flick latency testing on day 9, withdrawal was precipitated by the injection of 2mg/kg naloxone s.c. Of the withdrawal signs monitored (see Materials and methods section), escape jumps and teeth chattering occurred significantly more often in morphine-treated animals (Fig. 2). Analysis of variance performed on these two withdrawal signs showed that there was no significant difference between vehicle-morphine and SL327-morphine treatment groups,

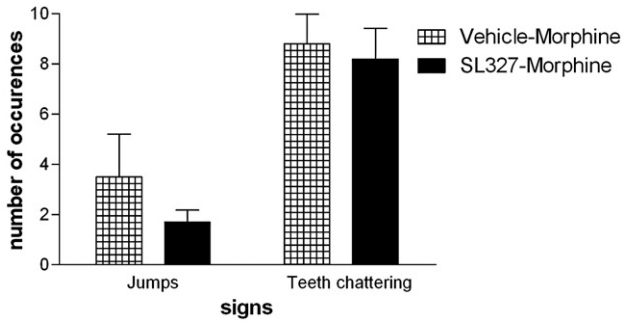

Fig. 4. The effect of chronic SL327 injection on the development of morphine dependence in CD-1 mice: withdrawal signs of CD-1 mice chronically treated with 5 mg/kg morphine preceded by vehicle or 50 mg/kg SL327, recorded for 15 min after acute injection of 2 mg/kg naloxone. SL327 or vehicle injection was omitted on the final day to control for any possible effect of SL327 on the expression of morphine physical dependence. Results are expressed as mean± SD. There was no statistically significant difference between vehicle/morphine and SL327/morphine groups (p=0.33 for escape jumps, p=0.73 for teeth chattering).

suggesting that SL327 co-administration also had no effect on the development of morphine dependence in this paradigm.

Given the prominent role played by ERK activation in other forms of neuronal plasticity (Atkins et al., 1998; Grewal et al., 1999; Ji and Woolf, 2001; Mazzucchelli and Brambilla, 2000; Selcher et al., 1999), and the demonstrated effect of SL327 in blocking cocaine place preference (Valjent et al., 2000), we found these results surprising. To determine whether this lack of effect of ERK inhibition on morphine tolerance and dependence was strain dependent, the experiment described above was repeated using CD-1 mice, the strain demonstrating inhibition of cocaine place preference by this dose of SL327 (Valjent et al., 2000). Tolerance developed slightly more slowly in the CD-1 mice, but they achieved the same degree of tolerance as the C57 mice (at day 10 vs. day 9) prior to withdrawal testing (Fig. 3). The same two signs (escape jumps and teeth chattering) that

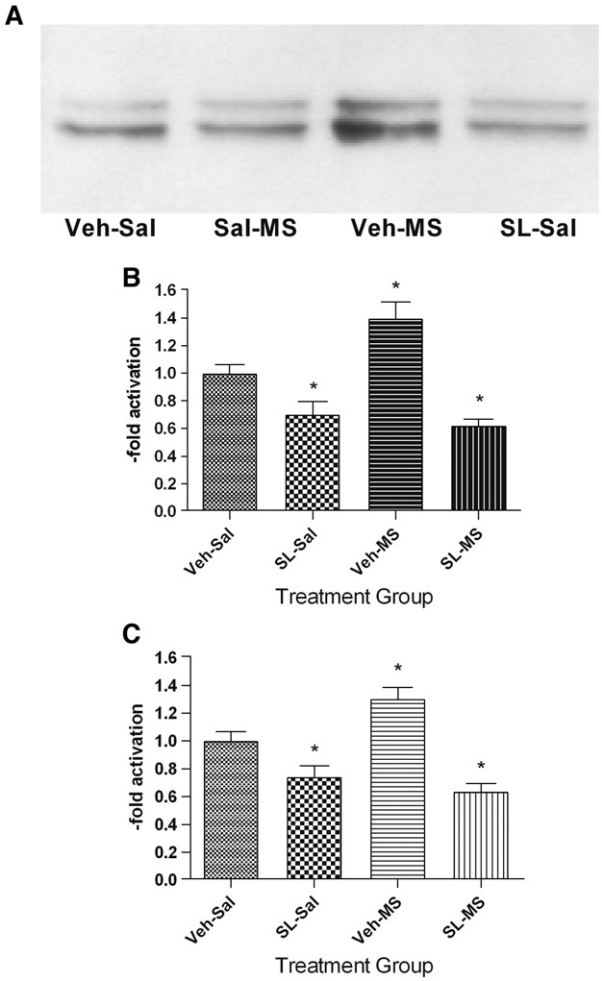

Fig. 5. SL-327 inhibited ERK activation by morphine in Cingulate Cortex: animals were treated and tissues harvested and processed as described in the Materials and methods section. A: Western blot representative of three replicate experiments. Veh–Sal — injection of vehicle and saline; SL–MS — injection of SL-327 and morphine; Veh–MS — injection of vehicle and morphine; SL–Sal — injection of SL-327 and saline. B: fold ERK 1 activation relative to Veh–Sal control (=1.0). *significantly different from Veh–Sal, P<0.05 by Dunnett's post-hoc test. C:-fold ERK 2 activation relative to Veh–Sal control (=1.0). *significantly different from Veh–Sal, p<0.05 by Dunnett's post-hoc test.

were significantly different between control and morphine-treated animals in the C57 mice were also investigated in the CD-1 mice (Fig. 4). As shown in Figs. 3 and 4, SL327 had no significant effect on the development of opioid tolerance or these signs of physical dependence in CD-1 mice. Thus, the lack of effect of SL327 on tolerance and dependence development did not appear to be strain dependent.

Even though we had used exactly the same SL-327 doses and injection protocol shown to block opioid-mediated ERK modulation in previous studies (Radwanska et al., 2005; Valjent et al., 2000), we still wished to thoroughly exclude the possibility that we were not effectively blocking ERK modulation. Hence, we acutely treated four groups of CD-1 mice (n = 5 per group) exactly as above, then sacrificed the animals 20min. after acute morphine or saline injection (Valjent et al., 2004). We then dissected the cingulate cortex from each animal by punch biopsy (Palkovits and Brownstein, 1983). Samples from each animal were individually processed and analyzed via immunoblotting. In the cingulate cortex, morphine induced significant activation of ERK 1 (ANOVA $F = 16.6$, $p < 0.0001$; Dunnett's test $p < 0.05$; Fig. 5A and B) as well as ERK 2 (ANOVA $F = 14.1$, $p < 0.0001$; Dunnett's test $p < 0.05$; Fig. 5A and C). SL-327 alone produced a significant reduction in ERK activation from the vehicle-saline control ($p < 0.05$). Co-administration of SL-327 and morphine resulted in reduction of ERK activation below baseline to a level similar to SL-327 administration alone. These data show that SL-327 at the 50mg/kg dose effectively blocked morphine-induced ERK activation (Fig. 5).

While the above results clearly demonstrate that ERK inhibition by SL327 did not inhibit the development of opioid tolerance or dependence, they do not provide any information about the expression of these behaviors. There have been observations that the development and expression of tolerance and physical dependence can be dissociated both behaviorally and neurochemically (Bhargava, 1994). Therefore, the development and expression of behaviors may represent two different types of neuronal plasticity, and ERK activation could possibly be involved in the expression of tolerance and physical dependence. Because SL327 would need to be injected only once to evaluate the expression of tolerance and dependence, we used a continuous morphine administration paradigm that has been shown to induce tolerance and robust signs of physical dependence more rapidly (Bohn et al., 2000). This paradigm was not used in the previous studies because we were concerned that continuous co-administration of SL327 could cause significant toxicity and morbidity. Fig. 6 shows that in C57 mice (n = 6 per group), tolerance rapidly developed after 75mg

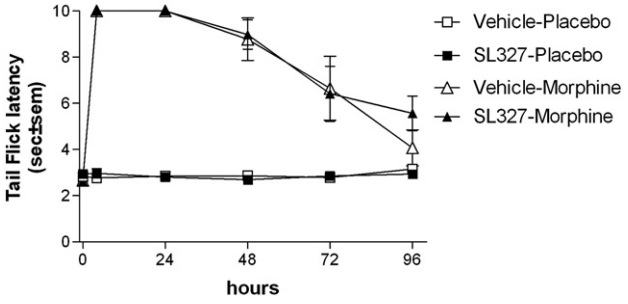

Fig. 6. The effect of acute SL327 injection on the expression of morphine tolerance in C57/BL6 mice: tail flick latency responses of C57BL6 mice chronically implanted with placebo or 75 mg morphine pellets. Tail flick latencies were recorded in triplicate daily. 96 h after pellet implantation, mice were injected i.p. with either 50% DMSO/saline vehicle or 50 mg/kg SL327, and underwent tail flick latency testing 30 min later. There was no statistically significant difference between placebo/vehicle (□) and placebo/SL327 (■) groups, and morphine/vehicle (△) and morphine/SL327 (▲) groups either during tolerance development or after acute SL327 injection, indicating that ERK inhibition did not significantly alter the behavioral expression of morphine tolerance. Results presented as mean±sem.

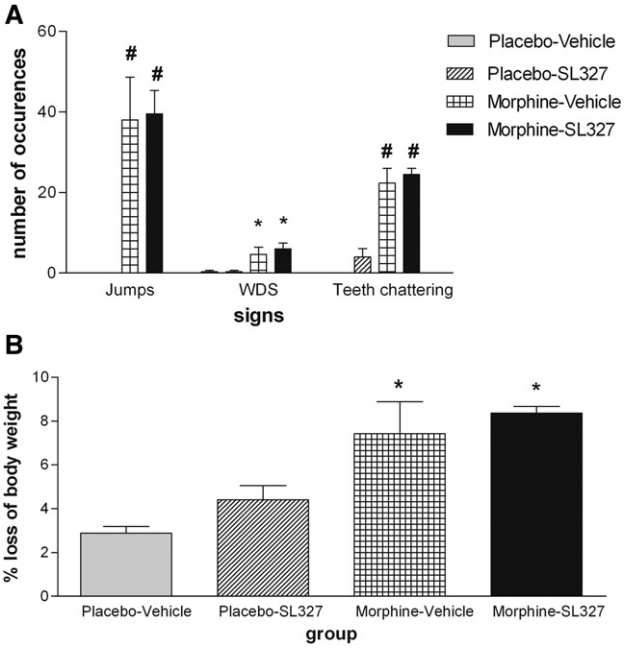

Fig. 7. The effect of acute SL327 injection on the expression of morphine dependence in C57/BL6 mice: groups of 6 C57/BL6 mice were implanted with placebo or 75 mg morphine pellets under general anesthesia. 96 h after pellet implantation, mice were injected i.p. with either vehicle or 50 mg/kg SL327 and underwent tail flick latency testing 30 min later. After tail flick testing, weights were determined and mice were injected i.p with 2 mg/kg naloxone. Withdrawal signs were recorded for 15 min after injection, and body weight determined 2 h. after naloxone injection. A) Escape jumps, wet dog shakes (WDS) and teeth chattering. B) Loss of body weight. All signs were significantly different between placebo and morphine groups (*$p<0.01$; #$p<0.001$). There was no statistically significant difference between morphine/vehicle and morphine/SL327 groups, indicating that ERK inhibition did not significantly affect the behavioral expression of morphine physical dependence. Results expressed as mean±SD.

Table 1
Withdrawal symptoms in placebo-and morphine-pelleted C57/BL6 mice

Treatment group	Freezing	Diarrhea	Lacrimation	Ptosis
Vehicle/placebo	0	0	0	0
SL327/placebo	0	0	0	0
Vehicle/morphine	83.3	100	100	83.3
SL327/morphine	100	100	100	100

Nominal variables. Each group is reported as the percentage of animals in the group exhibiting the behavior (n=6 animals per treatment group). SL327 did not significantly alter the occurrence of withdrawal signs in morphine-treated animals.

morphine pellet implantation. To determine the effect of ERK inhibition on the expression of tolerance, SL327 50mg/kg or vehicle was injected i.p. only once, 30min before tail flick testing on day 4. There was no significant difference in TFL between vehicle-morphine and SL327-morphine groups (Fig. 6), implying that acute SL327 administration had no effect on the expression of morphine tolerance.

To determine the potential effect of SL327 on the expression of physical dependence, immediately after tail flick testing on day 4, naloxone 2mg/kg was injected and withdrawal signs observed as previously described. In contrast to the intermittent morphine administration studies, all monitored withdrawal signs were greater in morphine pelleted than placebo pelleted animals (escape jumps: $F = 71.16$, $p < 0.001$; teeth chattering: $F = 58.59$, $p < 0.001$; wet dog shakes: $F = 8.68$; $p < 0.01$; weight loss: $F = 10.51$, $p < 0.01$; Fig. 7; $p < 0.005$; Table 1). Acute SL327 administration had no effect on naloxone-induced escape jumps, wet dog shakes or teeth chattering (Fig. 7A). SL327 also had no significant effect on the incidence of freezing, diarrhea, lacrimation or ptosis (Table 1) or on weight loss two hours after naloxone injection (Fig. 7B). Taken together, these results indicate that SL327 had no effect upon the expression of morphine tolerance or physical dependence.

3. Discussion

Our experiments demonstrated that the MEK inhibitor SL327 effectively blocked ERK activation by morphine, but did not prevent the development of morphine tolerance or physical dependence in two strains of mice. We also showed that acute administration of SL327 did not inhibit the expression of morphine tolerance or physical dependence in C57 mice. This dose (50 mg/kg) and mode of administration (i.p. injection) of SL327 has previously been shown to be efficient in acutely inhibiting ERK activation in mouse hippocampus (Selcher et al., 1999) and chronically inhibiting cocaine place preference (Valjent et al., 2000). Moreover, SL327 was shown to be efficient at inhibiting ERK activation for at least 3 h by Selcher et al. (Selcher et al., 1999), suggesting that it was unlikely that pharmacokinetic or pharmacodynamic factors accounted for the lack of observed behavioral effects of SL327 in our studies.

In contrast to the lack of involvement of ERK we have demonstrated in morphine tolerance and dependence behaviors, opioids have been shown to activate ERK both *in vitro* and *in vivo*. As described in the introduction, acute opioid administration has been shown to activate ERKs in non-neuronal cell lines (Fukuda et al., 1996; Gutstein et al., 1997). In vitro studies have also suggested a role for ERK activation in phosphorylation-mediated receptor desensitization (Polakiewicz et al., 1998), and opioid-induced adenylyl cyclase superactivation (Tso and Wong, 2001), processes that have been postulated to underlie tolerance and dependence behaviors (Taylor and Fleming, 2001). However, we have performed studies on a variety of neuronal cell lines that demonstrated that acute or chronic opioid administration, as well as withdrawal of opioids after chronic administration, did not activate ERK (Mouledous et al., 2004). In contrast with these results, ERK activation by opioids has been observed in the SK-N-SH cell line (Trapaidze et al., 2000). However, the SK-N-SH cell line is composed of at least two cellular phenotypes, neuroblastic and melanocytic/glial. ERK activation in SK-N-SH cells could be due to responses in cells with a melanocytic/glial phenotype. It is thus not clear that opioids can activate ERK in neuronal cells.

In vivo, both acute and chronic morphine administration and drug withdrawal after chronic morphine administration have been shown to modulate levels of activated ERK in specific brain regions. Previous work demonstrated that acute systemic morphine injection activated ERK in the cingulate, somatosensory, and association cortices, as well as the locus coeruleus, while decreasing ERK activity in the nucleus accumbens and the central nucleus of the amygdala (Eitan et al., 2003). These investigators also showed that chronic intermittent morphine administered to induce analgesic tolerance also caused tolerance to acute morphine-induced ERK activation in the anterior cingulate, somatosensory, and association cortices, as well as the locus coeruleus (Eitan et al., 2003). Another study demonstrated that chronic morphine administration increased ERK activation in the rat ventral tegmental area without increasing overall levels of ERK (Berhow et al., 1996). In contrast, Schulz and Hollt demonstrated that chronic morphine decreased basal levels of ERK activation observed in the median eminence, paraventricular hypothalamic nucleus, amygdaloid nuclei, cortical layers II and III, and the paragigantocellular nucleus (Schulz and Hollt, 1998). No change in ERK phosphorylation in the VTA was reported. However, this study used immunocytochemistry to detect ERK phosphorylation, which may be less sensitive than the western blotting technique employed in the Berhow et al. study. After 10 days of daily morphine injections, naloxone-precipitated withdrawal caused ERK activation to reappear in the brain regions mentioned above. In addition, after precipitated withdrawal ERK phosphorylation was abundant in several brain regions that did not previously demonstrate ERK activation, namely the locus coeruleus, nucleus of the solitary tract, spinal trigeminal nucleus, and the arcuate hypothalamic nucleus (Schulz and Hollt, 1998). They suggested that, at least in the locus coeruleus, ERK activation during opioid withdrawal might be caused by the cAMP/PKA burst previously noted to occur during withdrawal (Williams et al., 2001).

The findings summarized above are not inconsistent with either our *in vitro* findings showing a lack of effect of opioid treatment on neuronal cells or our *in vivo* results demonstrating no behavioral effect of ERK inhibition. Eitan et al. demonstrated that morphine-mediated ERK activation was predominantly not localized in cells expressing the mu opioid receptor (Eitan et al., 2003). However, these investigators did not determine the phenotypes of the cells expressing either ERK or the mu receptor. Even if under some circumstances ERK was activated in neurons, neural circuitry effects that cannot be replicated in a cell culture model could be mediating this activation indirectly. Prior *in vivo* results demonstrating ERK modulation in many relevant brain regions are also not inconsistent with the findings of the present study. The lack of effect of SL327 on morphine tolerance and physical dependence behaviors suggests that the

inhibition or induction of ERK activation by morphine administration or withdrawal in these brain regions does not mediate opioid tolerance or dependence behaviors, even though some of these regions have previously been implicated in the pathogenesis of these phenomena (Christie et al., 1997). The effects mediated by opioid-induced ERK activation in these brain regions remain unknown, but warrant further investigation. It should be noted that the present findings do not rule out the possibility that ERK activation could be involved in mediating the rewarding or addictive properties of morphine (Nestler and Aghajanian, 1997). While the findings of Berhow et al. demonstrating increased ERK activation in the ventral tegmental area after chronic morphine treatment are intriguing in this regard (Berhow et al., 1996), behavioral correlation of these anatomical findings is needed.

The fact that ERK inhibition did not prevent the development or the expression of opioid tolerance and physical dependence contrasts sharply with previous findings demonstrating the involvement of ERK in many other forms of neuronal plasticity (Kandel, 2001; Sweatt, 2001). At the cellular level, ERKs are involved in structural aspects of synaptic plasticity (Koh et al., 2002) as well as biochemical processes underlying learning and memory such as long-term potentiation and long-term facilitation (Mazzucchelli and Brambilla, 2000). ERK inhibition also impairs behavioral plasticity in animal models of contextual fear conditioning and spatial learning (Atkins et al., 1998; Ji and Woolf, 2001; Ji et al., 1999; Koh et al., 2002; Mazzucchelli and Brambilla, 2000; Mazzucchelli et al., 2002; Selcher et al., 1999; Valjent et al., 2000), nociceptive sensitization (Ji and Woolf, 2001; Ji et al., 1999) and cocaine-induced place preference (Valjent et al., 2000). The specific mechanisms by which ERK activation mediates these various forms of neural plasticity remain incompletely understood, but are the subject of intense investigation.

In conclusion, our findings demonstrate that inhibition of the ERK signaling cascade does not affect the development or the expression of tolerance to and physical dependence on morphine in the mouse. The fact that ERK inhibition alters the development of many other types of neural plasticity suggests that the neurochemical substrates of opioid tolerance and dependence may differ from other types of neural plasticity. Improved understanding of these differences may provide unique therapeutic opportunities to specifically prevent opioid tolerance and dependence without affecting other, more desirable, forms of neural adaptation.

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