In Vivo Regulation of Extracellular Signal-Regulated Protein Kinase (ERK) and Protein Kinase B (Akt) Phosphorylation by Acute and Chronic Morphine

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ABSTRACT

In vitro evidence suggests that extracellular signal-regulated protein kinases (ERKs) and Akt (also referred to as protein kinase B) are among the myriad of intracellular signaling molecules regulated by opioid receptors. The present study examined the regulation of ERK and Akt activation in the nucleus accumbens and caudate putamen following acute and chronic morphine administration in the rat. ERK and Akt are activated by phosphorylation, hence the levels of phosphorylated ERK (pERK) and Akt (pAkt) as well as total levels of ERK and Akt protein were measured by Western blot analysis. Male Sprague-Dawley rats received either a single injection of morphine or twice daily injections of morphine for 6 or 10 days. Following acute morphine, pERK levels were significantly decreased in the nucleus accumbens but not in the caudate putamen. Naltrexone pretreatment prevented both the morphine-induced pERK down-regulation and pAkt up-regulation. Although reductions in pERK levels were evident after 6 days of morphine administration, no differences were observed in pERK levels after 10 days. In contrast to the up-regulation seen after acute morphine, pAkt levels in the nucleus accumbens were significantly decreased after chronic morphine administration. Thus, the differential activation patterns of both ERK and Akt after acute and chronic morphine administration could have important implications for understanding additional pathways mediating opioid signaling in vivo.

It is well established that upon repeated exposure to drugs of abuse, including the opiates, profound long-lasting neurochemical alterations occur in discrete brain regions. Changes in gene expression likely mediate these enduring adaptations in brain neurochemistry thereby contributing to dependence and tolerance and enabling drug addiction (Nestler, 2001). What remain unclear are the key intracellular signaling molecules that participate in regulating the alterations in gene expression by chronic opiate exposure. In addition to directly activating opioid receptors, morphine indirectly facilitates dopaminergic transmission in the limbic and midbrain regions of the brain through a disinhibition mechanism (Johnson and North, 1992). Both the mesolimbic and nigrostriatal pathways are principal areas in the brain that mediate drug reward ( Koob, 1992) and addictive-related behaviors (Graybiel et al., 1990). Heightened dopaminergic neurotransmission is thought to trigger the induction of transient immediate early genes ( Liu et al., 1994) and more enduring transcription factors (Nye and Nestler, 1996) through intricate pathways involving numerous intracellular molecules.

ERKs, members of the mitogen-activated protein kinase (MAPK) family, are vital regulators of signal transduction and are involved in various central processes ( Seger and Krebs, 1995; Atkins et al., 1998) including drug addiction (Berhow et al., 1996; Mazzucchelli et al., 2002). Morphine regulates ERK both in vitro and in vivo ( Fukuda et al., 1996; Eitan et al., 2003). For example, activation of μ opioid receptors in vitro stimulates a rapid and short-lived increase in ERK activity ( Li and Chang, 1996). In the central nervous system, both acute and chronic morphine results in brain region-specific modulation of ERK activity (Berhow et al., 1996; Schulz and Hollt, 1998; Eitan et al., 2003). Increases in total ERK44/42 protein have been observed in rat caudate putamen and locus coeruleus but not in the nucleus accumbens after chronic morphine (Ortiz et al., 1995).

It is thought that MAPK signaling is initiated by growth factors as well as other extracellular signals (Cobb and Gold-
time course experiment, rats received a single injection of morphine sulfate (50 mg/kg, s.c.) (generously supplied by the National Institute on Drug Abuse) or saline (1 ml/kg, s.c.) and were euthanized 20, 60, or 240 min postinjection. In some experiments, animals received an injection of the opioid receptor antagonist naloxone (50 mg/kg, s.c.; Sigma-Aldrich, St. Louis, MO) or saline 30 min before a single dose of morphine (50 mg/kg, s.c.) and euthanized either 1 or 4 h later.

**Chronic Morphine Administration.** In the chronic morphine studies, rats were administered morphine in an escalating dosing schedule that consisted of twice daily s.c. injections for 6 or 10 days (all injections were given at 9:00 AM and 6:00 PM). The intermittent morphine dosing schedules were as follows: 1) 6-day schedule: day 1, 10 mg/kg; day 2, 20 mg/kg; days 3 and 4, 40 mg/kg; and days 5 and 6, 80 mg/kg; or 2) 10-day schedule: days 1 and 2, 10 mg/kg; days 3 and 4, 20 mg/kg; days 5 and 6, 40 mg/kg; days 7 and 8, 80 mg/kg; and days 9 and 10, 120 mg/kg. Rats were euthanized 4 h after the final injection, which was given on the morning of the 6th or 10th day. Control rats were injected with an equal volume of saline according to the same schedule.

**Preparation of Brain Extracts and Immunoblotting Procedure.** Following drug administration, rats were euthanized by brief exposure to CO2 and decapitated in an unconscious state. The nucleus accumbens and caudate putamen were carefully and rapidly dissected on ice. Each brain region was immediately sonicated in 1% sodium dodecyl sulfate (SDS) boiling buffer, boiled for 5 min, aliquotted, and stored at −80°C until assayed. This and other boiling extraction procedures have been used previously for successful detection of phosphorylated proteins, including pERK (Ortiz et al., 1995; Berhow et al., 1996). Protein concentrations were assessed using the method of Lowry et al. (1951). Protein extracts (40–50 μg) were then subjected to SDS-polyacrylamide gel electrophoresis (10% Tris-HCl BioRad Ready Gels; Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. Membranes were blocked for 1 h in blocking buffer containing 5% nonfat dry milk and Tween 20-TBS and then incubated in the following antibodies: an anti-phospho-ERK antibody (1:1000, catalog no. 9101; Cell Signaling Technology Inc., Beverly, MA), anti-ERK (1:2000, catalog no. 610123; BD Biosciences Discovery Labware, Bedford, MA), anti-phospho-Akt, anti-Akt (1:1000, catalog no. 9271/9172; Cell Signaling Technology Inc.), and anti-tubulin antibody (1:40,000, catalog no. T8535; Sigma-Aldrich). All blots were incubated in an anti-tubulin antibody to correct for any differences in protein loading. After washes, the membranes were incubated in either anti-mouse or anti-rabbit antibody conjugated to horseradish peroxidase (1:5000; Vector Laboratories, Burlingame, CA) for 1 h. Immunoreactivity was detected using chemiluminescence using Supersignal West Pico chemiluminescent substrate (Pierce Chemical, Rockford, IL). Specific bands were quantified using densitometry (Fujifilm System, Valhalla, NY).

**Data Analyses.** Immunoreactivity of phosphorylated ERK and Akt were expressed as a ratio to total ERK or Akt immunoreactivity, respectively. One-way analysis of variance (ANOVA) followed by Bonferroni’s test for multiple comparisons was used to analyze the antagonist studies. The time course experiments, as well as the chronic morphine studies, were analyzed using Student’s t test (each time point, saline versus morphine). Significance was set at p < 0.05.

**Results**

**Acute Morphine Administration Down-Regulates pERK Levels in the Nucleus Accumbens, but Not in the Caudate Putamen.** Time Course: pERK44/42. Phosphorylated ERK expression was measured in the nucleus accumbens and caudate putamen by Western blot analysis using an anti-phospho-ERK antibody. In both brain regions two protein bands were detected, phosphorylated ERK42 (ERK2, 42 kDa) and phosphorylated ERK44 (ERK1, 44 kDa) (see Fig. 1 for representative immunoblots).

**Materials and Methods**

**Animals.** Male Sprague-Dawley (200–250 g, Harlan, Indianapolis, IN) rats were housed in groups of two per cage in a standard animal facility for 5 to 7 days before the start of the experiments (12-h light/dark cycle, lights on 7:00 AM). All rats were weighed daily and had access to food and water ad libitum. Each treatment group consisted of 5 to 11 rats. All animal procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Acute Morphine Administration.** A single s.c. saline injection was given to each rat 48 h before the start of the acute morphine studies to acclimate the animals to the injection procedure. In the
proteins share 90% sequence homology (Seger and Krebs, 1995). A standard protein curve (10–60 µg of nucleus accumbens extract) for pErk44 and pErk42 was generated from quantified immunoblots. A linear relationship between the optical density readings and amount of protein was obtained with a correlation coefficient of 0.97 and 0.96 for pErk44 and pErk42, respectively (data not shown). Figure 2 illustrates the levels of phosphorylated ERK44 and ERK42 immunoreactivity in the nucleus accumbens 20, 60, or 240 min postinjection of morphine or saline. The amount of phosphorylated ERK44 was significantly reduced at 240 min postinjections (51.28 ± 10% of control) (Student’s t test, morphine versus saline; p = 0.0153), whereas no significant differences in pERK42 levels were observed at 60 or 20 min (p = 0.0565 and p = 0.1741; n = 9–11/group, respectively) (Fig. 2a). Phosphorylated ERK44 levels were not decreased at 20 or 60 min (p > 0.05) following the morphine injection (Fig. 2b). At 240 min there was a reduction in pERK44 levels, although this did not reach statistical significance (76.5 ± 6% of control, p = 0.0829) (Fig. 2b).

Total ERK44 and ERK42 protein (ERK44/42: tubulin) in the nucleus accumbens of rats treated with morphine did not significantly differ from that of saline-injected rats (data not shown) indicating that the amount of phosphorylated or activated protein, but not total protein, was decreased after morphine administration.

In contrast to the nucleus accumbens, the phosphorylation of ERK44/42 in the caudate putamen of rats administered an acute dose of morphine did not differ from saline controls 20, 60, or 240 min postinjection (p > 0.05, Fig. 3a and b). Total ERK levels in the caudate putamen were also unchanged after morphine (data not shown).

**Acute Morphine Administration Up-Regulates pAkt Levels in the Nucleus Accumbens, but Not in the Caudate Putamen.** *Time Course: pAkt.* Phosphorylated Akt levels were measured in the nucleus accumbens and caudate putamen by Western blot analyses. A single protein band was detected (60 kDa) (see Fig. 1 for representative Western blots). Figure 2c depicts the levels of phosphorylated Akt in the nucleus accumbens after acute morphine administration. The level of phosphorylated Akt was significantly increased at 60 min postmorphine injection compared with that in saline-injected subjects (151.19 ± 10% of control) (Student’s t test, morphine versus saline; p = 0.0025), whereas no differences in pAkt levels were observed at 20 or 240 min (p = 0.9206 and p = 0.2839; n = 5–6/group, respectively) (Fig. 2c).

Unlike the morphine-induced up-regulation of pAkt observed in the nucleus accumbens, the levels of phosphorylated Akt in the caudate putamen of rats administered an acute dose of morphine did not differ from saline controls 20, 60, or 240 min postinjection (p > 0.05, Fig. 3c).

Total Akt protein (Akt: tubulin) in the nucleus accumbens or caudate putamen of rats treated with morphine did not significantly differ from that of saline-injected rats (data not shown) suggesting that the amount of phosphorylated protein, but not total protein, was increased after morphine administration.

**Naltrexone Pretreatment Blocked Morphine-Induced Down-Regulation of pERK44/42.** To investigate whether morphine-induced down-regulation of phosphorylated ERK42 in the nucleus accumbens is mediated by activation of opioid receptors, the opioid receptor antagonist, naltrexone (50 mg/kg, s.c.) or saline, was given 30 min before acute morphine or saline. Figure 4a illustrates mean (+S.E.M.) ratios of phosphorylated ERK42 to ERK across the four treatment groups 4 h postinjection. A one-way ANOVA revealed a significant treatment effect for levels of pERK42 immunoreactivity [F(3,35) = 3.126, p = 0.0380]. Post hoc assessments revealed that rats given acute morphine displayed significantly lower levels of pERK42 immunoreactivity than animals receiving naltrexone followed by morphine.

**Naltrexone Pretreatment Blocked Morphine-Induced Up-Regulation of pAkt.** Figure 4b depicts mean (+S.E.M.) ratios of phosphorylated Akt to Akt across the four treatment groups 60 min after drug administration. A one-way ANOVA revealed a significant treatment effect for levels of pAkt immunoreactivity [F(3,31) = 2.98, p = 0.0465]. Post hoc assessments revealed that rats given acute morphine displayed significantly higher levels of pAkt immunoreactivity than saline-injected control animals (177.58 ± 21% of control, n = 12, p < 0.05). No other post hoc comparisons yielded a significant effect.

**Chronic Morphine Administration Altered Levels of pERK44/42 and Akt Immunoreactivity in the Nucleus Accumbens.** Figure 5 depicts the effects of repeated morphine administration on pERK44 and pERK42 levels in the nucleus accumbens. Animals were given twice daily injections of either morphine or saline for 6 days. Four hours following the morning injection on day 6, the nucleus accumbens was taken and prepared for Western blot analysis. An unpaired Student’s t test revealed that the levels of pERK42 were significantly decreased compared with saline-injected animals (p = 0.0324, Fig. 5a). Phosphorylated ERK44 levels in the nucleus accumbens, as well as the active form of Akt, appeared reduced after morphine, albeit not significantly (p = 0.0572 and p = 0.2216; Fig. 5b and c, respectively).

Figure 6 illustrates the effects of chronic administration of morphine for 10 days on pERK44, pERK42, and pAkt immunoreactivity in the nucleus accumbens. Rats were injected twice daily with morphine or saline in an escalating dosage paradigm for 10 days (see Materials and Methods for morphine dosing schedules). Four hours after the morning injection on day 10, the nucleus accumbens was removed and

![Fig. 1.](https://example.com/fig1.png) Representative immunoblots of nucleus accumbens tissue from saline- (S) and morphine- (M) injected animals at each time point (20, 60, or 240 min). Bands represent phosphorylated ERK44/42, total ERK, tubulin, pAkt, and total Akt (from top to bottom). The molecular weights of the proteins are given on the left (kilodaltons).
processed for measurement of phosphorylated ERK. Phosphorylated ERK44 and ERK42 levels in rats treated chronically with morphine did not differ from rats given saline for 10 days (all *p* values > 0.05; Fig. 6, a and b). In addition, no changes in total ERK44 or ERK42 protein were observed (data not shown).

Levels of total or phosphorylated ERK44/42 in the caudate putamen were not altered following 10 days of morphine administration (data not shown).

For pAkt, however, a significant morphine-induced downregulation was observed after 10 days of repeated morphine administration.

**Fig. 2.** Time course evaluating pERK44, pERK42, and pAkt levels in the nucleus accumbens after a single morphine (50 mg/kg) or saline injection. Each time point (20, 60, or 240 min) represents the mean (±S.E.M.) ratio of pERK44 to ERK (a), pERK44 to ERK (b), or pAkt to Akt (c) expressed as percentage of controls (n = 9–11/group). *, *p* < 0.05 and **, *p* < 0.01 when each time point was evaluated using Student's *t* test (saline versus morphine).
administration \((p = 0.0362, \text{Fig. 6c})\). Total Akt levels were unchanged after chronic morphine.

**Discussion**

ERK is abundantly expressed in the central nervous system (Ortiz et al., 1995). The highest levels of ERK protein are found in the nucleus accumbens, caudate putamen, hippocampus, and frontal cortex, and, therefore, it is not surprising that ERK signaling is involved in a number of central processes including drug addiction. Results from the present study demonstrate that acute morphine administration significantly suppressed the activation of ERK42 in the nucleus.
Regulation of Phosphorylated ERK and Akt by Morphine

Fig. 4. Effects of naltrexone pretreatment on pERK42 and pAkt levels in the nucleus accumbens following acute morphine administration. The opioid receptor antagonist naltrexone (NX, 50 mg/kg, s.c.) or saline (Sal, 1 ml/kg, s.c.) was given 30 min before a single injection of either morphine (Mor, 50 mg/kg, s.c.) or saline (1 ml/kg, s.c.), and tissue was taken 4 h postinjection for the pERK42 experiment and 1 h after morphine for the pAkt experiment. Data are expressed as mean (±S.E.M.) ratios of pERK42 to total ERK (a) or pAkt to total Akt (b) levels across treatment groups. Statistical analyses were carried out by a one-way ANOVA with Bonferroni post hoc analyses. *p < 0.05.

A number of in vitro reports have demonstrated that activation of μ opioid receptors expressed in transfected cell lines stimulates a rapid and transient increase in ERK activity (Fukuda et al., 1996; Li and Chang, 1996). These reports from cell culture studies are inconsistent with our in vivo findings of morphine-induced suppression of ERK activation in the nucleus accumbens. In support of our results, a recent report demonstrated that acute morphine treatment decreases phosphorylated ERK levels in the nucleus accumbens of mice using immunohistochemistry (Eitan et al., 2003). Our results from Western blots extend these observations by showing selective regulation of pERK42. One possible explanation for the discrepancy between in vitro and in vivo studies in regard to ERK regulation by morphine may be the time course of ERK activation. In a Chinese hamster ovary cell line expressing μ opioid receptors, ERK activity peaks within 4 to 8 min following the addition of morphine and returns to basal levels within 1 h (Li and Chang, 1996). In the present study, the earliest time point assessed was 20 min postinjection, a time when no significant effects were seen. It is unknown whether the pattern of in vivo ERK regulation by morphine would have differed if assessed at earlier time points. Another potential explanation for the discrepancy between in vitro and in vivo findings is that the reduction in pERK levels by morphine may not be the result of direct μ opioid receptor signaling. Confocal microscopy analysis revealed that morphine-induced ERK regulation in the nucleus accumbens does not occur in cells that express μ opioid receptors (Eitan et al., 2003). Therefore, it seems likely that other systems triggered by opioid receptor stimulation are involved in the down-regulation of pERK after morphine administration in the nucleus accumbens. Although some in vitro reports have shown that cAMP-mediated signaling activates ERK (Vossler et al., 1997; Vincent et al., 1998), others have found that elevations in cAMP levels inhibit ERK activation (Sevetson et al., 1993).

Another system that may be involved in ERK regulation by morphine is the Akt pathway since it has been demonstrated that Akt negatively regulates MAPK/ERK activation (Zimmerman and Moelling, 1999; Galetic et al., 2003). The present study found that acute morphine activated Akt in the nucleus accumbens. Akt activation was most pronounced 60 min postinjection, and naltrexone prevented the morphine-induced up-regulation of pAkt. Therefore, it is possible that suppression of ERK activity occurred as a result of morphine-induced Akt activation. Further studies are needed to test this hypothesis. Of note, phosphorylated Akt levels in the nucleus accumbens were down after repeated morphine administration at a time when ERK activity was no longer suppressed.

First discovered by its link to the pathogenesis of human malignancy, Akt is a serine/threonine protein kinase that plays an important role in neuroprotection. A recent report by Matsuzaki et al. (2004) demonstrates that Akt overexpression protects cortical neurons from methamphetamine-induced neuronal death. Likewise, antiepileptic drug-induced neurodegeneration in the developing rat brain is associated with decreased levels of proapoptotic proteins, including activated Akt (Bittigau et al., 2003). The results of the present study demonstrate that Akt activity in the nucleus accumbens was increased following acute morphine exposure. Given this protein’s involvement in cell survival, it may be the case that increased activity of Akt by acute morphine is serving a neuroprotective role. With continued morphine administration, however, Akt activity goes down potentially leading to an increased vulnerability to neurotoxicity. Although the latter is speculation, the exact role of Akt in opioid-mediated signaling is uncertain and requires further investigation.

Previous studies have demonstrated that chronic morphine administration alters intracellular signaling molecules.
in a brain region-specific manner. For example, Nestler and colleagues (Terwilliger et al., 1991) showed that continuous morphine exposure decreases levels of the G-protein subunit, \(G_i\), and increases adenyl cyclase and protein kinase A activity in the nucleus accumbens and amygdala, but not in the substantia nigra, ventral tegmental area, or hippocampus. Furthermore, chronic morphine has been shown to significantly reduce cAMP response element-binding protein expression in the nucleus accumbens, but not in the caudate putamen (Widnell et al., 1996). Consistent with the above reports, the results of the present study demonstrate that phosphorylated ERK42 and Akt levels were differentially regulated in distinct brain regions following acute morphine administration. Morphine-induced regulation of ERK and Akt activity was evident in the nucleus accumbens, but not in the caudate putamen. Consistent with the present findings, Eitan et al. (2003) also demonstrated a differential regulation of ERK activation. Although decreases in activated ERK...
were found in the nucleus accumbens and angular cortex, increases were observed in the cingulate cortex, locus coeruleus, lateral amygdala, and sensory cortex. Taken together, both acute and chronic morphine administration have been shown to differentially regulate a number of molecules in a brain region-specific manner.

In the present study, morphine administered either acutely or twice daily for 6 days resulted in a significant reduction in ERK42 activity in the nucleus accumbens. When morphine administration was continued for 10 days, ERK activation was no longer suppressed. Therefore, during repeated intermittent escalating dose morphine, tolerance develops to the suppression of ERK activation by morphine. This tolerance is probably unrelated to changes in μ opioid receptor density. In vivo studies examining opioid receptor binding following chronic morphine have failed, for the most part, to document changes in opioid receptor levels (Hitzemann et al., 1974; Simon and Hiller, 1978). Therefore, this is not a likely explanation for the development of tolerance to the effects of morphine on ERK or Akt phosphorylation. Changes in calcium levels may underlie the regulation of ERK following acute and chronic morphine. Calcium plays a role in morphine’s actions (Narita et al., 2002; Quillan et al., 2002) and calcium levels are affected by morphine exposure (Yamamoto et al., 1978). Rat synaptosomal calcium content is decreased after a single injection of morphine (25 mg/kg) but is markedly increased after chronic morphine administration (Yamamoto et al., 1978). Calcium also has been shown to activate ERK (Cobb and Goldsmith, 1995). Therefore, one potential mechanism for acute morphine-induced down-regulation of ERK activity may be the reduction in calcium concentrations. With repeated morphine exposure, calcium levels increase and phosphorylated ERK levels return to normal. As noted above, the regulation of ERK activity by morphine could also be due to changes in the activated form of Akt independent of calcium concentrations. Although these are possibilities, it is likely that several mechanisms account for the drug-induced changes following long-term opiate exposure.

In summary, the present findings demonstrate enhancement of Akt activity accompanied by suppression of ERK activity selectively in the nucleus accumbens following a single injection of morphine. During chronic morphine administration, these effects were reversed in that Akt activity became suppressed and ERK activity returned to normal. Given the regulation of Akt and ERK activation by acute and chronic morphine, it is likely that these signaling cascades play a critical role in mediating opiate-induced long-lasting changes in brain neurochemistry and, therefore, may be involved in the development of opiate tolerance and/or addiction. Further delineation of the molecules involved in the regulation of Akt and MAPK/ERK signaling pathways by morphine and examining the possible cross talk between these systems will give us a better understanding of the mechanisms mediating morphine-induced signaling in the brain.

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