Brief Research Report

Differential Effects of Oxycodone, Hydrocodone, and Morphine on Activation Levels of Signaling Molecules

Michael A. Emery, BA, M. L. Shawn Bates, MA, Paul J. Wellman, PhD, and Shoshana Eitan, PhD

Department of Psychology, Behavioral and Cellular Neuroscience Program, and the Interdisciplinary Program in Neuroscience (TAMIN), Texas A&M University, College Station, Texas, USA

Correspondence to: Shoshana Eitan, PhD, Department of Psychology, Texas A&M University, 4235 TAMU, College Station, TX 77843, USA. Tel: 001-979-845-2508; Fax: 001-979-845-4727; E-mail: seitan@tamu.edu

Funding sources: M.A.E. and M.L.S.B. were supported by the Heep fellowship in Neuroscience awarded by the Texas A&M Institute for Neuroscience (TAMIN). This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Disclosure and conflicts of interest: The authors have no financial interests to disclose.

Abstract

Background. Opioids alter the responses of D2-like dopamine receptors (D2DRs), known to be involved in the pathology of addiction and other mental illnesses. Importantly, our recent results demonstrated that various opioids differentially modulate the behavioral responses of D2DRs.

Objective. To examine the effect of various opioids on striatal activation levels of Akt and ERK1/2, as well as the signaling responses of D2DRs following opioid exposure.

Methods. Mice were pre-treated with 20 mg/kg morphine, hydrocodone, oxycodone, or saline for 6 days. Twenty-four hours later, mice were injected with vehicle or a D2/D3 receptor agonist, quinpirole.

Thirty minutes later, dorsal striatum was collected and analyzed using Western blot.

Results. In morphine-pretreated animals, baseline Akt activation level was unchanged, but was reduced in response to quinpirole. In contrast, baseline Akt activation levels were reduced in mice pretreated with hydrocodone and oxycodone, but were unchanged in response to quinpirole. In mice pretreated with all opioids, baseline ERK2 activation levels were unchanged and increased in response to quinpirole. However, quinpirole-induced ERK2 activation was significantly higher than drug naive animals only in the morphine-pretreated mice.

Conclusions. Various opioids differentially modulate the baseline activation levels of signaling molecules, which in turn results in ligand-selective effects on the responses to a D2/D3 dopamine receptor agonist. This demonstrates a complex interplay between opioid receptors and D2DRs, and supports the notion that various opioids carry differential risks to the dopamine reward system. This information should be considered when prescribing opioid pain medication, to balance effectiveness with minimal risk.

Key Words. Opioid; Pain Management; Addiction; Abuse; Akt; ERK1/2

Introduction

Opioids are widely used for the management of moderate-to-severe pain and are commonly misused and abused. Exposure to opioid analgesics has been associated with altered responses of D2-like dopamine receptors (D2DRs) [1–5]. Our previous studies demonstrated that various opioids modulate the responses of D2DRs in differential degrees. Specifically, mice administered methadone in clinically relevant doses demonstrated significantly greater locomotor supersensitivity to quinpirole, a D2/D3 receptor agonist, than drug-naive mice. However, mice administered buprenorphine did not, even at super-
therapeutic doses [6]. Moreover, mice pretreated with oxycodone showed significantly greater locomotor supersensitivity to quinpirole than did mice pretreated with equianalgesic doses of morphine, while mice pretreated with equianalgesic doses of hydrocodone showed sensitivity between that of mice treated with morphine and oxycodone [7].

Disturbances in the responses of the D2DRs would be expected to have important implications for the abuse potential of opioids and other drugs of abuse. The D2DRs are known to be involved in the reinforcing properties of morphine [8], and in mediating drug-seeking behaviors in mice [9] and rats [10] undergoing morphine withdrawal. Specifically, the D2L splice variant of the D2DRs was demonstrated to be essential for the rewarding properties of morphine [11,12]. In addition, an association has been observed between D2 receptor genes and the abuse of various illicit drugs, as well as between D2 receptor genes and alcoholism [13–15]. Moreover, the D2DRs were also suggested to be involved in the pathophysiology of affective and psychiatric disorders [16–18].

Opioid receptors and D2DRs are known to signal via changes in the activation levels (i.e., phosphorylation levels) of both extracellular-signal-regulated kinases (ERKs) 1 and 2, also known as p44/p42 mitogen-activated protein kinase (MAPK) [19–23], and Akt, also known as protein kinase B (PKB) [24–26]. Thus, in the present study, we examined the differential effects of repeated administration of oxycodone, hydrocodone, and morphine on the activation/phosphorylation levels of Akt and ERK1/2. Additionally, we examined the differential effects of repeated administration of oxycodone, hydrocodone, and morphine on altering the signaling responses to quinpirole, a D2/D3 dopamine receptor agonist. We used equianalgesic oral doses of oxycodone, hydrocodone, and morphine that were previously established to provide equianalgesia [7]. As in our previous studies, the oral route was chosen to provide a more accurate and realistic translational model of human administration of oxycodone and hydrocodone and abuse of these prescription opioid medications.

Methods

Animals

All animal procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and were approved by the Texas A&M Institutional Animal Care and Use Committee. Male C57BL/6 mice, purchased from Harlan Laboratories (Houston, TX) were housed 3–5 per cage with food and water ad libitum. They were acclimated to the temperature-controlled (21 ± 2°C) vivarium with a 12 hour/12 hour light/dark cycle (lights on at 07:00) for 5–7 days prior to the start of experiments. Mice were treated and tested from postnatal day (PND) 27–35, because previous studies indicate that the effect on D2DR responses is more pronounced at these ages [27,28].

Drugs

Mice were administered oxycodone, hydrocodone, morphine (20 mg/kg, 10 mL/kg) or saline (10 mL/kg) once daily (9 AM) for six days via gavage. Drugs were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). These doses represent base plus salt concentrations. For oxycodone these doses roughly correspond to 18 mg/kg base. For hydrocodone, these doses roughly correspond to 13 mg/kg base. For morphine, these doses roughly correspond to 15 mg/kg base. These doses were selected to represent equianalgesic doses as was confirmed by tail withdrawal test in our previous study [7]. Please note that there are species- and age-dependent differences in the pharmacokinetics of opioids (absorbance, metabolism, etc.), as well as in the levels and distribution of opioid receptors, which are likely involved in the underlying response for these oral doses to represent equianalgesic doses.

Experimental Design

Mice (n=9–14 per group) were pre-treated with morphine, hydrocodone, oxycodone (20 mg/kg, 10 mL/kg) or saline (10 mL/kg) via gavage once daily for 6 days. Twenty-four hours following the final opioid/saline administration, mice were injected with quinpirole hydrochloride (10 mg/kg, 10 mL/kg, i.p.) or vehicle (100 µM hydrochloric acid, 10 mL/kg). Thirty minutes following quinpirole/vehicle injection, mice were deeply anesthetized with sodium pentobarbital (120 mg/kg, 10 mL/kg), brains were extracted, and flash-frozen in a bath of 2-methylbutane cooled on dry ice. Dorsal striatum tissue was dissected out of the flash-frozen brains and stored at −80°C until processed for Western blot. The number of animals in each experiment group was as follows: Saline-Vehicle n = 10; Saline-Quinpirole n = 9; Morphine-Vehicle n = 9; Morphine-Quinpirole n = 14; Hydrocodone-Vehicle n = 9; Hydrocodone-Quinpirole n = 11; Oxycodone-Vehicle n = 9; Oxycodone-Quinpirole n = 10.

Western Blots

Striatum tissue was homogenized in boiling 1% SDS plus 2 µM okadaic acid. Homogenized samples were then boiled for 10 minutes. Total protein concentration was determined for each sample using DC Protein Assay (Bio-Rad). Samples were diluted to 200 µg total protein in loading buffer and boiled for an additional 10 minutes. Samples were then loaded and resolved on 10% SDS-PAGE then transferred to PVDF membrane. Blots were probed using antibodies to p-P44/42 MAPK (pERK) (T202/Y204, 1:1,000, Cell Signaling Technology), P44/42 MAPK (Total ERK) (1:1,000, Cell Signaling Technology), pAkt (S473, 1:1,000, Cell Signaling Technology), and Akt (total Akt) (1:1,000, Cell Signaling Technology). Membranes were then incubated with.
HRP-conjugated secondary antibody (1:5,000, Bio-Rad), developed with ECL, and imaged using AlphaView software (Cell Biosciences). Phospho-protein signals were normalized to total protein signals on the same membrane.

**Data Analysis**

For each mouse’s striatum, the ratio of phospho-protein:total protein was calculated for Akt, ERK1, and ERK2, and expressed as a percentage. For each experimental group, the percent change was averaged for each protein. Data for the between-subjects factors of pretreatment (morphine, hydrocodone, oxycodone, or saline) and treatment (quinpirole vs vehicle) were analyzed using two-way ANOVA. Post hoc contrasts between each treatment group were computed using Bonferroni procedure. Differences with $P$-values of less than 0.05 were deemed statistically significant. Results are presented as mean ± SEM.

**Results**

**Akt**

Two-way ANOVA revealed a main effect of pretreatment with the various opioids ($F[3, 71] = 16.73, P < 0.0001$), a main effect of treatment with quinpirole ($F[1, 71] = 24.20, P < 0.0001$), and a significant interaction between pretreatment and quinpirole treatment ($F[3, 71] = 4.10, P < 0.01$). Bonferroni post hoc comparison revealed that morphine pretreatment did not alter Akt activation levels (i.e., phospho-Akt/total Akt) in the dorsal striatum as compared to levels observed in drug-naïve animals ($P > 0.05$, n.s.). In contrast, pretreatment with both oxycodone and hydrocodone significantly decreased Akt activation levels in the dorsal striatum ($P < 0.001$) (Figure 1).

Additionally, differential responses to quinpirole were also observed in mice pretreated with various opioids. Quinpirole administration did not significantly alter Akt activation levels in drug-naïve animals ($P > 0.05$, n.s.). In contrast, quinpirole administration reduced Akt activation levels in morphine pretreated animals ($P < 0.001$). However, no further reduction in Akt activation levels (from baseline) was observed in mice pretreated with hydrocodone or oxycodone in response to quinpirole ($P > 0.05$, n.s.).

**ERK1/p44 MAPK**

Two-way ANOVA revealed a main effect of pretreatment with the various opioids ($F[3, 73] = 3.02, P < 0.05$). However, there was no main effect of treatment with quinpirole ($F[1, 73] = 1.24, P > 0.05$), and no significant interaction between pretreatment and quinpirole treatment ($F[3, 73] = 0.89, P > 0.05$). Bonferroni post hoc comparison revealed that opioid pretreatment did not significantly alter ERK1 activation levels (i.e., phospho-ERK1/total ERK1) in the dorsal striatum as compared to levels observed in drug-naïve animals ($P > 0.05$, n.s.). Additionally, quinpirole administration did not significantly alter ERK1 activation levels in either the drug-naïve or in the opioid-pretreated animals ($P > 0.05$, n.s.) (Figure 2).

**ERK2/p42 MAPK**

Two-way ANOVA revealed a main effect of pretreatment with the various opioids ($F[3, 73] = 10.86, P < 0.0001$), a
main effect of treatment with quinpirole ($F_{[1, 73]} = 27.04, P < 0.0001$), but no significant interaction between pretreatment and quinpirole treatment ($F_{[3, 73]} = 1.21, P > 0.05$). Bonferroni post hoc comparison revealed that opioid pretreatment did not alter ERK2 activation levels (i.e., phospho-ERK2/total ERK2) in the dorsal striatum as compared to levels observed in drug-naive animals ($P > 0.05$, n.s.). Although visually there seems to be a tendency towards increased ERK2 activation levels in morphine-pretreated animals, and a tendency towards decreased ERK2 activation levels following oxycodone pretreatment, nonetheless even a statistical trend was not observed.

Quinpirole administration did not significantly alter ERK2 activation levels in drug-naive animals ($P > 0.05$, n.s.). In contrast, increased ERK2 activation in response to quinpirole was observed in mice pretreated with opioids ($P < 0.05$). However, ERK2 activation level was significantly higher than drug naïve animals following quinpirole only in the morphine-pretreated mice ($P < 0.001$).

Discussion

In the current study, we observed that pretreatment with equianalgesic doses of oxycodone, hydrocodone, and morphine results in differential patterns of phosphorylation (activation) of the cellular signaling proteins Akt and ERK1/2. Moreover, pretreatment with these opioids have differential effects on the signaling responses to quinpirole, a D2/D3 receptor agonist. These findings suggest a complex interplay of signaling between opioid receptors and D2DRs within the striatum, and provide further evidence supporting the notion that various opioids carry differential risks to the dopamine reward system.

Various opioids display differential affinities to the three opioid receptors, μ-, δ-, and κ-opioid receptor (MOR, DOR, and KOR, respectively). Specifically, while all three drugs are most selective for MOR, morphine’s affinity for DOR and KOR, while lower than its affinity for MOR, is notably higher than either hydrocodone or oxycodone, which have very low affinities for DOR and KOR [29,30]. Additionally, various opioids were demonstrated to differentially modulate downstream signaling even when binding to and activating the same receptor [31–33], a phenomenon termed ligand-directed signaling or biased agonism. This is likely related to the ability of G-protein coupled receptors to signal via different mechanisms and the differential abilities of various opioids to activate those various mechanisms. These mechanisms include the canonical G-protein coupled signaling (GPCS) and the non-canonical G-protein independent signaling which involves the scaffolding proteins beta-Arr2 ($\beta$-Arr2). For example, mice lacking $\beta$-Arr2 show no development of tolerance to morphine, as well as an attenuation of naloxone-induced withdrawal [34]. These mice showed normal tolerance and withdrawal when treated with oxycodone, methadone, or fentanyl, which are known to signal via the same receptor. Different opioids have been shown to activate ERK differently [35]. Additionally, oxycodone and morphine have been demonstrated to differentially activate MOR and have differential pain relieving efficacies in various conditions [36]. Moreover, various opioids can have very different levels of side effects, despite equal potency to relieve pain [7,37]. Thus, the differential effects of the various opioids on activation levels of downstream signaling molecules might be attributed to differential effects at the various opioid receptors as well as the differential effects of various opioids on activation of GPCS and β-Arr-dependent signaling at the same receptor.

The differential modulation of activation levels of signaling molecules by various opioids are likely to account for the differential effects of various opioids on the signaling responses to a D2DR agonist. Like opioid receptors, D2DRs are G protein-coupled receptors that can signal through both GPCS and β-Arr-dependent signaling. β-Arr is known to be involved in the desensitization of the ability of D2DRs to signal via GPCS [38–40]. However, β-Arr2 was also demonstrated to play a role in D2DR signaling by forming a complex that inactivates (decreases phosphorylation of) the cell signaling protein Akt [25,41,42]. This study shows that pretreatment with oxycodone or hydrocodone is accompanied by a decreased phosphorylation of Akt. However, quinpirole did not alter Akt activation state in the oxycodone- or hydrocodone-pretreated animals. This might suggest that prior treatment with oxycodone or hydrocodone decreases the ability of the D2DRs to signal via β-Arr-dependent mechanism. GPCS through D2DRs is subject to a faster desensitization rate as compared to a β-Arr-dependent mechanism [25]. Thus, this result suggests that pretreatment with oxycodone or hydrocodone will lead to an increase in the desensitization rate of the signaling through D2DRs. Importantly, morphine did not affect Akt activation levels, which might preserve D2DRs’ ability to signal via β-Arr-dependent mechanism, i.e. quinpirole inactivated Akt in morphine-pretreated mice. Moreover, the effect of quinpirole to reduce Akt phosphorylation levels was significantly enhanced in the morphine-pretreated animals as compared to drug-naive mice. This suggests that morphine pretreatment not only did not decrease, but might have enhanced the ability of D2DRs to signal via β-Arr, in a mechanism yet to be identified.

In this study, repeated administration of opioids alone did not significantly alter phosphorylation of ERK1 or ERK2 in the dorsal striatum. Although it should be noted that a trend toward increased ERK2 activation levels was observed in morphine-pretreated animals, and a trend toward decreased ERK2 activation levels was observed following oxycodone pretreatment, neither reached statistical significance. None of the opioids, repeatedly administered followed by an acute challenge with quinpirole resulted in activation of ERK1. However, all of the opioids followed by quinpirole resulted in activation of ERK2, relative to levels seen in opioid-treated animals that were not administered...
quinpirole. In morphine-pretreated animals, this was also increased relative to opioid-naïve protein levels following quinpirole.

D2DRs were shown to activate ERK [43–45]. Activation of ERK by D2DRs was reported to be almost exclusively in a rapid, G-protein-dependent manner, that returns to baseline within 15 minutes [46,47]. However, in this study, significant activation of ERK2 was observed in morphine-pretreated mice at 30 minutes post-quinpirole. This suggests that the quinpirole-induced ERK activation observed in this study might be via a yet to be identified βArr-dependent mechanism. Importantly, although quinpirole-induced ERK2 activation was also observed in oxycodone and hydrocodone pretreated animals, it was not significantly above control levels. Thus, further studies are needed to elucidate potential differences in the effect of various opioids on the abilities of D2DRs to signal via GPCs and βArr.

This study demonstrates that, at equianalgesic dosages, various opioids differentially alter striatal Akt and ERK activation levels in ways unique to individual opioids. These differential modulations of signaling are suggested to account for the distinct effects of various opioids on the signaling responses, as well as the locomotor response of D2DRs [7]. The general risks associated with opioid use and abuse have long been understood, and various opioids carry differing levels of efficacy and abuse potential. Here, we show differential effects of various opioids on striatal signaling molecules and the dopamine reward pathway. This information should be considered when prescribing opioid pain medication, to balance effectiveness with minimal risk.

References
16 Seeman P. All roads to schizophrenia lead to dopamine supersensitivity and elevated dopamine D2(high) receptors. CNS Neurosci Ther 2011;17:116–32.


Emery et al.


