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Tolerance to the anti-nociceptive and hypothermic effects of morphine are mediated by multiple isoforms of c-Jun N-terminal Kinase

Matthew B Yuill^{1,2,3,†}, Michael L Zee^{1,†}, David Marcus¹, and Daniel J Morgan^{1,2,3,*}

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Abstract

The abuse and overdose of opioid drugs are growing public health problems, globally. While progress has been made towards understanding the mechanisms governing tolerance to opioids, the exact cellular machinery involved remains unclear. However, there is growing evidence to suggest that c-Jun N-terminal Kinases (JNKs) play a major role in mu opioid receptor regulation and morphine tolerance. In this study, we aimed to determine the potential roles of different JNK isoforms in development of tolerance to the anti-nociceptive and hypothermic effects of morphine. We used the hotplate and tail-flick tests for thermal pain to measure tolerance to the anti-nociceptive effects of once daily sub-cutaneous injections with 10 mg/kg morphine. Body temperature was also measured to determine tolerance to the hypothermic effects of morphine. Tolerance to morphine was assessed in wild-type mice and compared to single knockout (KO) mice each lacking of the JNK isoforms (JNK1, JNK2, or JNK3). We found that loss of each individual JNK isoform causes impairment in tolerance for the anti-nociceptive and hypothermic effects of daily morphine. However, disruption of JNK2 seems to have the most profound effect on morphine tolerance. These results demonstrate a clear role for JNK signaling pathways in morphine tolerance. This compliments previous studies suggesting that the JNK2 isoform is required for morphine tolerance, but additionally presents novel data suggesting that additional JNK isoforms also contribute to this process.

Keywords

tolerance; morphine; JNK; anti-nociception; opioids; mu opioid receptor; desensitization; GPCR

*To whom correspondence should be sent: dmorgan1@hmc.psu.edu.

†These authors contributed equally

Conflicts of interest

There are no conflicts of interest.

Introduction

Opioid drugs such as morphine, fentanyl, and oxycodone, remain a preferred and commonly prescribed class of drug for pain management [1]. While they demonstrate remarkable efficacy for treating acute pain, there are several limitations to their use. Opioid drugs are associated with rapid development of tolerance and also high abuse potential. Despite this, they remain the default approach to treatment of many chronic pain conditions. As a result, abuse and overdose of opioids are the fastest growing issues for narcotic drugs in the United States. This is punctuated by a tripling in the rate of prescription opioid overdose in just two decades [1].

The opioid system is comprised of multiple opioid receptors, each with a unique distribution and function. The anti-nociceptive effects of many opioid drugs are mediated through the mu-opioid receptor (MOR, [2]), making this receptor one of the most extensively studied G protein-coupled receptors (GPCRs, [3]). MOR is expressed in numerous regions of the central nervous system, among them: the dorsal horn of the spinal cord, the periaqueductal gray, and the cortex. As a result, it is a crucial receptor in the modulation of pain circuitry at both the supra-spinal and spinal level. Activation of MOR also causes euphoria, which plays a role in the high addictive liability of opiates [4, 5]. Opioid dependence is compounded by the development of tolerance, which can cause an escalation of dose and a progression to dependence [6]. Due to the diminishing efficacy of repeated opioid use and the resulting prevalence and severity of opioid drug dependence, understanding the mechanisms behind tolerance to these drugs is of significant interest.

There are multiple mechanisms that may act to mediate opioid tolerance [5, 6]. One process termed desensitization occurs with the loss of MOR-effector coupling following opioid administration, and appears to be mediated through phosphorylation of the receptor and subsequent recruitment of β -arrestin [7]. Several previous studies have demonstrated the loss of MOR-effector coupling following agonist treatment [6, 8]. This phenomena appears to result from phosphorylation of MORs at C-terminal threonine 370 and/or serine 375 [9]. These phosphorylation events are thought to be the result of G-protein coupled receptor kinase (GRK)2 and/or GRK3 activity, and cause β -arrestin 2 recruitment [7]. β -arrestin 2 causes both the uncoupling of MOR from its associated G proteins, and can result in endocytosis [6, 10]. This sequence of events occurs in an agonist-specific manner, and some studies suggest that internalization of MOR does not occur efficiently in response to morphine [10]. Several studies have demonstrated that GRK is responsible for fentanyl but not morphine tolerance [11, 12].

Recent work has shown that tolerance to morphine is attenuated through the use of the JNK inhibitor SP600125 [13–16]. SP600125 is an anthrapyrazolone capable of inhibiting JNK1, JNK2, and JNK3 with high affinity [17]. It has been demonstrated that systematic administration of sp600125 prevents morphine-stimulated phosphorylation of JNK in the spinal cord, resulting in attenuation of tolerance to the anti-nociceptive and anti-allodynic effects of morphine [14, 15]. However, use of this JNK inhibitor is non-selective for the different JNK isoforms and does not allow determination of which isoform(s) are responsible for morphine tolerance. It has been suggested by recent studies that JNK2 is

required for tolerance to the anti-nociceptive effects of morphine [18]. However, no studies thus far have compared morphine tolerance in JNK1, JNK2, and JNK3 Knock-Out (KO) mice.

Therefore, this novel work examined tolerance to the anti-nociceptive effects of morphine in JNK1, JNK2, and JNK3 KO mice. We tested the hypothesis that tolerance to morphine would be disrupted in JNK2 KO mice. While this was indeed the case, we also found that JNK1, (and to a lesser extent) JNK3 also contribute to morphine tolerance. This finding represents a novel and significant addition to recent work demonstrating the role of JNK signaling in morphine tolerance and MOR regulation.

Methods

Subjects

Experiments were carried out with wild-type C57BL6/J mice obtained from Jackson Laboratories (Bar Harbor, Maine), and three strains of JNK mutant mice. Mice lacking either JNK1, JNK2, or JNK3 were generously provided by Dr. Charles Chavkin at the University of Washington School of Medicine. The generation of JNK1 KO [19], JNK2 KO [20], and JNK3 KO mice [21] have been described previously.

All mice were kept on a standard 12:12h light-dark cycle with *ad libitum* access to standard mouse chow and water. All animal care and procedures conformed to the guidelines of the National Institutes of Health on the Care and Use of Animals, and were approved by the Institutional Animal Care and Use Committee of the Penn State University College of Medicine.

Drugs

Morphine sulfate was obtained from the National Institute on Drug Abuse Drug Supply (Bethesda, MD). Morphine was dissolved in isotonic 0.9% saline and administered subcutaneously in an injection volume of 10 mL/kg body weight.

Tail-flick and hotplate anti-nociception

Tail-flick anti-nociception was assessed with a Columbus Instruments TF-1 analgesia meter (Columbus, OH). The apparatus was calibrated to elicit an average tail-flick latency of 3–4 s in wild-type mice. A cutoff time of 10 seconds was used to prevent tissue damage. Mice were restrained, allowing their tail to be exposed to the radiant heat source. The latency until reflexive tail withdrawal from the heat source was recorded.

Hotplate anti-nociception was measured with a Columbus Instruments hotplate set to 55° C (Columbus, OH). A 30s cutoff was used to avoid paw damage. The latency between an animal being placed on the hotplate and withdrawal from the heated surface (jumping, licking of paws) was recorded. Both the hotplate and tail-flick responses were used to calculate the percentage of maximal possible effect (%MPE). This value was calculated using the formula $\%MPE = [(post\text{-}drug\text{ latency} - pre\text{-}drug\text{ latency}) / (cutoff\text{ time} - pre\text{-}drug\text{ latency})] \times 100$. These procedures and calculations have been described previously [13, 22].

Measurement of body temperature

Body temperature was measured using a mouse rectal thermometer probe (Physitemp, Clifton, NJ). Hypothermia was reported as a % change in body temperature between pre-drug and post-drug measurements, as demonstrated by the formula: (% BT)=[(pre-morphine temperature)–(post-morphine temperature)]/[pre-morphine temperature] x 100.

Procedures

Anti-nociception and hypothermia were measured in groups of mice [wild-type (n=17), JNK1 KO (n=15), JNK2 KO (n=14), and JNK3 KO (n=20)] receiving daily sub-cutaneous (s.c.) injections of morphine (10 mg/kg × 10 days). Each day mice were tested for body temperature, tail-flick and hotplate latency immediately prior to, and one hour after morphine injection.

Data analysis

Anti-nociception and hypothermia values were expressed as mean ± SEM. Values were analyzed using two-way ANOVA (genotype x day) followed by Bonferroni *post-hoc* testing. Differences in baseline tail-flick and hotplate latencies and basal body temperatures were analyzed by one-way ANOVA. Analyses of the initial responses to the first injection of morphine were also analyzed by one-way ANOVA. Analyses were performed using PRISM6 statistical software (Graphpad, La Jolla, CA). P<0.05 was considered significant.

Results

Tolerance to the anti-nociceptive effects of 10 mg/kg morphine

Baseline tail-flick latencies differed between WT (3.35±0.09 sec) and JNK1 KO (4.39±0.28 sec; p<0.001), JNK2 KO (4.04±0.20 sec; p<0.001), and JNK3 KO (4.34±0.16 sec; p<0.0001) mice. However, the response to the first injection of morphine was not different ($F_{3,70} = 1.164$, P=0.33) between WT (90.9±4.6% MPE), JNK1 KO (90.0±3.4% MPE), JNK2 KO (98.7±1.2% MPE), and JNK3 KO (94.4±2.4% MPE) mice.

Wild-type mice rapidly developed tolerance to the anti-nociceptive effects of daily s.c. morphine (10 mg/kg) injections in the tail-flick test (Fig 1). Tolerance to the anti-nociceptive effect of 10 mg/kg morphine, in the tail-flick test, developed in a time-dependent manner ($F_{9,637} = 12.77$, P<0.0001) that was also dependent on genotype ($F_{3,637} = 105.6$, P<0.0001). There was also a significant day x genotype interaction effect ($F_{27,637} = 3.26$, P<0.0001). Bonferroni *post-hoc* tests show that tolerance to morphine, in the tail-flick test, was different between wild-type mice and JNK1 KO (p<0.0001), JNK2 KO (p<0.0001), and JNK3 KO mice (p<0.0001). However, post-hoc testing also indicated that JNK2 KO mice developed less tolerance than either JNK1 KO (p<0.0001) or JNK3 KO (p<0.001) mice. There was no difference in morphine tolerance between JNK1 KO and JNK3 KO mice.

Baseline hotplate latencies differed between WT (7.88 ± 0.57 sec.) and JNK1 KO (11.48±0.77 sec; p<0.001), JNK2 KO (6.84±0.57 sec; p<0.001), and JNK3 KO (5.96±0.26 sec; p<0.0001) mice. However, the response to the first injection of morphine was not

different ($F_{3,64} = 1.747$, $P=0.17$) between WT ($67.7 \pm 6.1\%$ MPE), JNK1 KO ($83.9 \pm 5.7\%$ MPE), JNK2 KO ($67.1 \pm 8.9\%$ MPE), and JNK3 KO ($65.3 \pm 2.4\%$ MPE) mice.

Similar results were observed for tolerance to anti-nociceptive effects of 10 mg/kg morphine, in the hotplate test (Fig 2). All mutant mouse groups showed delayed onset of tolerance to the anti-nociceptive effects of morphine, relative to wild-type mice. Two-way ANOVA analysis reveals main effects of genotype ($F_{3,577} = 39.39$, $P<0.0001$) and time ($F_{9,577} = 32.2$, $P<0.0001$). However, no significant day x interaction effect was detected ($F_{27,577} = 1.28$, $P=0.155$).

Tolerance to morphine-induced hypothermia

Basal body temperature was also different between WT (38.6 ± 0.1 °C), and JNK1 KO (37.7 ± 0.1 °C; $p<0.001$), JNK2 KO (36.8 ± 0.1 °C; $p<0.0001$), and JNK3 KO (37.1 ± 0.1 °C; $p<0.0001$) mice. There were differences in basal body temperature between JNK1 and JNK2 KO mice ($p<0.01$) as well as JNK1 KO and JNK3 KO mice ($p<0.05$). There were also genotype differences in the hypothermic response to morphine ($F_{3,68} = 6.537$, $P=0.0006$), with Bonferroni post-tests revealing that the main effect of genotype was due to differences between WT ($-4.2 \pm 0.7\%$ change) and JNK3 KO ($-1.6 \pm 0.5\%$ change) mice ($p<0.01$) and also between JNK1 ($-4.6 \pm 0.4\%$ change) and JNK3 KO mice ($p<0.01$). There were no differences between JNK2 KO mice ($-2.5 \pm 0.7\%$ change) and the other genotypes.

Rapid tolerance developed to the hypothermic effects of once daily 10 mg/kg morphine in wild-type mice (Fig 3). Tolerance to the hypothermic effect of 10 mg/kg morphine developed in a time-dependent manner ($F_{9,607} = 18.44$, $P<0.0001$) that also depended on genotype ($F_{3,607} = 31.24$, $P<0.0001$). There was also a significant day x genotype interaction effect ($F_{27,607} = 3.33$, $P<0.0001$). Bonferroni *post-hoc* tests show that tolerance to morphine was different between wild-type mice and JNK1 KO ($p<0.001$), JNK2 KO ($p<0.0001$), and JNK3 KO mice ($p<0.05$). However, post-hoc testing also indicated that JNK 2 KO mice also developed less tolerance to morphine hypothermia than JNK3 KO ($p<0.05$) mice. There was also a difference in tolerance to the hypothermic effects of morphine between JNK1 KO and JNK3 KO mice ($p<0.0001$). However, there was no difference in morphine tolerance between JNK1 KO and JNK2 KO mice.

Discussion

The primary finding of this study is that all three isoforms of JNK (JNK1, JNK2, and JNK3) are involved in tolerance to the anti-nociceptive and hypothermic effects of morphine. Although deletion of JNK2 had the greatest impact on morphine tolerance, the loss of JNK1 or JNK 3 in KO mice also attenuated morphine tolerance. Our results suggest that JNK3 plays the least prominent role in morphine tolerance. These findings are novel in showing that all three forms of JNK contribute to morphine tolerance, and they are consistent with previous work demonstrating that JNK2 can mediate tolerance to morphine [11, 18].

Interestingly, disruption of JNK2 appears to have a greater impact on tolerance than the other isoforms in the tail-flick but not the hotplate test. This may be due to the mediation of tail-flick response primarily by spinal circuitry, versus the mediation of hotplate response by

both spinal and supraspinal circuitry [23]. It should also be noted that hotplate responses are not reflexive, and thus there is the possibility that they might have been altered by a learning effect due to repeated testing. While the JNK3 KO mice showed a significant diminishment in hypothermic tolerance relative to wild-type mice, it is important to note that JNK3 KO mice exhibit a profound difference in the hypothermic response to the first morphine injection. Further investigation is warranted to determine the cause for reduced morphine-induced hypothermia in JNK3 KO mice.

Morphine appears to be different from many other opioids, as it does not produce MOR desensitization through the common GRK/ β -arrestin pathway [11, 12]. As such, the question of how JNK is mechanistically involved in morphine tolerance is of significant interest. Morphine results in an increase in spinal JNK phosphorylation through a protein kinase C (PKC)-dependent process [11]. Furthermore, this effect was abolished in JNK2 KO, but not JNK1 KO or JNK3 KO mice [18]. Both of these studies also demonstrated that JNK2 KO mice have a reduction in acute morphine tolerance. Despite these results, it should not be concluded that morphine tolerance is strictly the result of JNK2 phosphorylation by PKC. While other opioids such as fentanyl also cause JNK2 phosphorylation, tolerance to fentanyl is JNK-independent [13, 18]. Moreover, while spinal MOR-desensitization by morphine requires JNK2, the desensitization of MOR in the locus coeruleus appears to be JNK-independent [24].

Our finding that all three JNK isoforms impact morphine tolerance suggests that morphine tolerance is not always mediated by JNK2 alone. This raises the question of how the other two JNK isoforms might be involved. While all three JNK isoforms are expressed in parts of the CNS, they have distinct localization patterns and levels of expression *in vivo* [25]. It is an intriguing possibility that tolerance to specific effects of morphine might be mediated to varying extents by different JNK isoforms. However, it should also be noted that knockout of individual JNK isoforms may result in compensatory upregulation of the remaining isoforms. Moving forward, experiments comparing JNK expression and phosphorylation in wild-type and knockout mice would help shed light on potential compensatory effects. Additional investigation is needed to fully understand how JNK signaling pathways contribute to morphine tolerance and MOR desensitization.

Conclusions

Our findings suggest a possible role for multiple isoforms of JNK on the development of tolerance to morphine. We found a diminishment of tolerance to the anti-nociceptive and hypothermic effects of morphine not only in JNK 2 KO mice, but also JNK 1 KO and JNK 3 KO mice. This may be a significant factor in the developing picture regarding the mechanisms responsible for morphine tolerance. Determining the nature through which these signaling pathways act on MOR will be an important task if JNK pathways are to become a therapeutic target in pain management and opioid abuse.

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Figure 1.

Tolerance to the anti-nociceptive effects of morphine in the tail-flick test is altered in mutant mice lacking JNK 1, JNK 2, or JNK 3.

Wild-type (WT; squares and line), JNK1 KO (circles and dotted line), JNK2 KO (triangles and dashed line), and JNK3 KO (diamonds and dotted line) mice were injected (s.c.) with 10 mg/kg morphine once daily for ten days. All three knockout mouse lines showed impaired tolerance to the anti-nociceptive effects of morphine, in the tail-flick test, relative to wild-type animals ($p < 0.0001$). Data are expressed as mean $\pm$ SEM ($n = 15-20$ per group).

Figure 2.

Tolerance to morphine-induced anti-nociception, in the hotplate test, is altered in mutant mice lacking JNK 1, JNK 2, or JNK 3.

Wild-type (WT; squares and line), JNK1 KO (circles and dotted line), JNK2 KO (triangles and dashed line), and JNK3 KO (diamonds and dotted line) mice were injected (s.c.) with 10 mg/kg morphine once daily for ten days. All three knockout mouse lines showed impaired tolerance to the anti-nociceptive effects of morphine, in the hotplate test, relative to wild-type animals ($p < 0.0001$). Data are expressed as mean $\pm$ SEM ($n = 15-20$ per group).

Figure 3.

Tolerance to morphine-induced hypothermia is altered in mutant mice lacking JNK 1, JNK 2, or JNK 3.

Wild-type (WT; squares and line), JNK1 KO (circles and dotted line), JNK2 KO (triangles and dashed line), and JNK3 KO (diamonds and dotted line) mice were injected (s.c.) with 10 mg/kg morphine once daily for ten days. All three knockout mouse lines showed impaired tolerance to the hypothermic effect of morphine relative to wild-type animals ($p < 0.0001$). Data are expressed as mean $\pm$ SEM ($n = 15$ – 20 per group).