Ligand-directed c-Jun N-terminal kinase activation disrupts opioid receptor signaling

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Ligand-directed signaling has been suggested as a basis for the differences in responses evoked by otherwise receptor-selective agonists. The underlying mechanisms are not understood, yet clearer definition of this concept may be helpful in the development of novel, pathway-selective therapeutic agents. We previously showed that κ-opioid receptor activation of JNK by one class of ligand, but not another, caused persistent receptor inactivation. In the current study, we found that the μ-opioid receptor (MOR) could be similarly inactivated by a specific ligand class including the prototypical opioid, morphine. Acute analgesic tolerance to morphine and related opioids (morphine-6-glucuronide and buprenorphine) was blocked by JNK inhibition, but not by G protein receptor kinase 3 knockout. In contrast, a second class of μ-opioids including fentanyl, methadone, and oxycodone produced acute analgesic tolerance that was blocked by G protein receptor kinase 3 knockout, but not by JNK inhibition. Acute MOR desensitization, demonstrated by reduced D-Ala^2-Met^5-Glyol-enkephalin–stimulated [^{35}S]GTPγS binding to spinal cord membranes from morphine-pretreated mice, was also blocked by JNK inhibition; however, desensitization of D-Ala^2-Met^5-Glyol-enkephalin–stimulated [^{35}S]GTPγS binding following fentanyl pretreatment was not blocked by JNK inhibition. JNK-mediated receptor inactivation of the κ-opioid receptor was evident in both agonist-stimulated [^{35}S]GTPγS binding and opioid analgesic assays; however, gene knockout of JNK 1 selectively blocked κ-receptor inactivation, whereas deletion of JNK 2 selectively blocked MOR inactivation. These findings suggest that ligand-directed activation of JNK kinases may generally provides an alternate mode of G protein–coupled receptor regulation.

acute analgesic tolerance | biased agonism | collateral agonist | desensitization

The concept of ligand-directed signaling, also called "biased agonism" or "functional selectivity," at G protein-coupled receptors (GPCRs) has been developing since it was originally shown that two different agonists can induce different conformations of the receptor and activate distinct signaling pathways (1–5). This concept has been extended to opioid receptor signaling in a study defining the mechanism responsible for long-lasting κ-opioid receptor (KOR) antagonism (6) and in studies of ligand-directed μ-opioid receptor (MOR) trafficking (7). Three classes of KOR ligands have been distinguished: typical agonists that activate G protein signaling to produce analgesia and other G protein-mediated actions, conventional antagonists (e.g., naloxone and buprenorphine) that competitively inhibit agonist binding, and a third class of highly selective KOR ligands (e.g., norBNI, JDTic, and GNTI) that activate the JNK family of MAPKs to produce long-lasting inactivation of KOR signaling that persists weeks after the drug is cleared (6, 8). How JNK activation disrupts KOR signaling is under active investigation and not yet known, but we asked if this form of ligand-directed signaling might also be responsible for effects at other GPCRs. The MOR is structurally similar to KOR (9), and we predicted that MOR inactivation by this JNK mechanism might resemble receptor desensitization. Although many of the μ-selective opioid agonists desensitize MOR through G protein receptor kinase (GRK) and β-arrestin–dependent internalization (10, 11), earlier studies have noted that the prototypical μ-opioid agonist morphine fails to efficiently internalize MOR, yet still produces acute analgesic tolerance (7, 12–14).

In this study we examined the role of JNK in regulating opioid receptor signaling and found that activation of the JNK pathway is required for acute tolerance to morphine and related opioids, but not fentanyl or other agonists that efficiently activate GRK/β-arrestin–dependent internalization. These results extend the concept of ligand-directed signaling by distinguishing two classes of MOR ligands that inactivate MOR through either GRK3 or JNK2 mechanisms.

Results

GRK3$^{-/-}$ Mice Develop Acute Analgesic Tolerance to Morphine but Not Fentanyl. We previously showed that analgesic tolerance caused by prolonged treatment with fentanyl, but not morphine was attenuated in GRK3-KO mice ($^{-/-}$) (15). To determine whether GRK3 also mediated acute analgesic tolerance, male WT C57BL/6 mice were challenged twice with agonist and tail-withdrawal latencies measured. Fentanyl produced a significant, but submaximal, increase in tail-withdrawal latencies that persisted for approximately 120 min (Fig. 1*A*). A second fentanyl challenge 180 min after the first injection produced a strongly attenuated response, demonstrating acute analgesic tolerance. GRK3$^{-/-}$ mice showed an initial analgesic response that was not significantly different from littermate WT controls (GRK3$^{+/+}$); however, the response to the second fentanyl injection was not significantly different from the first ($P > 0.05$; Fig. 1*A*), indicating that GRK3$^{-/-}$ mice do not show acute analgesic tolerance to fentanyl. Similar to fentanyl, treatment with morphine of either GRK3$^{+/+}$ or GRK3$^{-/-}$ littermates produced equivalent, submaximal increases in tail-withdrawal latencies that lasted approximately 180 min (Fig. 1*B*). However, the responses of WT and GRK3$^{-/-}$ mice to the second challenge with morphine at 240 min after the first injection were equally attenuated, and both showed equivalent acute analgesic tolerance (Fig. 1*B*). Acute tolerance to fentanyl and morphine is equivalent in magnitude, and both fully recovered by 24 h (Fig. S1 *A* and *B*).

Pretreatment with JNK Inhibitor Blocks Acute Tolerance to Morphine. The mechanism by which morphine produces acute analgesic tolerance is unclear, but we recently found that inactivation of KOR signaling can be produced by activation of JNK in a ligand-dependent manner (6). To determine if a similar mechanism mediated acute tolerance to morphine, we pretreated animals with the potent and selective small-molecule JNK inhibitor SP600125 (16). Pretreatment with SP600125 (3–30 mg/kg) had

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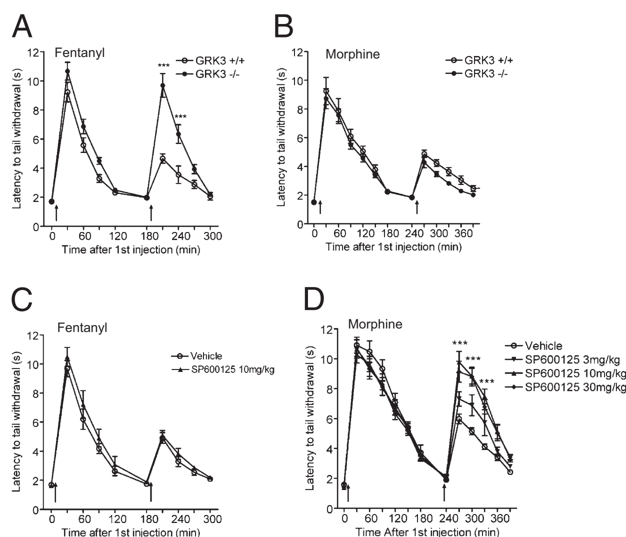

Fig. 1. GRK3 is required for tolerance to fentanyl but not morphine. WT C57BL/6 mice and littermate GRK3$^{-/-}$ mice were tested for acute analgesic tolerance to morphine and fentanyl in the warm-water (52.5 °C) tail-withdrawal assay. Data are presented as latency to tail withdrawal as a function of time after initial drug administration. Animals were injected with morphine (10 mg/kg s.c.) or fentanyl (0.3 mg/kg s.c.) at time points indicated by the arrows. GRK3$^{-/-}$ mice showed significantly reduced acute analgesic tolerance to the second dose of fentanyl compared with WT controls at 30 min and 60 min after the second injection ($P < 0.001$) (*A*). In contrast, both WT and GRK3$^{-/-}$ mice showed significantly reduced analgesic response to the second dose of morphine (*B*), indicating acute tolerance. Pretreatment of WT mice with the JNK inhibitor SP600125 (3 mg/kg, 10 mg/kg, or 30 mg/kg i.p. 60 min before opioid agent) had no effect on acute tolerance to fentanyl (*C*), but dose-dependently reduced analgesic tolerance to morphine compared with vehicle controls ($P < 0.001$) (*D*); n = 6–9, data analyzed by two-way ANOVA using Bonferroni post hoc tests.

no effect on the amplitude or kinetics of the initial analgesic response to fentanyl or morphine; neither vehicle nor SP600125 affected the initial analgesic responses (Fig. 1 *C* and *D*); and SP600125 did not block the acute analgesic tolerance to fentanyl (Fig. 1*C*). However, pretreatment with the JNK inhibitor dose-dependently blocked the acute analgesic tolerance to morphine (Fig. 1*D*). In contrast, the selective MEK inhibitor SL327 had no effect on acute tolerance to morphine (Fig. S1*C*). The selective effect of SP600125 on the amplitude of the second response to morphine, but not fentanyl, and the lack of effect of SL327 suggest that the acute analgesic tolerance to morphine is specifically mediated by JNK activation.

The site of JNK-mediated inactivation of MOR signaling could be at the receptor level or at a downstream effector. To distinguish these alternatives, we investigated the effect of JNK inhibition on cross-tolerance between the different agonists. Mice given fentanyl 240 min after an initial injection of morphine showed a diminished analgesic response consistent with cross-tolerance between morphine and fentanyl (Fig. 2*A*). Pretreatment with SP600125 before the initial morphine dose significantly increased the subsequent response to fentanyl (Fig. 2*A*), indicating that the JNK inhibitor blocked cross-tolerance. Cross-tolerance was also evident when morphine was administered after fentanyl (Fig. 2*B*); however, pretreatment with SP600125 before fentanyl did not significantly increase the diminished response to morphine (Fig. 2*B*). As previously seen, pretreatment with the JNK inhibitor in this experiment had no effect on the magnitude or kinetics of the initial response to either morphine or fentanyl.

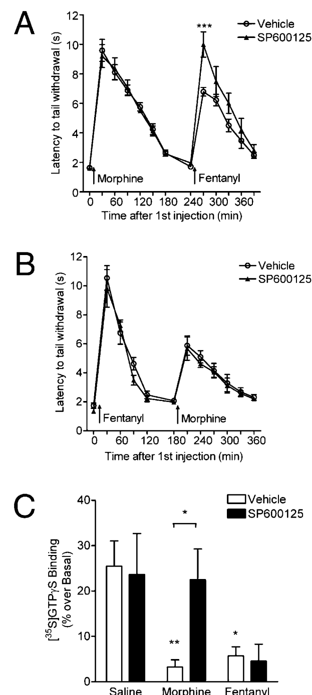

Fig. 2. JNK is required for uncoupling of MOR following morphine. (*A*) WT mice were injected with vehicle or SP600125 at 10 mg/kg i.p. 60 min before opioid treatment. Animals given an initial dose of morphine (10 mg/kg s.c.) followed by a challenge dose of fentanyl (0.3 mg/kg s.c.) showed reduced analgesic response to fentanyl not significantly different from tolerance following an initial dose of fentanyl (6.20 ± 0.30 s latency compared with 4.91 ± 0.37 s latency, respectively). In contrast, the increased latency of tail withdrawal in response to fentanyl following an initial dose of morphine was significantly greater in mice pretreated with SP600125 (10.22 ± 1.78 s) compared with vehicle controls ($P < 0.001$), and was not significantly different from the initial analgesic response to fentanyl (9.89 ± 0.50 s). (*B*) Animals given an initial dose of fentanyl (0.3 mg/kg s.c.) followed by a challenge dose of morphine (10 mg/kg s.c.) showed a reduced analgesic response to morphine that was insensitive to SP600125 (10 mg/kg i.p.) pretreatment. (*C*) DAMGO (1 μM)-stimulated [^{35}S]GTPγS binding was assayed in membranes from spinal cords of mice pretreated with saline solution, 10 mg/kg morphine, or 0.3 mg/kg fentanyl. Both morphine ($P < 0.01$) and fentanyl ($P < 0.05$) treatment significantly decreased subsequent DAMGO [^{35}S]GTPγS binding compared with binding to membranes from saline solution–treated mice. Pretreatment with SP600125 prevented the morphine-induced reduction in binding ($P < 0.05$), but had no effect on fentanyl-induced reduction in binding (n = 5–8, data analyzed by two-way ANOVA in *A* and *B*; by one-way ANOVA using Bonferroni post hoc tests in *C*).

These results suggest that JNK activation blocked MOR signaling at a target common to both morphine and fentanyl action, but to determine if the site of JNK action was at the MOR signaling complex, we measured agonist-stimulated [^{35}S]GTPγS binding to membranes isolated from spinal cords of mice treated in vivo with drug or saline solution 3 h before membrane isolation. In membranes of saline solution–treated mice, the selective MOR agonist D-Ala^{2}-Met^{5}-Glyol-enkephalin (DAMGO) increased [^{35}S] GTPγS binding 20% to 30% versus basal level (Fig. 2*C*). In vivo pretreatment with SP600125 alone had no effect on subsequent basal or DAMGO-stimulated [^{35}S]GTPγS binding. As expected, both morphine and fentanyl pretreatment significantly reduced subsequent DAMGO-stimulated [^{35}S]GTPγS binding, consistent with the acute analgesic tolerance results and expected receptor desensitization. Interestingly, pretreatment with the JNK inhibitor blocked the reduction of binding in membranes from morphine-treated animals, but not in those from fentanyl-treated animals (Fig. 2*C*). These data suggest that JNK activation by

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morphine produced receptor desensitization and acute analgesic tolerance by a direct effect on the MOR signaling complex.

JNK Inhibition Does Not Affect Tolerance to Other MOR Agonists. We next investigated whether JNK activation resulted in acute analgesic tolerance induced by other MOR agonists. Both vehicle- and JNK inhibitor–pretreated mice showed increased tail-withdrawal latencies following an initial dose of methadone that recovered by 120 min. The second dose of methadone evoked a blunted analgesic response that was unaffected by pretreatment with the JNK inhibitor (Fig. 3*A*). Similarly, JNK inhibitor pretreatment had no effect on the magnitude or kinetics of the initial analgesic response to oxycodone or on the acute tolerance following a second challenge (Fig. 3*B*). Whereas JNK inhibitor pretreatment had no effect on the initial analgesic response to the partial agonist buprenorphine, acute analgesic tolerance was significantly reduced (Fig. 3*C*). Although buprenorphine is a mixed μ-agonist/κ-antagonist opioid (17), the analgesic effects of buprenorphine in this assay were not evident in $MOR^{-/-}$ mice (Fig. 3*C*). Morphine-6-glucuronide (M6G; 10 mg/kg), the principal active metabolite of morphine (18), also produced acute analgesic tolerance that was blocked by the JNK inhibitor (Fig. 3*D*). Thus, acute analgesic tolerance to morphine, buprenorphine, and M6G may occur although a JNK-mediated pathway, whereas fentanyl, methadone (Fig. S2*A*), and oxycodone (Fig. S2*B*) desensitize MOR through the GRK3/β-arrestin pathway, independent of JNK activation.

This grouping is consistent with the differing abilities of these opioids to induce GRK/β-arrestin–dependent MOR internalization (7, 13). To confirm that JNK sensitivity correlates with the lack of internalization, we directly compared each of these opioids under identical treatment/expression conditions. rMOR-GFP stably transfected in HEK293 cells was significantly internalized within 10 min after application of fentanyl, methadone, or oxycodone (Fig. 4*A*). In contrast, neither morphine, buprenorphine, nor M6G caused significant internalization of rMOR-GFP even after 60 min of exposure (Fig. 4*A*). Quantification of the fluorescence showed significantly more GFP-tagged receptor inside the cell following fentanyl, methadone, and oxycodone treatments compared with vehicle controls (Fig. 4*B*). These data are generally consistent with previous reports of ligand-specific receptor internalization in various other expression systems (7, 13, 19–23) and support the grouping of opioid agonists into those that inactivate MOR by GRK/β-arrestin–dependent internalization or JNK-dependent receptor desensitization.

Both Morphine and Fentanyl Activate JNK Through a Pertussis Toxin-Sensitive, Gαi/o Mechanism. To assess possible differences between morphine and fentanyl activation of JNK, spinal cord proteins were isolated after in vivo pretreatment, and phospho-JNK immunoreactivity (pJNK-ir) was resolved by Western blot. Morphine significantly increased pJNK-ir at 30 and 60 min after treatment, and the increased pJNK-ir returned to baseline 2 h after morphine administration (Fig. S3 *A* and *B*). Interestingly, fentanyl also increased pJNK-ir for 2 h (Fig. S3 *A* and *B*), even though this increase was not accompanied by JNK-dependent MOR desensitization. Neither morphine nor fentanyl increased pJNK-ir in spinal cords of $MOR^{-/-}$ mice. The three isoforms of JNK can be partially resolved by differences in gel mobility; both morphine and fentanyl treatment significantly increased both pJNK1-ir and pJNK2-ir (approximately 46 kDa) without significantly affecting pJNK3-ir (approximately 54 kDa; Fig. S3*A*).

To determine the mechanism of JNK activation, we pretreated HEK293 cell stably transfected with MOR-GFP with saline solution or 0.2 μg/mL pertussis toxin (PTX) overnight. Following 10 min stimulation with morphine or fentanyl, pJNK-ir was sig-

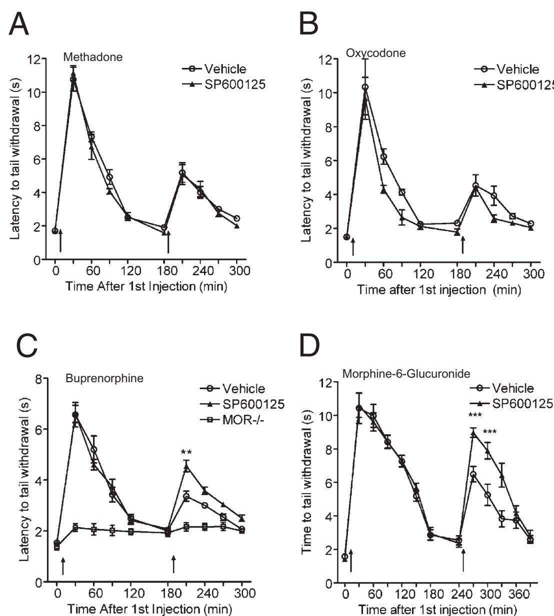

Fig. 3. JNK inhibition has no effect on acute tolerance to non–morphine-like opioids. Acute analgesic tolerance was assayed as before. (*A*) Pretreatment with SP600125 (10 mg/kg i.p.) had no effect on acute tolerance to methadone (10 mg/kg s.c.) or (*B*) oxycodone (3 mg/kg s.c.). In contrast, SP600125 significantly reduced acute analgesic tolerance to the partial agonist buprenorphine (3 mg/kg s.c.) compared with vehicle-treated animals ($P < 0.01$), but failed to completely attenuate it in WT mice (*C*). Buprenorphine also binds to the KOR; however, the analgesic effects of buprenorphine were not evident in $MOR^{-/-}$ mice. (*D*) Tolerance to the principal active morphine metabolite M6G (10 mg/kg, s.c.) was completely blocked by SP600125 compared with vehicle controls ($P < 0.001$); $n = 5–6$; data analyzed by two-way ANOVA using Bonferroni post hoc tests.

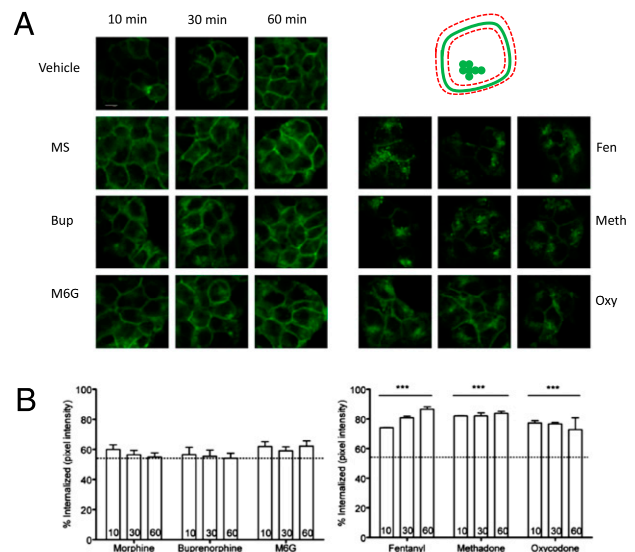

Fig. 4. MOR agonists display different internalization profiles. (*A*) HEK293 cells stably transfected with rMOR-GFP were treated with vehicle or 1 μM morphine sulfate (MS), buprenorphine (Bup), M6G, fentanyl (Fen), methadone (Meth), and oxycodone (Oxy) for 10, 30, and 60 min. Representative images are shown for each data set. (Scale bar: 10 μm.) Semiquantitative analysis of internalization was performed by Metamorph software and is based on three cells per plate for three plates for each data set. Dashed line represents vehicle treated controls. Fentanyl, methadone, and oxycodone treatment produced significantly more internalization of the GFP-tagged receptor than vehicle treatment ($P < 0.001$) (*B*). Data analyzed by one-way ANOVA using Bonferroni post hoc tests.

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nificantly increased in MOR-GFP–transfected HEK293 cells (Fig. S4 *A* and *B*). The increase in pJNK-ir caused by morphine or fentanyl was not evident in MOR-GFP cells pretreated with PTX (Fig. S4 *A* and *B*). These results indicate that both morphine and fentanyl increase pJNK-ir through a MOR-dependent, PTX-sensitive mechanism; however, JNK activation has ligand-specific consequences on receptor coupling to G protein activation. To further define mechanism of JNK activation, HEK293 cells stably expressing the rMOR-GFP were pretreated with the small molecule PKC inhibitor Gö6976, which was previously shown to block morphine tolerance (24). The increase in pJNK-ir caused by 60 min treatment with morphine was significantly reduced by Gö6976 (Fig. S5 *A* and *B*). Similarly, treatment of rKOR-GFP–expressing HEK293 cells increased pJNK-ir in response to norBNI, and this increase was also significantly blocked by pretreatment with Gö6976 (Fig. S5 *C* and *D*). These results suggest that mu-opioid receptor activation of JNK occurs through a Gαi/o–mediated activation of a Gö6976-sensitive form of PKC.

JNK Inhibition Blocks Long-Lasting Antagonism of KOR by norBNI. As stated previously, morphine-induced inactivation of MOR signaling strongly resembles the inactivation of KOR produced by selective κ-antagonists norBNI, JDtic, and GNTI (6, 8). Consistent with those studies, pretreatment with norBNI blocked the increase in tail-withdrawal latencies produced by the selective κ-opioid agonist U50,488 given 7 d after norBNI (Fig. 5*A*). Pretreatment with a moderate dose of the JNK inhibitor (10 mg/kg) before norBNI completely blocked the long-lasting antagonistic effects of norBNI. On day 7 following norBNI treatment, U50,488 increased the tail-withdrawal latencies in mice pretreated with SP600125 to the same extent as in mice that did not receive norBNI (Fig. 5*A*). In a separate group of WT mice, pretreatment with SP600125 before norBNI did not block the acute competitive antagonism of the U50,488 analgesic effect (Fig. S6). This result supports the conclusion that SP600125 blocks the long-lasting effects of norBNI through JNK inhibition rather than a nonspecific reduction in norBNI binding to KOR. In addition, these results show that norBNI may act both as an acute competitive antagonist and as a long-lasting noncompetitive antagonist of KOR signaling.

To extend these findings to the molecular level, we isolated spinal cord membranes and measured stimulation of $[^{35}S]GTP\gamma S$ binding by the KOR agonist U69,593. $[^{35}S]GTP\gamma S$ binding to spinal cord membranes from saline solution–treated animals increased 15% to 25% with U69,593 over basal (Fig. 5*B*); pretreatment with SP600125 had no effect on U69,593-stimulated $[^{35}S]GTP\gamma S$ binding. In vivo pretreatment with norBNI 7 d before spinal cord membrane isolation significantly suppressed U69,593-stimulated $[^{35}S]GTP\gamma S$ binding, and pretreatment with SP600125 before norBNI completely blocked the antagonistic effects of norBNI on subsequent U69,593-stimulated $[^{35}S]GTP\gamma S$ binding (Fig. 5*B*). Although in vivo pretreatment with the KOR agonist U50,488 also increases pJNK-ir (8), U50,488 (10 mg/kg) pretreatment on day 0 did not significantly affect U69,593-stimulated $[^{35}S]GTP\gamma S$ binding on day 7. These $[^{35}S]GTP\gamma S$ binding results extend our prior analysis and suggest that ligand-directed JNK activation by some opioids can inactivate both MOR and KOR.

JNK Knockout Blocks Opioid Receptor Inactivation. Although pharmacological inactivation of JNK signaling by SP600125 is reportedly selective (16), we sought to validate these results and identify the JNK isoforms responsible by using genetic inactivation. $JNK1^{-/-}$ mice show a normal increase in tail-withdrawal latency following U50,488 treatment compared with $JNK1^{+/+}$ littermates (Fig. 5*C*); however $JNK1^{-/-}$ mice injected with norBNI do not exhibit long-lasting antagonism of U50,488-induced analgesia (Fig. 5*C*). Similarly, the increase in tail-withdrawal latency in response to the first injection of morphine

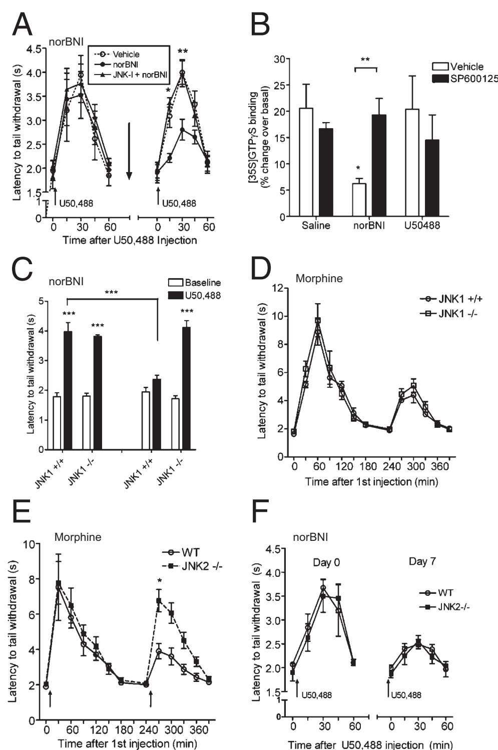

Fig. 5. JNK1 is required for the long lasting effects of norBNI. WT mice were tested in the tail-withdrawal assay after administration of U50,488 (15 mg/kg i.p.) before (*Left*) and 7 d after (*Right*) norBNI (10 mg/kg i.p.). SP600125 significantly blocked the long-lasting antagonistic effects of norBNI on U50,488-induced analgesia compared with vehicle 15 min and 30 min after U50,488 ($P < 0.05$ and $P < 0.01$, respectively) (*A*). U69,593-stimulated $[^{35}S]GTP\gamma S$ binding was measured in spinal cord membranes from animals treated with saline solution, U50,488, and norBNI and dissected 7 d later. Only norBNI showed significant long-lasting reduction in $[^{35}S]GTP\gamma S$ binding ($P < 0.05$ vs. saline solution treatment), an effect that was significantly reversed by pretreatment with the JNK inhibitor ($P < 0.01$) (*B*). $JNK1^{+/+}$ and $JNK1^{-/-}$ mice showed equivalent analgesia to U50,488 prior to norBNI (*Left*), but 7 d later (*Right*) norBNI still blocked U50,488 responses in $JNK1^{+/+}$ but not $JNK1^{-/-}$ mice. ($P < 0.001$), indicating that the JNK1 isoform is required for norBNI antagonism (*C*). In contrast, $JNK1^{-/-}$ animals showed normal development of acute tolerance to morphine, indicating that the JNK1 isoform is not required for morphine tolerance (*D*). By comparison, $JNK2^{-/-}$ mice, although they show similar analgesic response to an initial dose of morphine as WT controls, do not show significant analgesic tolerance following a second challenge dose of morphine versus controls ($P < 0.05$) (*E*). $JNK2^{-/-}$ mice showed similar analgesia following U50,488 (15 mg/kg i.p.) compared with WT mice. Both WT and $JNK2^{-/-}$ animals injected with norBNI (10 mg/kg i.p.) and challenged with U50,488 7 d later showed significant reduction in U50,488-induced analgesia compared with the initial assay (*F*); n = 4–6; data analyzed by two-way ANOVA using Bonferroni post hoc tests.

was not affected by JNK1 gene knockout (Fig. 5*D*). However, in contrast to norBNI, both $JNK1^{-/-}$ and littermate controls showed equivalently blunted analgesic responses to the second challenge dose of morphine (Fig. 5*D*). These results suggest that JNK1 selectively mediates ligand-induced inactivation of KOR, but not MOR signaling.

$JNK2^{-/-}$ mice also show normal analgesic responses to initial doses of both morphine and U50,488 (Fig. 5 *E* and *F*). However, JNK2 gene knockout significantly blocked the acute analgesic tolerance to morphine as demonstrated by increased tail-withdrawal latencies following the second dose of morphine compared with WT controls (Fig. 5*E*), but had no effect on the long-lasting antagonism of U50,488 analgesia produced by norBNI (Fig. 5*F*). These complementary results suggest that JNK2 se-

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lectively mediates ligand-induced inactivation of MOR, but not KOR signaling. The specificity of JNK1 for KOR and JNK2 for MOR was a surprise. The basis for this specificity is not known, but is consistent with the different cellular distributions of these opioid receptors.

Discussion

The principal findings of this study are that both MOR and KOR demonstrate ligand-directed signaling, and these two receptor types can be inactivated by JNK-dependent mechanisms. This work supports the burgeoning idea in the field of receptor pharmacology that there are multiple classes of agonists that can be distinguished by downstream effectors and internalization profiles. In the MOR system, it has been shown that a class of drugs including morphine, M6G, and buprenorphine do not induce robust internalization of the receptor in heterologous expression systems, whereas a second class of drugs including methadone (7, 22, 23) and fentanyl (19, 20) produce strong internalization and desensitization. However, because internalization profiles may be influenced by the cell expression system used (7, 25), here we corroborate these findings in a direct comparison in the same heterologous expression system. Interestingly, classification of MOR agonists by their internalization profile correlates tightly with our observed dependence on JNK activation for the development of analgesic tolerance, a MOR-dependent phenomenon as determined by knockout studies (26). Although less well studied, such arrangement also applies to JNK-dependent inactivation of KOR by norBNI, which does not produce receptor internalization, whereas the JNK-insensitive desensitization by the KOR agonist U50,488 does.

The difference between the morphine-like and fentanyl-like opioid agents in their abilities to induce MOR internalization has been attributed to differences in their intrinsic efficacies (10, 24, 27). Opioid agents with low intrinsic efficacies fail to efficiently activate GRK, which is a Gβγ-dependent kinase (28), and thus fail to efficiently recruit the β-arrestin–dependent internalization machinery (29). However, closer inspection of the relationship between intrinsic efficacies as measured by the [^{35}S]GTPγS binding assay showed a ranking of methadone > morphine > fentanyl > oxycodone > buprenorphine (30, 31) that does not correlate with the agonist groupings based on internalization efficiencies. Although intrinsic efficacy measures may depend on the expression system or tissue response studied, fentanyl and oxycodone were consistently found to have lower efficacy than morphine in this assay (27, 31), yet still induce GRK/β-arrestin–dependent acute analgesic tolerance and internalization. Our results suggest that intrinsic efficacy alone is insufficient to account for differences in desensitization, and that other characteristics of the ligand-directed receptor conformation determines whether the opioid activates JNK-dependent or GRK/β-arrestin–dependent receptor desensitization.

In this study we have focused on acute desensitization mechanisms, but recognize that opiate tolerance is a complex and multidimensional process. Sustained exposure to morphine produces compensatory adaptations that may include PKC-dependent or ERK processes (14, 24, 32). The present study suggests a crucial regulatory process mediated by JNK and potentially involving PKC. Beyond implicating PKC, the intermediate steps linking GPCR and JNK have not been defined, but small G proteins and upstream kinase pathways have been suggested in other systems (33). For example, the MOR agonist DAMGO activates JNK in an Src-dependent manner, whereas activation by the KOR agonist U50,488 is dependent on the PI3-kinase and Akt pathways (34). Additionally, the downstream JNK substrates responsible for opioid receptor inactivation are not yet defined. Because our studies show that JNK activation blocks G protein coupling to the opioid receptors, we hypothesize that a JNK substrate may physically bind to the cytoplasmic face of the receptor and sterically interfere with G protein association.

The JNK isoform selectivity identified in the present study was unexpected. MOR desensitization by morphine selectively required JNK2, whereas KOR inactivation by norBNI required the JNK1 isoform. Prior studies showed that activation of either MOR or KOR could cause JNK phosphorylation (34), but isoform selectivity has not previously been appreciated and the basis for this specificity is not yet clear. Our data on pJNK-ir following morphine and fentanyl, as well as the JNK isoform KO data, suggest that the JNK3 isoform does not play a significant role in receptor desensitization. This was unexpected as the JNK3 isoform is expressed exclusively in the central nervous system, but may be related to the specific cell types in which the opioid receptors are expressed and the relative expression levels of the JNK isoforms in those tissues (33).

JNK-mediated receptor inactivation may not be a unique property of the opioid receptor family and may regulate other GPCRs. The long-lasting effects of norBNI made JNK-inactivation of KOR more obvious, but GRK-independent receptor desensitization may possibly control cannabinoid receptor (CB1) function as well. Similar to differences between MOR agonists, previous studies showed that the full CB1-selective agonist WIN 55,212–2 induces receptor desensitization and internalization (35, 36), whereas the less efficacious agonist Δ9-THC desensitizes CB1 without causing GRK-dependent internalization (36). Collateral agonism and ligand-directed signaling following stimulation of the same receptor has also been described for numerous other GPCR systems, including dopamine receptors, serotonin receptors, and adrenergic receptors (4, 5). In the case of the 5-HT7 receptor system, risperidone-like ligands produce persistent inactivation of G protein–mediated 5-HT signaling in a manner that resembles norBNI and morphine inactivation of agonist signaling at the KOR and MOR (37, 38). Therefore, we suggest JNK-activated GPCR regulation may be a more general mechanism of GPCR desensitization.

In conclusion, results from the present study suggest that the JNK MAPK system may have important functions beyond its well defined role in regulating transcription factors and gene expression important to cell survival, apoptosis, and chemotaxis. Key differences in signal transduction effects caused by different ligands binding to the same receptor supports the concept that the ligand-directed conformational state of the receptor can initiate different signaling cascades. For the opioid receptors, one ligand class initiates GRK-dependent signaling whereas other initiates distinctly different JNK cascades. These distinctions may be a general property of GPCR signaling, and in the future may be deliberately exploited for therapeutic advantage.

Materials and Methods

Reagents. Morphine sulfate, fentanyl, methadone, oxycodone, buprenorphine, M6G, norBNI, U50,488H, and U69,593 were provided by the National Institute of Drug Abuse. DAMGO was from Bachem. SP600125 was from Calbiochem. The MEK1/2 inhibitor SL327 was from Tocris Biosciences. MOR and KOR agonists were dissolved in 0.9% NaCl. SP600125 and SL327 were dissolved in 5% DMSO and 20% Cremophor (Sigma-Aldrich). Drugs were administered at a volume of 10 mL/kg.

Animals. Male C57BL/6 mice (20–25 g) were purchased from Charles River Laboratories. GRK3$^{-/-}$ mice were provided by Marc Caron and Robert Lefkowitz (Duke University, Durham, NC) and bred within the University of Washington vivarium under specific pathogen-free conditions. JNK1$^{-/-}$ and JNK2$^{-/-}$ mice were purchased from Jackson Laboratories on a C57BL/6 background. Homozygous MOR-KO mice were prepared by homologous recombination as described (39). For additional details on housing and genotyping procedures, see *SI Materials and Methods*.

Acute Analgesic Tolerance. Antinociceptive responses were measured using the warm-water tail-withdrawal assay modified as previously described (6).

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Briefly, the latency to tail withdrawal from immersion in a 52.5 °C water bath was measured before agonist administration and every 30 min thereafter until responses returned to baseline. To investigate the development of acute tolerance, a second administration of agonist was given after responses returned to baseline and the tail-withdrawal latency measures were repeated.

Cell Culture. HEK293 cells transfected with rKOR-GFP or rMOR-GFP were cultured in DMEM/nutrient mixture F-12 with L-glutamine and 15 mM Hepes (Invitrogen) with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C and 5% CO_2, and 200 μg/mL G418.

Immunoblotting. rMOR-GFP and rKOR-GFP HEK293 cells were serum-starved in DMEM/F-12 overnight and pretreated with indicated compounds before opioid treatment. Cells were treated with MOR or KOR agonists for time points indicated. For spinal cord samples, mice were injected i.p. with drug as indicated. Tissue was dissected after injection and was homogenized in lysis buffer using a 2-mL Dounce homogenizer. Samples were analyzed by Western blot using antibodies against phosphorylated JNK protein. Relative intensities of fluorescence bands were determined by Odyssey quantification (Li-Cor Biosciences).

$[^{35}S]GTP\gamma S$ Binding. DAMGO- and U69,593-stimulated $[^{35}S]GTP\gamma S$ binding was assayed in spinal cord cell membrane homogenates as described (40). Briefly, animals were treated with vehicle or drug, and spinal cords were dissected and homogenized as described in supporting information.

Data Analysis. Data are expressed as mean ± SEM. Differences between groups were determined using one-way ANOVAs followed by post hoc Bonferroni comparisons using GraphPad Prism (version 4.0) if the main effect was significant at $P < 0.05$.

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