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

Erica J. Melief, Mayumi Miyatake, Michael R. Bruchas, and Charles Chavkin

Department of Pharmacology, University of Washington, Seattle, WA 98195

Edited by Susan G. Amara, University of Pittsburgh School of Medicine, Pittsburgh, PA, and approved May 4, 2010 (received for review January 20, 2010)

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 (morphone-6-glucuronide and buprenorphine) was blocked by JNK inhibition, but not by G protein receptor kinase 3 (GRK3) 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-Ala2-Met5-Glyol-enkephalin-stimulated [35S]GTPγS binding to spinal cord membranes from morphine-pretreated mice, was also blocked by JNK inhibition; however, desensitization of D-Ala2-Met5-Glyol-enkephalin-stimulated [35S]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 [35S]GTPγS binding and opioid analogical 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.

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. 1A), 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. 1B). 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. 1B). 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 no effect on the acute analgesic response of morphine (Fig. 2 A and B, and Fig. S1 C).


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1000751107/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1000751107

PNAS Early Edition | 1 of 6
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. 1C). However, pretreatment with the JNK inhibitor dose-dependently blocked the acute analgesic tolerance to morphine (Fig. ID). In contrast, the selective MEK inhibitor SL327 had no effect on acute tolerance to morphine (Fig. S1C). 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. 2A). Pretreatment with SP600125 before the initial morphine dose significantly increased the subsequent response to fentanyl (Fig. 2A), indicating that the JNK inhibitor blocked cross-tolerance. Cross-tolerance was also evident when morphine was administered after fentanyl (Fig. 2B); however, pretreatment with SP600125 before fentanyl did not significantly increase the diminished response to morphine (Fig. 2B). 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.

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 [35S]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-Ala²-Met³-Glyol-enkephalin (DAMGO) increased [35S]GTPγS binding 20% to 30% versus basal level (Fig. 2C). In vivo pretreatment with SP600125 alone had no effect on subsequent basal or DAMGO-stimulated [35S]GTPγS binding. As expected, both morphine and fentanyl pretreatment significantly reduced subsequent DAMGO-stimulated [35S]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. 2C). These data suggest that JNK activation by
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. 3A). Similarly, JNK inhibitor pretreatment had no effect on the magnitude of kinetics of the initial analgesic response to oxycodone or on the acute tolerance following a second challenge (Fig. 3B). Whereas JNK inhibitor pretreatment had no effect on the initial analgesic response to the partial agonist buprenorphine, acute analgesic tolerance was significantly reduced (Fig. 3C). 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. 3C). 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. 3D). Thus, acute analgesic tolerance to morphine, buprenorphine, and M6G may occur although a JNK-mediated pathway, whereas fentanyl, methadone (Fig. S2A), and oxycodone (Fig. S2B) 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. 4A). In contrast, neither morphine, buprenorphine, nor M6G caused significant internalization of rMOR-GFP even after 60 min of exposure (Fig. 4A). Quantification of the fluorescence showed significantly more GFP-tagged receptor inside the cell following fentanyl, methadone, and oxycodone treatments compared with vehicle controls (Fig. 4B). 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. S3A and B). Interestingly, fentanyl also increased pJNK-ir for 2 h (Fig. S3A and B), even though this increase was not accompanied by JNK-dependent MOR desensitization. Neither morphine nor fentanyl increased pJNK1-ir, but pJNK2-ir (approximately 46 kDa) without significantly affecting pJNK3-ir (approximately 54 kDa; Fig. S3A).

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.](image)

![Fig. 4. MOR agonists display different internalization profiles.](image)
significantly 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 the 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 Goi/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, JDtc, 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 [35S]GTPγS binding by the KOR agonist U69,593 ([35S]GTPγS 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 [35S]GTPγS binding. In vivo pretreatment with norBNI 7 d before spinal cord membrane isolation significantly suppressed U69,593-stimulated [35S]GTPγS binding, and pretreatment with SP600125 before norBNI completely blocked the antagonistic effects of norBNI on subsequent U69,593-stimulated [35S]GTPγS 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 [35S]GTPγS binding on day 7. These [35S]GTPγS 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 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-
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 [35S]GTPyS 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 determine 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 multi-dimensional 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).

Animals. Male C57BL/6 mice (20–25 g) were purchased from Charles River Laboratories. GRK3−/− mice were provided byMarc 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).
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 10% FBS, 50 μU/ml penicillin, and 50 μg/ml streptomycin at 37 °C and 5% CO₂, and 200 μg/ml G418.

Immunoblotting. rMOR-GFP and rKOR-GFP HEK293 cells were serum-starved for 16 h. Differences between rMOR-GFP and rKOR-GFP HEK293 cells 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.

**ACKNOWLEDGMENTS.** We thank Dr. John Pintar (University of Medicine and Dentistry of New Jersey) for the MOR (~77) mice and Drs. Marc Caron and Robert Lefkowitz (Duke University Research Institute) for their generous gift of the GRK3 (~95) mice. We thank Dr. Nephé Stella for help with the [3H]GTPγS studies. Dan Messinger monitored the mouse breeding and genotyped the mice. Funding was provided by US Public Health Service Grants R37-DA11672 and K99-DA025182 and National Institute on Drug Abuse Grant K05 DA20570.

6 of 6 | www.pnas.org/cgi/doi/10.1073/pnas.1000751107

Melief et al.
Supporting Information

Melief et al. 10.1073/pnas.1000751107

SI 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 obtained from Bachem. The JNK inhibitor SP600125 was obtained from Calbiochem. The MEK1/2 inhibitor SL327 was obtained 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+/− and WT littermates were generated by homologous recombination as described (2). Animals were backcrossed for more than 10 generations with C57BL/6 mice, and heterozygous breeding pairs were used to generate homozygote mice and paired littermate controls for this study. Mice were genotyped using DNA extracted from tail samples as described previously (1). All mice were group housed and kept on a 12-h light/dark cycle with food and water available ad libitum. Animal procedures in this study were approved by the Animal Care and Use Committee of the University of Washington and conform to the guidelines of the National Institutes of Health on the care and use of animals.

Genotyping. GRK3 mice were genotyped using a common primer in conjunction with a WT and NEO primer mixed them at a concentration of 7.5 µg/uL. DNA was extracted using a DNEasy kit (Qiagen). Primers used were as follows: GRK3 common, CAGGGC-TAGGGTGTACGTCTAGT; GRK3 WT, CTGACTAGGG-GAGGATAGAAAGGT; GRK3 NEO, CTTCCACAGCTGA-GCATGAAAGAC. MOR mice are genotyped using the DNA using Extract-N-AMP kits (Sigma) and the attendant PCR reagents. Primers are at 50 µM in separate tubes as follows: MOR WT 5′, CAACCTTGTCCACGTGTAGT; MOR WT 3′, AGGTTCCCTTCTCAGTGT; MOR NEO 5′, AGGAAAGAAAAAGCCTTCTC; MOR NEO 3′, CTGTGCTTCGACTGTATCATTCA.

JNK1 mice were genotyped as GRK3 mice as follows: JNK1 common, CCA GCC TCT CCT CAT CTT CA; JNK1 KO, CCA GCT CAT TCC TCC ACT CAT G; JNK1 WT, TCA CCA CAT AAG GCC TCA TC.

Acute Analgesic Tolerance. Antinociceptive responses were measured using the warm-water tail-withdrawal assay modified as previously described (3). Briefly, the latency to tail withdrawal after immersion in a 52.5 °C water bath was measured before agonist administration and every 30 min thereafter until responses returned to baseline. To prevent tissue damage, a cut-off of 15 s was used. To investigate the development of acute tolerance, a second administration of the same agonist was given after responses returned to baseline and the measures were repeated. Drugs investigated included morphine (10 mg/kg, s.c.), fentanyl (0.5 mg/kg, s.c.), methadone (10 mg/kg, s.c.), oxycodone (10 mg/kg, s.c.), buprenorphine (3 mg/kg, s.c.), and M6G (10 mg/kg, s.c.). Doses were determined by previous studies and pilot trials to be nearly equivalently effective at increasing tail-withdrawal latencies. To determine the effect of the MAPK inhibitors on the development of acute tolerance, animals were pretreated with SP600125, SL327, or vehicle 60 min before testing.

Cell Culture. HEK293 cells transfected with rKOR-GFP or rMOR-GFP were grown as previously described (4) in DMEM/nutrient mixture F-12 with L-glutamine and 15 mM Hepes (Invitrogen) with 10% FBS, 50 µ/mL penicillin, and 50 µg/mL streptomycin at 37 °C and 5% CO2, and 200 µg/mL G418 to maintain selective pressure.

Immunoblotting. rMOR-GFP and rKOR-GFP HEK293 cells were cultured as described earlier. For PTX experiments, cells were serum-starved in DMEM/F-12 with or without PTX (0.2 µg/mL) for 18 h before drug treatment. For PKC experiments cells were pretreated with Go6976 or DMSO (0.1% DMSO final concentration) at indicated concentrations for 1 h. rMOR-GFP cells were treated with morphine (10 µM), fentanyl (10 µM), anisomycin (10 µM), or vehicle control whereas rKOR-GFP cells were treated with norBNI (1 µM) or vehicle control for indicated time points and then immediately harvested in lysis buffer [50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, 10% glycerol, 1:100 phosphatase inhibitor mixture set 1 (Calbiochem), and 1:100 protease inhibitor mixture set 1 (Calbiochem)]. Lysates were sonicated (twice for 5 s at 4 °C) and centrifuged (15,000 × g, 15 min, 4 °C), and the supernatant was stored at −20 °C until use. For spinal cord samples, mice were injected i.p. with drug as indicated. Tissue was dissected at time indicated after injection and was homogenized in lysis buffer using a 2-mL Dounce homogenizer (as described earlier). Total protein concentration was determined using bicinchoninic acid colorimetric assay (Pierce) with BSA standards as described (3). Thirty micrograms of each sample was boiled at 100 °C for 10 min in Laemmli buffer before loading onto nondenaturing SDS–PAGE gels. Lysates were blocked with 5% BSA in TBS (5 min), washed with TBS (5 min) and then scanned on the Odyssey Infrared Imaging System (Li-Cor Biosciences). The blots were washed with Tris-buffered saline (TBS) solution (5 min), blocked with 5% BSA/TBS (60 min), and incubated overnight at 4 °C in phospho-stress–activated protein kinase/JNK (Thr-183/Tyr-185) rabbit antibody diluted 1:1,000 in 5% BSA/TBS (Cell Signaling). After overnight incubation, the blots were washed with TBS with Tween-20 (TBS; 5 × 10 min) and incubated for 60 min at room temperature in anti-rabbit IRDye800 diluted 1:10,000 in a 1:5 mixture of 5% milk/TBST and Li-Cor blocking buffer (Li-Cor Biosciences). The blots were washed with TBS (5 × 10 min) and TBS (5 min) and then scanned on the Odyssey Infrared Imaging System (Li-Cor Biosciences). Relative intensity of fluorescence bands was determined by Odyssey quantification. pJNK fluorescence was normalized to actin loading control and then expressed as change over vehicle treatment.

[^35]S GTPγS Binding. DAMGO- and U69,593-stimulated[^35]S GTPγS binding was assayed in spinal cord cell membrane homogenates as previously described (4). Briefly, animals were treated with vehicle or drug and spinal cords were dissected and...
homogenized as described earlier. Supernatant was discarded and the pellet was washed in membrane buffer, rehomogenized, and centrifuged two more times before freezing the pellet at −80 °C before use. For DAMGO-stimulated GTPγS assay, 20 mg membrane proteins were incubated with 1 μM DAMGO in 50 mM binding buffer (50 mM Hepes, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 0.1% BSA, and 1 mM DTT, pH 7.4) at 30 °C for 1 h in the presence of 0.1 nM [35S]GTPγS and 10 mM GDP. For U69,593-stimulated GTPγS binding, 10 μg membrane proteins were incubated with 1 μM U69,593 and reagents as described earlier. Bound [35S]GTPγS was separated from free [35S]GTPγS by rapid filtration using a Brandel cell harvester onto GF/B filters (Whatman). Filters were washed three times using binding buffer. Bound [35S]GTPγS was measured using liquid scintillation counter at approximately 50% efficiency. Data were normalized to percent of binding in vehicle-treated samples.

**Data Analysis.** Data are expressed as mean ± SEM. Specific analytical tests used are described in figure legends. Statistical analyses were conducted using GraphPad Prism (version 4.0; GraphPad).


Fig. S2. GRK3 is required for acute analgesic tolerance to methadone and oxycodone. Tail-withdrawal latencies of GRK3−/− and WT littermate controls on C57BL/6 background were measured following methadone (10 mg/kg) or oxycodone (3 mg/kg) administration. Data are tail-withdrawal latencies responses to the second dose of agonist expressed as a percentage of the maximal effect of the first dose. GRK3−/− mice show significantly less acute analgesic tolerance to methadone compared with WT mice (A). Similarly, GRK3−/− mice show significantly less acute analgesic tolerance to a second dose of oxycodone than did WT controls (B). (n = 4–5; data analyzed by Student t test; **P < 0.01, *P < 0.05 vs. initial analgesic response.)

Fig. S3. Both morphine and fentanyl activate the JNK pathway. WT animals were administered saline solution, morphine, or fentanyl, and then spinal cords were dissected at times indicated. Tissue extracts were resolved on a 4% to 12% Mops NuPAGE gel and transferred to a nitrocellulose membrane. Membranes were probed using antibodies for phospho-JNK (pJNK) and β-actin (A). Band intensities were normalized to β-actin and quantified using the Odyssey imaging system. pJNK1/2-IR (approximately 46 kDa) was significantly increased at 30 and 60 min following morphine compared with saline solution control, whereas pJNK1/2-IR was significantly increased at 30, 60, and 120 min following fentanyl (Top) (*P < 0.05). In contrast, pJNK3-IR (approximately 54 kDa) was not significantly increased at any time point for either drug (Bottom). In addition, pJNK-IR was not increased in MOR−/− mice following either drug (B). (n = 3–4; data analyzed by Student t test; *P < 0.05 vs. vehicle control.)
Fig. S4. The increase in pJNK-IR was blocked by PTX pretreatment of HEK293 cells stably transfected with rMOR-GFP. Cells were pretreated with PTX for 18 h and then treated with morphine (1 μM) (M) or fentanyl (1 μM) (F) for 10 min before harvesting. Blots were immunoprobed for pJNK-IR and β-actin-IR (A). Band intensities were normalized to β-actin-IR and quantified using the Odyssey imaging system. Both morphine and fentanyl significantly increased pJNK1-IR (approximately 46 kDa) versus vehicle controls (*P < 0.05), and these increases were blocked by PTX pretreatment (B); n = 3; data analyzed by Student t test.

Fig. S5. PKC inhibition blocks JNK activation by morphine and norBNI in HEK293 cells expressing rMOR-GFP and KOR-GFP, respectively. HEK293 cells expressing rMOR-GFP were pretreated with the PKC inhibitor Gö6976 (1 μM, 60 min) or 0.1% DMSO and then treated with morphine (10 μM) for 30 or 60 min (A and B). Pretreatment with Gö6976 blocked morphine-induced increase in pJNK-IR at 60 min (***P < 0.01). HEK293 cells expressing rKOR-GFP were pretreated with Gö6976 (0.1 μM or 1 μM, 60 min) or 0.1% DMSO and then treated with norBNI (1 μM, 60 min). Pretreatment with either concentration of Gö6976 blocked norBNI-induced increase in pJNK-IR (C and D) (***P < 0.01); n = 6–7; data analyzed by ANOVA using Bonferroni post hoc comparisons.
In contrast to its effect on long-lasting antagonism, SP600125 has no effect on acute norBNI antagonism. WT animals were pretreated with vehicle or SP600125 (10 mg/kg i.p.) 60 min before acute administration of norBNI (10 mg/kg i.p.). Sixty minutes after norBNI, U50,488-induced analgesia was tested in comparison with vehicle pretreated controls. Control animals showed a significant increase in tail-withdrawal latency following U50,488 (**P < 0.01). U50,488 did not significantly increase in tail-withdrawal latency in animals acutely treated with norBNI. Similarly, U50,488 did not significantly increase in tail-withdrawal latency in animals acutely treated with both norBNI and SP600125. Thus, SP600125 did not block the acute antagonist effects of norBNI (n = 4–6; data analyzed by one-way ANOVA using Bonferroni post hoc tests).