LONG-ACTING KAPPA OPIOID ANTAGONISTS DISRUPT RECEPTOR SIGNALING AND PRODUCE NONCOMPETITIVE EFFECTS BY ACTIVATING C-JUN N-TERMINAL KINASE

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NorBNI, GNTI, and JDTic are selective kappa opioid receptor (KOR) antagonists having very long durations of action in vivo despite binding non-covalently in vitro and having only moderately high affinities. Consistent with this, we found that antagonist treatment significantly reduced the subsequent analgesic response of mice to the KOR agonist U50,488 in the tail-withdrawal assay for 14-21 days. Receptor protection assays were designed to distinguish between possible explanations for this anomalous effect, and we found that mice pretreated with the readily reversible opioid antagonists, naloxone or buprenorphine, prior to norBNI responded strongly in the tail-flick analgesia assay to a subsequent challenge with U50,488 one week later. Protection by a rapidly cleared reagent indicates that norBNI was not persisting at the site of action. In vitro binding of [³H]-U69,593 to KOR showed that Kd and Bmax values were not significantly affected by prior in vivo norBNI exposure, indicating that the agonist binding site was intact. Consistent with the concept that the long-lasting effects might be caused by a functional disruption of KOR signaling, both norBNI and JDTic were found to stimulate c-Jun N-terminal kinase (JNK) phosphorylation in HEK293 cells expressing KOR-GFP, but not in untransfected cells. Similarly, norBNI increased phospho-JNK in both the striatum and spinal cord in wild-type mice, but not in KOR knockout mice. Pretreatment of mice with the JNK inhibitor SP600125 prior to norBNI partially reversed the long acting antagonism. Together, these results suggest that the long duration KOR antagonists disrupt KOR signaling by activating JNK.

NorBNI, GNTI, and JDTic are the selective KOR antagonist norbinaltorphine (norBNI) two decades ago, and this ligand has been the most commonly used KOR antagonist since (1,2). NorBNI has greater than 100-fold selectivity for KOR over the µ or δ opioid receptors (MOR, DOR) (3). KOR is a G-protein coupled receptor (GPCR) that is widely expressed throughout the nervous system and is activated by endogenous opioid peptide agonists derived from prodynorphin (4,5). Several reports have shown that agonist occupation of the KOR leads to the pertussis toxin sensitive inhibition of adenylate cyclase, increase in potassium conductance, decrease in calcium conductance, and mobilization of intracellular calcium (6). Recently, KOR activation has also been shown to stimulate the mitogen activated protein kinase pathways (MAPK), including extracellular signal-regulated kinase (ERK 1/2), p38, and c-Jun N-terminal Kinase (JNK) (7-11).

Two other KOR antagonists GNTI and JDTic were subsequently generated (12,13), and all 3 share two distinct pharmacological properties: slow onset of antagonist activity and exceedingly long durations of action in vivo (12-19). Reports in rhesus monkeys have shown kappa-antagonist effects up to 21 days following a single injection of norBNI (14). A single injection of norBNI in mice maintains continual blockade of KOR even after three weeks (17). GNTI and JDTic have similar long-lasting effects and produce antagonism for at least ten to 14 days (12,13). These findings are surprising because these antagonists do not covalently bind to KOR (20). The basis for this long-duration of action is not clear. One explanation is that these drugs become physically trapped in the lipid membrane and do...
not clear easily from the nervous system. A second possibility is that these drugs are biotransformed in vivo to long-lasting metabolites that covalently bind to the receptor. An alternative hypothesis is that NorBNI, GNTI, and JDTic produce their long lasting effects by acutely uncoupling the KOR signaling complex such that agonists can no longer activate the receptor to stimulate G-protein signaling.

To distinguish these mechanisms, we first compared the duration of actions in mice for norBNI, GNTI, and JDTic. Building on these findings, we used receptor protection experiments and looked at both the functional and binding properties of KOR ligands. If transient occupancy of KOR by a readily reversible ligand could protect against receptor inactivation, the long-lasting antagonist must also be producing its effects by transiently occupying the same binding site rather than by forming a drug depot in the brain. Using this strategy, we found that the readily reversible opioid antagonists naloxone and buprenorphine were able to protect KOR signaling. We further found that the long-lasting antagonists activate JNK in a KOR-dependent manner, and we found that blockade of JNK activation partially prevented the long lasting antagonism.

Understanding how kappa antagonists produce long lasting effects has important implications for the ultimate utility of these agents as therapeutic tools. Recent studies have suggested that the antagonists might have antidepressant activity and also be useful in preventing relapse of drug abuse (21-23). In addition, understanding how JNK activation by these drugs disrupts KOR signaling would provides new insight to opioid and GPCR signal transduction events.

**EXPERIMENTAL PROCEDURES**

**Chemicals.** (-)U50,488, norbinaltorphimine HCl (norBNI), and guanidinonaltindole (GNTI) were obtained from Tocris (Ellisville, MO). Buprenorphine was obtained from the NIDA Drug Program (Bethesda, MD) and naloxone from Sigma (St Louis, MO). JDTic was provided by Dr. F.I. Carroll (Research Triangle Institute, NC). All other drugs were purchased from Calbiochem (La Jolla, CA). Drugs were dissolved in water, or saline (for in vivo experiments) unless otherwise indicated.

Animals and housing. Male C57Bl/6 mice (Charles River Laboratories, Wilmington, MA) weighing 20-30g (8-12 weeks old) were used in these experiments. Mice were maintained in a specific pathogen free housing unit in the core animal facility at the University of Washington. Housing rooms were illuminated on a 12-hr light-dark cycle with lights on at 7am. Food pellets were available ad libitum. All animal procedures were approved by the institutional Animal Care and Use Committee in accordance with NIH guidelines.

Breeding and Genotyping of MOR and KOR knockout mice. Homozygous mu-opioid receptor (MOR) and KOR knockout (-/-) mice were prepared by homologous recombination as described (24,25) and provided for this study. Animals were backcrossed for >10 generations with C57Bl/6 mice, and heterozygote breeding pairs were used to make homozygote MOR (-/-) mice and paired littermate controls for this study. Mice were genotyped using DNA extracted from tail samples as described previously (21).

Cell Culture. HEK293 cells were grown as previously described (26) in Dulbecco’s Modified Eagle Medium / Nutrient Mixture F12 with L-glutamine and 15 mM HEPES (DMEM/F12) (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin at 37°C and 5% CO2. HEK293 cells transfected with green fluorescent protein tagged KOR-GFP or MOR-GFP were maintained in the above media with an additional 200 µg/ml G418 to maintain selective pressure. Untransfected HEK293 cells for control experiments were grown in the absence of G418.

Immunoblotting. KOR-GFP, MOR-GFP, and untransfected HEK293 cells were cultured as described above. Cells were serum starved in DMEM/F12 for 18 hours prior to drug treatment. Cells were treated with U50,488 (10 µM), norBNI (1 µM), anisomycin (100 µM), naloxone (1 µM), GNTI (100 nM), or vehicle control for the appropriate time periods and then immediately lysed in: 50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, 10% Glycerol, 1:100 Phosphatase Inhibitor Cocktail Set 1 (Calbiochem, La Jolla, CA), and 1:100 Protease Inhibitor Cocktail Set 1 (Calbiochem, La Jolla, CA). Lysates were sonicated (20 sec, 4°C),
centrifuged (15,000 x g, 15 min, 4°C), and the supernatant was stored at -20°C. For spinal cord and striatal samples, mice were injected intraperitoneally (i.p.) with drug as indicated, tissue was dissected (60 min following final injection) and tissue was homogenized/lysed using a 2ml dounce homogenizer in buffer (as above). Total protein concentration was determined using Pierce bicinchoninic assay with bovine serum albumin (BSA) standards, before loading 40 µg onto non-denaturing 10% bisacrylamide precast gels (Invitrogen, Carlsbad, CA), and running at 140V for 1.5 to 2 hrs. Blots were transferred to nitrocellulose (Whatman, Middlesex, UK) for 1.5 to 2 hrs at 30V. The nitrocellulose was then washed with TBS (5 min), blocked with 5% milk/TBST (60 min), washed with TBST (3 x 5 min), and incubated overnight at 4°C in phospho-SAPK/JNK (Thr183/Tyr185) rabbit antibody or phospho-ERK1/2 (T202/Y204) rabbit antibody diluted 1:1000 in 5% BSA/TBST (Cell Signaling, Beverly, MA). After overnight incubation, the blots were washed with TBST (3 x 10 min) and incubated for 60 min at room temperatures in anti-rabbit IRDye800 diluted 1:10,000 in a 1:1 mixture of 5% milk/TBST and Li-Cor Blocking Buffer (Li-Cor Biosciences, Lincoln, NE). The blots were washed with TBST (3 x 5 min) and TBS (5 min), and then scanned on the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). The blots were re-probed with rabbit anti-β-actin diluted 1:2500 in 5% milk/TBST (2hrs, room temperature) and secondary antibody (as above) to confirm equal protein loading.

**In vivo antinociceptive testing.** The response latency for the mouse to withdraw its tail after being immersed in 52 ± 1°C water was taken as the end point. A cut-off time of 15 sec was used to prevent heat-related tissue damage.

**Radioligand Binding.** Whole mouse brains with cerebellum removed were homogenized in ice cold 50 mM Tris Buffer, pH 7.5, followed by centrifugation at 26,000 x g for 30 min at 4°C. Three brains were pooled for one N of each treatment group, with the pellet collected as the membrane fraction. Homogenization and centrifugation were repeated twice more to wash the membrane fractions, and any residual ligand present in the preparation. After the final centrifugation step, excess buffer was removed and the membranes stored at -80°C until use. Membranes were resuspended in ice cold Tris Buffer. Protein concentrations were determined by BCA assay. Samples were incubated for 90 min at room temperature with the KOR ligand [3H]U69,543. Nonspecific binding was determined in the presence of 10 µM U50,488. GF/B glass fiber filters (Brandel) were incubated 90 min at room temperature with Tris Buffer, 0.3% polyethyleneimine. After 90 min incubation at room temperature, samples were placed on ice and collected with the filters a Brandel 24 well harvester. Filters were washed 3x with cold Tris Buffer and counted in 5 ml Ecoscint scintillation fluid. For saturation binding, [3H]U69,543 was tested at concentrations ranging from 0.156-20nM. Radioligand concentrations were confirmed by scintillation counts of free ligand.

**Data analysis.** Immunoblots were scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Band intensity of both phospho-p54, and phosphop46 (JNK 1 and 2) was measured together using the Odyssey software, which subtracts background, and calculates band density in pixels. Data were normalized to a percentage of control sample band intensity (Basal, 100 %) and plotted using GraphPad (GraphPad Prism 4.0, San Diego, CA) software. Concentration-response curves, and saturation binding isotherms were fit using non-linear regression (Prism 4.0), Scatchard plots were fit using linear regression analysis. Statistical significance was taken as p < 0.05, or p < 0.01 as determined by the Student’s t test or ANOVA followed by Bonferroni post-hoc test where appropriate.

**RESULTS**

**NorBNI, GNTI, and JDTic produce long lasting antagonism for 14-21 days.** Treatment of mice with a single dose of norBNI (10 mg/kg, i.p.) resulted in significant blockade of U50,488 (KOR agonist) mediated analgesic responses 24hrs through 21 days after initial injection, as measured by tail withdrawal assay (Fig. 1A and B). However, 28 days after injection, residual norBNI effects were absent, with no difference between norBNI and saline injected groups evident at this time point. This slow rate of recovery of analgesic activity of U50,488 is the same as following
treatment of mice with the receptor alkylating drug β-chloronaltrexamine (27), and the results suggest that recovery of response requires new receptor synthesis. Further characterization of other KOR antagonists showed similar long durations of action in the analgesic assay. Single injection of GNTI (10 mg/kg) resulted in sustained and significant KOR antagonism that persisted for 21 days (Fig. 1B). JDTic (10 mg/kg, i.p.), also showed robust KOR antagonism for 14 days, and its effect reversed only at day 21 (Fig. 1B). Naloxone, a non-selective, competitive opioid receptor antagonist, produced transient KOR antagonism and did not block U50,488 analgesia on day 2 or during the duration of the experiment (Fig. 1B). For comparison, a single injection of the agonist U50,488 (10 mg/kg, i.p.) was also found not to produce long lasting effects, demonstrating that U50,488 tolerance did not underlie the long-lasting antagonism by norBNI, JDTic and GNTI. These results are consistent with prior reports and demonstrate that norBNI, GNTI, and JDTic all have long durations of antagonist activity in mice.

Protection of KOR with Competitive Antagonists Prevents NorBNI’s Long Duration of Action. To assess whether the long duration of action of norBNI was due to slow clearance of the drug, we used a receptor protection strategy. Mice were pretreated with naloxone (30 mg/kg, i.p.) (non-selective competitive opioid receptor antagonist) to block available KOR binding sites prior to injection of norBNI. If norBNI remained in the brain, then once naloxone cleared the system, residual norBNI will again have access to KOR and cause blockade. Surprisingly, in this experiment, naloxone given 15 min prior to norBNI (10 mg/kg, i.p.) significantly blocked the reduction in U50,488 response measured on Day 8 after norBNI injection (Fig. 2A). To further test this result, we next used buprenorphine, which is a mixed acting opioid ligand with partial agonist activity at MOR and competitive antagonist activity at KOR (28,29). To eliminate the potential confounding effects of partial agonist activity at MOR, we assessed the protective effects of buprenorphine in MOR knockout mice (MOR -/-). Injection of mice with buprenorphine (3 mg/kg, i.p) 60 min prior to injection of norBNI (10 mg/kg) significantly protected KOR from norBNI antagonism (Day 8) (Fig. 2B). Mice pretreated with buprenorphine prior to norBNI responded to U50,488 challenge with the same increase in tail-flick latency as mice not pretreated with norBNI or pretreated with buprenorphine alone. Buprenorphine alone did not produce analgesia in MOR(-/-) mice and did not produce sustained KOR antagonism measured on day 8. Together, these results suggest that the long duration of action of norBNI was not due to norBNI’s pharmacokinetic properties, but instead by norBNI acting at the receptor to produce changes in KOR functioning.

NorBNI Does Not Alter KOR Binding Characteristics. Single injections of norBNI followed by saturation binding experiments one week later were performed to assess whether norBNI exposure affected KOR receptor agonist affinity or KOR receptor density. Mice were injected with norBNI (10 mg/kg, i.p.) and brain membranes were prepared on day 8 for radioligand binding. Using the selective agonist [3H]-U69,593, we found no significant difference in the total receptor density (Bmax) or the affinity for kappa agonist (Kd) between norBNI treated (Bmax = 21±2 fmol/mg protein, Kd = 1.65±0.4 nM) and saline treated (Bmax = 21±1 fmol/mg protein, Kd = 1.0±0.2 nM) mouse brain membranes taken 7 days after injection of norBNI (Fig. 3A and Table 1). These data are consistent with previous reports demonstrating that norBNI does not form a covalent bond to the receptor and does not decrease apparent receptor number (20,30).

The effect of receptor protection on membranes from norBNI treated mice was also measured. In this experiment mice were pretreated with buprenorphine (3 mg/kg, i.p) 1hr prior to injection of norBNI (10 mg/kg, i.p.), and membranes were prepared on day 8 after these initial injections. There was also no significant change in either the Bmax or Kd of [3H]-U69,593 (Fig. 3B and Table 1) further supporting the conclusion that the long duration of action of norBNI cannot be attributed to changes in receptor density or affinity.

NorBNI Activates c-Jun N-terminal kinase in a KOR-dependent Manner. Previous studies have shown that norBNI acts as an antagonist for KOR-mediated signal transduction (6,8,10). For example norBNI has been shown to block the KOR-mediated inhibition of cAMP, activation of inward rectifying potassium channel, and increased ERK1/2 and p38 MAPK phosphorylation (6,8,10). While performing pilot
MAPK studies in KOR-transfected cells, we were surprised to find that norBNI treatment activated JNK and hypothesized that JNK activation may be involved in its long-lasting effects.

In KOR-GFP expressing HEK293 cells, we found that treatment with U50,488 (1hr, 37°C) caused a concentration-dependent ($EC_{50} = 1240\pm 180$ nM) increase in phospho-JNK activity. Maximal activation of JNK by U50,488 was evident at the 1hr time point, with the response returning to basal levels at 3hrs (data not shown). The endogenous kappa-opioid peptide dynorphin (1hr, 37°C) also increased phospho-JNK in a concentration-dependent manner ($EC_{50} = 87\pm 13$ nM) (Fig. 4A and C, Table 1), although with a much lower efficacy than U50,488. Unexpectedly, norBNI treatment (1hr, 37°C) also caused a concentration-dependent increase ($EC_{50} = 158\pm 18$ nM) in phospho-JNK in KOR-GFP expressing HEK293 cells (Fig. 4B and C, Table 1). JDTic also caused a concentration-dependent ($EC_{50} = 5\pm 3$ nM) increase in phospho-JNK (Table 1) in KOR-GFP HEK293 cells. The competitive KOR antagonist, buprenorphine, however did not elicit any JNK response in these cells, suggesting that this effect was characteristic of long acting KOR antagonists (Fig. 4B and C, Table 1).

To confirm that norBNI would produce KOR antagonism under these conditions, we measured U50,488-induced phospho-ERK1/2 activation in KOR-GFP expressing HEK293 cells. In these experiments, norBNI treatment completely blocked KOR-dependent (U50,488 treated) phospho-ERK1/2 activation, but in the same cells, increased phospho-JNK activity (Fig. 5A). In addition, selectivity for KOR-induced phospho-JNK was confirmed by stimulating untransfected HEK293 cells with KOR ligands under the same conditions. NorBNI, JDTic, GNTI, and U50,488 (10 µM, 1hr, 37°C) did not increase phospho-JNK in untransfected HEK293 cells (Fig. 5B).

Anisomycin (100 µM, 15 min, 37°C), the stress-kinase activator, increased phospho-JNK by greater than 2-fold, as expected, confirming that JNK signaling is intact in these untransfected HEK293 cells. Additionally, we stimulated MOR-GFP expressing HEK293 cells with norBNI (10 µM, 1hr, 37°C) (Fig. 5B, inset), and found that there was no effect on phospho-JNK by possible norBNI binding to MOR. These data support the conclusion that norBNI and related ligands induce JNK activation in a KOR-dependent manner.

**NorBNI-induced phospho-JNK is not pertussis toxin sensitive.** NorBNI has been previously found to be unable to induce GTPγS binding or GTP hydrolysis (31). Consistent with these observations, we found that pertussis toxin (200 ng/ml, 18hrs, 37°C) pretreatment of KOR-GFP expressing HEK293 cells failed to block norBNI-induced (10 µM, 1hr, 37 °C) increase in phospho-JNK (Fig. 6A and B). In contrast, U50,488-induced phospho-ERK 1/2 activation was blocked by pertussis treatment (Fig. 6C). These data support the conclusion that norBNI-induced phospho-JNK activation was independent of Goxi-activation. In addition, the blockade of two classical mediators of MAPK signaling Src and Raf, did not alter the norBNI-induced phospho-JNK activation (data not shown). The mechanism of JNK activation in vitro was not further defined.

**NorBNI induces phospho-JNK activation in vivo, in mouse spinal cord and striatal tissue.** Since transfected cell systems may have limited physiological relevance, we next did in vivo studies to assess the relationship between JNK activation and the long-lasting antagonist effects. NorBNI treatment (10 mg/kg, i.p., 1hr) caused an increase in phospho-JNK in both the mouse spinal cord and striatum (Fig. 7A and B). Further, norBNI treatment did not significantly increase phospho-JNK in KOR knockout mice (KOR $^{-/-}$) (Fig. 7A and B). These data support the conclusion that norBNI activates JNK in vivo in a KOR-dependent manner, further corroborating the results from HEK293 cells.

**JNK antagonist SP600125 reverses the NorBNI Long Duration of Action.** To assess the role of JNK activation in norBNI’s long duration of action, we treated mice with JNK inhibitor prior to injection of norBNI, and measured U50,488 (25 mg/kg, i.p.) analgesic responses 7 days after the initial injection. Treatment of mice with SP600125 (30 mg/kg, i.p., 2 times: 24 hrs and 30 min before norBNI), a potent and selective JNK inhibitor (32,33) prior to injection of norBNI (10 mg/kg, i.p) significantly blocked the norBNI antagonist effect on 8 day (Fig. 8). SP600125 alone had no effect on U50,488-induced analgesic responses or basal tail-withdrawal latencies; there was no significant difference in U50,488 response in mice pretreated with vehicle, untreated or SP600125.
alone (Fig. 8). These data support the hypothesis that the long duration of norBNI action is caused by JNK activation.

**DISCUSSION**

The principal finding of this study was that long acting KOR antagonists cause lasting changes in signaling that block KOR agonist responses. The KOR antagonists, norBNI, JDTic, and GNTI all produced long lasting antagonism of the kappa analgesic effects in mice, consistent with findings reported previously (12-19). This long lasting effect was not due to the deposition of antagonist at lipid depot sites because receptor protection assays with competitive, rapidly cleared ligands, blocked the long lasting antagonist effects. Furthermore, norBNI did not alter the affinity of the radiolabeled KOR agonist or decrease the total KOR density in mouse brain membranes, indicating that the antagonists did not down-regulate receptor or covalently occlude the agonist binding pocket. Instead, the results suggest that the long-active effects were caused by JNK activation.

The potential therapeutic effects of KOR antagonists have generated considerable interest recently because the receptor was identified as a novel target for the treatment of drug abuse, depression, and chronic pain-related disorders (21-23). Portoghese et al. (1987) initially described the potent and selective KOR antagonist norbinaltorphimine (norBNI) and also synthesized the antagonist GNTI, an indolomorphinan also having high affinity and selectivity for KOR (1,2,34). In addition, a novel phenylpiperidine-based KOR antagonist was developed called JDTic, 4-diethylaminotrans-(3R,4R)-dimethyl-4-(3-hydroxyphenyl) piperidine (35). A common feature of all three of these KOR antagonists is that when injected in vivo they produce sustained KOR antagonism for several days to weeks (12-19). NorBNI, GNTI, and JDTic have some common features including 2 basic nitrogens and a second hydroxyl group necessary for high binding affinity (35,36). However, the structural differences of these compounds makes it unlikely that they share common active metabolites. Because both buprenorphine and naloxone can block agonist effects without inactivating the receptor, long-action is not an intrinsic property of KOR antagonism, and it will be interesting to learn how the new class of selective KOR antagonists based on the salvinorin A core act (37-39).

The duration of action of the long-acting antagonists was equivalent to β-chlornaltrexamine suggesting that recovery requires new kappa receptor synthesis (27). Inactivation of the KOR-signaling complex was also observed during studies of analgesic tolerance mechanisms; repeated injection of U50,488 produced KOR-phosphorylation and receptor inactivation that required 2 weeks for recovery (27). These prolonged inactivation results suggest that the kappa opioid signaling complex expressed in neurons is a macromolecular entity assembled by appropriate chaperones in the golgi then inserted as a functional unit at the site of action. One interpretation of our results is that activation of JNK inactivates a key component of this structure and restoration of function requires regeneration of the whole structure. Examples of this type of signaling partner would be a JNK-sensitive scaffold protein. Possible regulation by partners in the cellular protein sorting machinery, including the ubiquitin family proteins, and small ubiquitin-related modifiers (SUMO) that have been shown to interact with stress kinases in other systems (40), are potential mechanisms to consider. While this explanation seems plausible there are several issues that are not fully resolved.

First, although both the untransfected HEK cell and KOR(−/−) control experiments establish the requirement for KOR, how antagonist binding to KOR activates JNK in a pertussis-toxin insensitive manner is not clear. Second, the conclusion depends on assumptions of the in vivo specificity of the JNK inhibitor SP600125. This drug does not block JNK phosphorylation but instead inhibits the kinase activity of the activated enzyme (32,33). Nonspecific effects of this compound were not evident in the experiments done, but more confidence in the interpretation of the experiment would be possible if a critical JNK substrate was known. Future studies designed to identify the basis for KOR inactivation will be important to resolve this issue.

Third, while JNK activation seems to be required for KOR inactivation since kappa ligands that failed to activate JNK (i.e., buprenorphine) do not produce long-lasting antagonism, JNK activation alone is not sufficient since U50,488 and dynorphin also activate JNK in a KOR-
dependent manner. The basis for this difference is not clear, but we propose that it may be related to the pharmacological difference between JNK-sensitivities of the agonist-activated conformation of KOR versus the antagonist conformation of KOR. This concept seems reasonable since we know that agonist-activation of KOR results in receptor phosphorylation, down-regulation, and activation of p38 and ERK 1/2 MAPKs (10,11,27) all of which may be distinct from the JNK signaling pathway. We also know these conformations are likely to be different because this is one of the primary bases for intrinsic efficacy (42). However, the JNK substrates and sites of JNK activation are not yet defined. Resolution of this mechanism will not be a trivial exercise, because the structure and constituents of the KOR signaling complex in vivo are not presently understood. In any case, an important result of the present study is the strong suggestion that JNK activation regulates the function of this KOR signaling complex.

Ligand specific activation of MAPK signaling pathways by GPCRs has been proposed for several GPCR classes in a number of cell and tissue types (43). U50,488 stimulation of KOR activates ERK1/2 and p38 MAPK via different signal transduction pathways (10,11). ERK1/2 activation in C6 glioma cells occurs through pertussis toxin sensitive G protein activation (7), and p38 MAPK activation occurs through an alternative, arrestin-independent mechanism (11). The mechanism of KOR antagonist-induced JNK activity was not defined but may be mediated by an alternative G-protein, because other reports have identified several pertussis-insensitive proteins, including Gα12 and Gα16, and others in GPCR-mediated MAPK activation (43). However, this hypothesis requires further validation.

The JNK MAPK pathways have been demonstrated to play a major role in environmental stress and inflammatory signals, including cytokine activation, and more recently this pathway has been implicated in both mouse models of pain and mouse behavioral responses (44). It is unclear whether KOR-mediated JNK phosphorylation by norBNI and other KOR ligands represents an untoward effect of these antagonists or whether these mixed agonist effects are useful in the development and characterization of KOR ligands for therapeutic benefit.

Studies using heterologous expression systems have important limitations for studies of signal transduction mechanisms because receptors are typically not expressed at physiological densities, and immortalized cell lines do not always contain all the necessary cellular proteins that interact with a given receptor signaling complex. Since we found mixed agonist-antagonist properties of norBNI in HEK293 cells that express a high density of KOR, it was possible that this could be an artifact of this expression system. Confirmation of norBNI effects in wildtype mice, but not in mice lacking KOR was an essential step, and future studies defining the components of the proposed functional signaling complex in neurons will be an important extension of these studies.

Finally, the type of long-acting effects seen for these KOR antagonists may also be evident in other classes of GPCRs. For example, some muscarinic receptor antagonists have been shown to have pseudo-noncompetitive characteristics (45). In addition, respiradone, a 5-HT7-selective antagonist has also been shown to have “pseudo-irreversible” characteristics that was suggested to depend on cytosolic-receptor interactions (46). The idea that certain classes of GPCR antagonists may have the unique ability to alter receptor conformations is consistent with mathematical models (42), but will require additional characterization in vitro and in vivo. Recent reports have also suggested that some GPCR antagonists possess a characteristic termed, collateral agonist efficacy which may result from a ligand stabilizing an intermediate receptor state that possesses distinct signaling properties (47). Our present results support the classification of long acting KOR antagonists as collateral agonists for the c-Jun N-terminal kinase cascade. Future studies with other KOR antagonists will help to validate these classifications.

In conclusion, the studies presented suggest a novel mechanism of kappa opioid antagonism. Insights gained from this approach are likely to provide a new understanding of the structural and functional properties of the kappa opioid signaling complex. These insights may be relevant to other GPCRs.
REFERENCES


FOOTNOTES

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+ The abbreviations used are: ANOVA, analysis of variance; BCA, Bicinchoninic acid; BSA, bovine serum albumin; ERK, Extracellular signal-regulated kinase; GFP, green fluorescent protein; GNTI, Guanidinaltrindole; GPCR, G-protein coupled receptor; JDTic, atrans-(3R,4R)-dimethyl-4-(3-hydroxyphenyl) piperidine; KOR, Kappa opioid receptor; MOR, Mu opioid peptide receptor; JNK, c-Jun N-terminal Kinase; MAPK, Mitogen-activated protein kinase; MEM, Minimal essential media; norBNI, norbinaltorphimine HCl; PBS, Phosphate-buffered saline; p-p38, Phospho-p38 MAPK; SAPK, stress-activated protein kinase; TBS, Tris-buffered saline; TBST, Tris-buffered saline with tween-20
**Figure Legends**

**Figure 1**: NorBNI, JDTic, and GNTI produce long lasting antagonism in mice. A, Mice were given a single injection of norBNI (10 mg/kg, i.p.), or saline, and tail withdrawal latencies were measured as described in “experimental procedures” at the specified time points 30 min after an injection of U50,488 (15 mg/kg, i.p.). NorBNI significantly antagonized U50,488-induced analgesic responses through day 21 following initial injection. Data are the mean ± S.E. of tail flick latencies expressed as ± S.E. n = 8-12 where each n is a separate animal. *, significantly different than U50,488 +saline group, p < 0.05 using one-way ANOVA followed by Bonferroni post-hoc test. B, Mice were given a single injection of norBNI (10 mg/kg, i.p.), GNTI (10 mg/kg, i.p.), JDTic (10mg/kg, i.p.), Naloxone (30 mg/kg, i.p.) or U50, 488 (15 mg/kg, i.p.) and then the tail withdrawal latencies were measured at the designated time points 30 min after injection of U50,488 (15 mg/kg, i.p.). NorBNI, and GNTI had similar time courses for KOR antagonism. JDTic produced significant KOR antagonism for 14 days. Naloxone and U50,488 did not produce long lasting effects on KOR-mediated analgesic responses. Data are the mean ± S.E. of tail flick latencies. n = 8, where each n is a separate animal. *, significantly different than saline control, p < 0.05, using one way-ANOVA, followed by Bonferroni post-hoc test.

**Figure 2**: Kappa opioid receptor protection by competitive antagonist, reverses the sustained antagonism by norBNI. A, Mice were injected with naloxone (30 mg/kg, i.p.) (non-selective opioid receptor antagonist) to occlude the available KOR binding pocket to norBNI, saline, or U50,488 (15 mg/kg, i.p.) prior to injection with norBNI (10 mg/kg, i.p.), and on Day 8 tail “flick” latencies were measured as described, over a 1hr time course following U50,488 injection. Naloxone significantly protected the KOR from norBNI antagonism at the day 8 time point. *, significantly different from saline/saline group and naloxone/norBNI group, n = 4-8, p < 0.05 using one-way ANOVA, followed by Bonferroni post-hoc test. B, MOR knockout (MOR-/-) mice were injected with buprenorphine (3 mg/kg, i.p.) (KOR-selective competitive antagonist) to occlude the available KOR binding pocket to norBNI, prior to injection with norBNI (10 mg/kg, i.p.), and on Day 8 tail “flick” latencies were measured as described, over a 1hr time course. Buprenorphine significantly protected the KOR from norBNI antagonism at the day 8 time point. *, significantly different from Bup/Saline (MOR -/-), Bup/NorBNI, or Bup/Saline (MOR +/+). p < 0.05 using one-way ANOVA, followed by Bonferroni post-hoc test, n = 8, where each n is a different animal.

**Figure 3**: NorBNI does not alter KOR binding characteristics. A, Mean saturation binding isotherms for specific [3H]-U69,593 (a KOR-selective agonist) in membranes from mouse brain on Day 8 after norBNI (10 mg/kg, i.p.) or saline. Concentrations of total [3H]-U69,593, ranging from 0.156-20nM were incubated with membranes in the presence (non-specific binding) and absence (total binding) of 10 µM U50,488 as described in “Experimental Procedures.” Specific binding was determined by subtracting non-specific from total binding counts, and converted into fmol/mg protein. Non-linear regression of five individual binding curves show that [3H]-U69593 bound to a homogenous receptor population, and had similar affinity and receptor density in both the norBNI group (Bmax = 21 ± 1 fmol/mg protein, KD = 1.00 ± 0.20 nM) and the saline treated group (Bmax = 21 ± 2 fmol/mg protein, KD = 1.65 ± 0.40 nM). Inset shows the mean data ± S.E. in Scatchard plots that were derived from the saturation isotherms. n = 5, where each n is a different experiment. B, Mean saturation binding isotherms for specific [3H]-U69,593, and measured as indicated above. Non-linear regression of five individual binding curves show that under conditions known to protect KOR from norBNI (Fig 2), [3H]-U69,593 bound to a homogenous receptor population in both the buprenorphine (BPN, Bmax = 19 ± 1 fmol/mg protein , KD = 0.73 ± 0.16 nM) and BPN + NorBNI (Bmax = 23 ± 3 fmol/mg protein, KD = 2.50 ± 0.78 nM) groups with similar affinity and total receptor number. Inset shows the mean data ± S.E. in Scatchard plots that were derived from the saturation isotherms. n = 5, where each n is a different experiment.
Figure 4: NorBNI increases phospho-JNK in a concentration-dependent manner. A, Phospho-c-Jun N-terminal Kinase (Phospho-JNK) concentration response curves for the KOR agonists U50,488 and the endogenous KOR-agonist peptide dynorphin B in HEK293 cells expressing KOR-GFP. B, Phospho-JNK concentration responses curves for the KOR ligands norBNI and buprenorphine in HEK293 cells expressing KOR-GFP. All ligand treatments were performed at the 1hr time point. n = 3-5, with each n taken from a separate cell culture and experiment. C, Representative western blots for phospho-JNK concentration response data, in KOR-GFP expressing HEK cells for U50,488 (top), Dynorphin B (Middle, Dyn B), and NorBNI (bottom).

Figure 5: NorBNI blocks KOR-induced ERK 1/2 activation, but increases phospho-JNK in KOR expressing HEK293 cells. A, Mean band intensities expressed as a percentage of basal-untreated control (dashed line) ± S.E. of KOR-GFP mediated ERK 1/2 and JNK phosphorylation from HEK293 cells expressing KOR-GFP. Phospho-JNK but not phospho-ERK 1/2 levels were significantly increased following norBNI treatment (10 µM, 1hr, 37°C). As expected U50,488 caused a robust increase in both phospho-JNK and phospho-ERK 1/2 over control. NorBNI treatment blocked U50-induced phospho-ERK 1/2 activity but had no effect on U50-mediated phospho-JNK activity. * or **, significantly different than basal, *p < 0.05, or **p < 0.01 using the Student’s t test. n = 3-5 where each n is taken from an independent experiment. B, Mean band intensities expressed as a percentage of basal-untreated control (dashed line) ± S.E. in untransfected HEK293 cells stimulated with the stress kinase activator Anisomycin (Anis, 100 µM), or the KOR ligands: NorBNI (10 µM), JDTic (10 µM), GNTI (10 µM), and U50,488 (10 µM) for 1hr at 37°C. As predicted none of the KOR-selective ligands caused activation of JNK in cells that do not express KOR. Anisomycin treatment (50 µM, 15 min, 37°C) increased phospho-JNK, confirmed that JNK signaling was intact in the untransfected cells. n = 3-7, where each n represents an individual experiment. *, significantly different from basal, p < 0.05 using the student’s t test. Inset, Mean band intensities expressed as a percentage of basal-untreated control (dashed line) ± S.E. in MOR-GFP expressing HEK293 cells stimulated with either Anis or norBNI under the same conditions as above. n = 3-4, where each n represents an individual experiment.

Figure 6: NorBNI-induced phospho-JNK is pertussis toxin insensitive. A, Mean band intensities expressed as a percentage of basal-untreated control (dashed line) ± S.E. of KOR-GFP mediated JNK phosphorylation from HEK293 cells expressing KOR-GFP treated with pertussis toxin (200 ng/ml, 18hrs, 37°C). Phospho-JNK levels were significantly (*) increased 1hr after norBNI treatment, and were not reversed by Gα(i) inhibition by pertussis toxin. *, significantly different from basal, p < 0.05, using the Student’s t test. n = 3-4, where each n represents an individual experiment. B, Representative western blot of phospho-JNK bands in norBNI treated KOR-GFP expressing HEK293 cells treated with and without pertussis toxin (Ptx). β-Actin western blot below confirms equal protein loading in each lane. C, Representative western blot of phospho-ERK 1/2 bands in U50,488 (U50) treated KOR-GFP expressing HEK293 cells treated with and without pertussis toxin (Ptx). β-Actin western blot below confirms equal protein loading in each lane.

Figure 7: NorBNI induces JNK phosphorylation in wild type mouse spinal cord and striatum, but not in KOR (-/-) mice. A, Mean phospho-JNK band intensities expressed as a percentage of basal-untreated control (dashed line) ± S.E. taken from spinal cord or striatal cell lysates of KOR wild type (KOR +/+ ) or KOR knockout (KOR -/- ) mice treated with norBNI (10 mg/kg, i.p., 1hr). NorBNI caused a significant (*) increase in phospho-JNK in both mouse striatum and spinal cord that was absent in samples taken from norBNI-treated KOR(-/-) mice. n = 5-9, where each n is taken from a separate animal, *, significantly different than basal, p < 0.05 using the student’s t test. B, Representative western blot of phospho-JNK in spinal cord cell lysate samples taken from KOR(+/-) and KOR(-/-) norBNI, or saline (SA) treated mice. β-actin blot below confirms equal protein loading in each lane.
Figure 8: Blockade of JNK reverses the long lasting antagonism of norBNI. Wildtype mice were treated with the selective JNK inhibitor SP600125 (30 mg/kg, i.p., 2 times: 24hrs and 30 min before norBNI) prior to injection with the KOR antagonist norBNI and tail flick analgesic responses were determined 7 days after injection. For injections SP600125 (JNK-I) was solubilized in vehicle (V) containing 5% Me$_2$SO, 20% Cremophor EL, 75% Saline. U50,488 caused a significant (**) increase in the tail flick analgesic response in vehicle treated SP600125 (###) alone (JNK-I), and no vehicle (Control) groups. Inhibition of JNK prior to norBNI injection significantly (*) reversed the long lasting antagonist effect on U50,488-induced analgesia. *, p < 0.05 for V/NorBNI groups vs. JNK-I, ** p < 0.01 for V vs. V + NorBNI groups, ###, p < 0.01 for Control vs. V + NorBNI groups, ####, p < 0.001 JNK-I vs. V +NorBNI groups; using one-way ANOVA followed by Bonferroni post-hoc test. n = 12, where each n is a separate animal.

Table 1: Affinity values, total binding site densities, and pharmacological data for KOR ligands in mouse brain tissue and HEK293 cells. All values are expressed as ± S.E.. Binding data and concentration response data were analyzed using non-linear regression curve fitting in Graph Pad Prism (4.0). Bmax (fmol/mg protein), total receptor density in mouse brain membranes; Kd, equilibrium dissociation constant in nanomolar (nM) (affinity) for specific [H]-U69,593 binding. EC$_{50}$ (nM), half maximal effective concentration (potency) of KOR ligands in stimulation phospho-JNK in HEK293 cells; IA, relative intrinsic activity of KOR ligands for causing JNK activation, as compared to the full agonist, U50,488.
Figure 2

**A**

- **Saline + Saline**
- **Naloxone + NorBNI**
- **Saline + NorBNI**
- **U50 + NorBNI**

*Time after U50,488 Injection (min)*

*Tail withdrawal latency (sec)*

**Day 8**

* * *

**B**

- **Bup + Saline (MOR -/-)**
- **Bup + NorBNI (MOR -/-)**
- **Saline + NorBNI (MOR -/-)**
- **Bup + Saline (MOR +/+)**

*Time after U50,488 Injection (min)*

*Tail withdrawal latency (sec)*

**Day 8**

* * *
Figure 3

A

Specific $[^3]H$U69,593 Bound (fmol/mg protein)

- Saline + Saline
- Saline + NorBNI

B

Specific $[^3]H$U69,593 Bound (fmol/mg protein)

- BPN + Saline
- BPN + NorBNI
Figure 4

A

% Increase over Basal (pJNK)

log M [Agonist]

B

% Increase over Basal (pJNK)

log M [Agonist]

C

U50,488

pJNK

-9 -8 -7 -6 -5 M

Dyn B

pJNK

-9 -8 -7 -6 -5 M

NorBNI

pJNK

-9 -8 -7 -6 -5 M
Figure 6

(A) Bar graph showing the % of Control (pJNK) for KOR-GFP and UT-HEK with NorBNI and NorBNI + Ptx. The graph includes error bars.

(B) Western blot images for pJNK, Actin, NorBNI, and Ptx conditions.

(C) Western blot images for pERK 1/2, U50, and Ptx conditions.
A

![Bar graph showing % of Basal (Control pJNK) for Spinal Cord and Striatum in KOR (+/+) and KOR (-/-) groups.](Image 1)

B

![Western blot images for pJNK and Actin with Saline and NorBNI treatments for KOR (+/+) and KOR (-/-) groups.](Image 2)

**Figure 7**

- **A**
  - Bar graph with % of Basal (Control pJNK) for Spinal Cord and Striatum in KOR (+/+) and KOR (-/-) groups.
  - Significant differences marked with asterisks.

- **B**
  - Western blot images for pJNK and Actin with Saline and NorBNI treatments for KOR (+/+) and KOR (-/-) groups.
Figure 8

Tail withdrawal latency (sec)

V  V  JNK-I  JNK-I  Control
+NorBNI  +NorBNI

Basal U50,488

**  ###  *  ##
Table 1: Affinity values, total binding site densities, and pharmacological data for KOR ligands in mouse brain tissue and HEK293 cells.

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<tr>
<th>Group</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$ (fmol/mg)</th>
<th>$EC_{50}$ (nM)</th>
<th>$IA$</th>
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<td>Saline</td>
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<td>-</td>
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Long-acting kappa opioid antagonists disrupt receptor signaling and produce noncompetitive effects by activating C-jun N-terminal kinase

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