Physical Presence of Nor-Binaltorphimine in Mouse Brain over 21 Days after a Single Administration Corresponds to Its Long-Lasting Antagonistic Effect on \( \kappa \)-Opioid Receptors

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ABSTRACT

In the mouse 55°C warm-water tail-withdrawal assay, a single administration of nor-binaltorphimine (nor-BNI; 10 mg/kg i.p.) antagonized \( \kappa \)-opioid receptor (KOR) agonist-induced antinociception up to 14 days, whereas naloxone (10 mg/kg i.p.)-mediated antagonism lasted less than 1 day. In saturation binding experiments, mouse brain membranes isolated and washed 1 or 7 (but not 14) days after nor-BNI administration demonstrated a significant time-dependent decrease in maximal KOR agonist \([3H]U69,593\) binding. To determine whether brain concentrations of nor-BNI were sufficient to explain the antagonism of KOR-mediated antinociception, mouse brain and perfused brain were harvested at time points ranging from 30 minutes to 21 days after a single administration and analyzed for the presence of nor-BNI using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Nor-BNI was detected in the perfused brain homogenate up to 21 days after administration (30 nmol i.c.v. or 10 mg/kg i.p.). Subsequent experiments in which nor-BNI was administered at doses estimated from the amounts detected in the brain homogenates isolated from pretreated mice over time demonstrated significant antagonism of U50,488 antinociception in a manner consistent with the magnitude of observed KOR antagonism. The dose (1.4 nmol) approximating the lowest amount of nor-BNI detected in brain on day 14 did not antagonize U50,488-induced antinociception, consistent with the absence of U50,488 antagonist observed in vivo at this time point after pretreatment. Overall, the physical presence of nor-BNI in the mouse brain paralleled its in vivo pharmacological profile, suggesting physicochemical and pharmacokinetic properties of nor-BNI may contribute to the prolonged KOR antagonism.

Introduction

\( \kappa \)-Opioid receptor (KOR) selective antagonists have been traditionally used as pharmacological tools to study \( \kappa \) receptors. However, recent reports suggest these compounds may possess a number of therapeutic applications (Aldrich and McLaughlin, 2009), as they exhibit antidepressant-like effects (Mague et al., 2003; Beardsley et al., 2005; Zhang et al., 2007), anxiolytic-like effects (Knoll et al., 2007; Wittmann et al., 2009), efficacy against opiate addiction (Rothman et al., 2007), anxiolytic-like effects (Knoll et al., 2007; Wittmann et al., 2009), efficacy against opiate addiction (Rothman et al., 2007), anxiolytic-like effects (Knoll et al., 2007; Wittmann et al., 2009), efficacy against opiate addiction (Rothman et al., 2007), anxiolytic-like effects (Knoll et al., 2007; Wittmann et al., 2009), efficacy against opiate addiction (Rothman et al., 2007) broventricular (1 nmol i.c.v.) treatment with nor-BNI produced significant antagonism of the KOR agonists U69,593 and bremazocine in the mouse tail-flick test lasting 21 days and significantly reduced the affinity of \([3H]U69,593\) binding to isolated brain membranes for over 28 days. A number of in vivo studies subsequently confirmed that nor-BNI produces an unusually long antagonism of KOR after a single administration (Jones and Holtzman, 1992; Butelman et al., 1993; Broadbear et al., 1994; Metcalf and Coop, 2005; Potter et al., 2011). This prolonged antagonism of KOR in animal models by nor-BNI presents difficulties in using nor-BNI as an efficient, reversible pharmacological tool (Aldrich and McLaughlin, 2009). Moreover, a number of KOR selective antagonists, including 5′-guanidinonaltrindole (Jones et al., 1998) and the phenylpiperidine JDTic (Thomas et al., 2001), also exhibit an exceptionally long duration of antagonism.
described as “pseudoirreversible,” lasting weeks in vivo after a single administration (Carroll et al., 2005; Metcalf and Coop, 2005).

The mechanism by which nor-BNI and similar ligands produce their prolonged KOR antagonism has been a subject of considerable study (Metcalf and Coop, 2005). A number of long-lasting opioid antagonists identified previously (Ward et al., 1982; Aldrich and Vigil-Cruz, 2002) contain a reactive electrophilic functional group (such as a Michael acceptor) that can form an irreversible covalent bond with the opioid receptor protein, producing a prolonged antagonism of the opioid-mediated effects by preventing interaction of other opioid ligands with the receptor binding site (Chen et al., 1996). Nor-BNI does not contain such a reactive functionality capable of forming an irreversible covalent bond with KOR, precluding such an interaction as a mechanism for its long-lasting KOR antagonism. More recently, the extended KOR-selective antagonism in animal models produced by nor-BNI has been attributed to the ligand-induced activation of the c-Jun N-terminal kinase (JNK) family of mitogen-activated protein kinases, leading to prolonged inactivation of KOR signaling persisting for weeks after a single exposure (Bruchas et al., 2007; Melief et al., 2010, 2011). However, the mechanism by which the KOR-selective antagonists might mediate JNK activation leading to the inactivation of KOR signaling has not been fully elucidated.

Pharmacological properties of nor-BNI have been extensively studied, but few detailed pharmacokinetic studies of nor-BNI in vivo are available. One of the important pharmacokinetic parameters of a drug that affects its duration of action is the distribution of the drug in the body. To evaluate the presence of nor-BNI in brain tissue for an extended period requires a highly selective and sensitive method capable of detecting nor-BNI in minute quantities. Liquid chromatography coupled with mass spectrometry (LC-MS/MS) offers a suitable tool to detect nor-BNI in mouse brain with selectivity and high sensitivity. Mass spectrometry selectively detects nor-BNI based on its mass, which is a fundamental characteristic of a molecule, and the consistent chromatographic retention time in each sample provides a means for confident quantitation of nor-BNI in the samples. We used LC-MS/MS to assess whether a single administration of nor-BNI to C57BL/6J mice, through either the intracerebroventricular or intraperitoneal route, results in its prolonged retention in the brain in the concentrations that correlate with the long-lasting antagonism of U50,488-induced antinociception measured in the 55°C warm-water tail-withdrawal assay. In a separate experiment, we evaluated if nor-BNI produced an equivalent extended inhibition of the binding of KOR agonist [3H]U69,593 to murine brain membranes containing KOR.

Materials and Methods

Animals

All the experiments were carried out using male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) of ages 8–14 weeks old. Mice were kept in groups of four in a temperature-controlled room with 12-hour light/dark cycle. Food and water were available ad libitum until the time of the experiment. All mice were housed, tested, and cared for in accordance with the 2002 National Institutes of Health Guide for the Care and Use of Laboratory Animals and as approved by the Institutional Animal Care and Use Committee.

Chemicals and Reagents

Nor-binaltorphimine (nor-BNI)-dihydrochloride, naloxone-hydrochloride, (2-)-trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzenacetic acid methylene-sulfonate hydrate (U50,488), ammonium acetate, and analytical grade acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO). (1H) (+) (5α,7α,8α)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzenacetic acid ([3H]U69,593, 37.4 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

Preparation of Test Solutions and Standards for LC-MS/MS Analysis

Nor-BNI and naloxone stock solutions were made in water and diluted to the desired concentrations in saline (0.9%) before administration. Nor-BNI or naloxone was administered via intracerebroventricular or an intraperitoneal injection. LC-MS/MS standard curves of nor-BNI concentration in extracted brain homogenate were generated ex vivo by adding nor-BNI (0.3–6 nmol) directly to weighed, homogenized brains from naive mice, facilitating the normalization of response to brain mass. LC-MS/MS-determined concentrations of nor-BNI in experimental brain homogenates were then calculated using these standard curves. Naloxone (0.12 or 1.2 μg/ml in acetonitrile) was used as the internal standard for all LC-MS/MS assays unless otherwise stated.

Injection Techniques

Intracerebroventricular injections were made directly into the lateral ventricle according to the modified method of Haley and McCormick (1957). The volume of all intracerebroventricular injections was 5 μl using a 10-μl Hamilton microsyringe. The mouse was lightly anesthetized with isoflurane, an incision made in the scalp, and the injection made freehand 2 mm lateral and 2 mm caudal to bregma at a depth of 3 mm.

Mice were administered nor-BNI or naloxone through the intracerebroventricular or intraperitoneal routes 80 minutes or 6, 23.3, 47.3, 167.3, 335.3, or 503.3 hours before administration of U50,488 (see below) or euthanasia for serum and brain harvest post-injection (see below).

In Vivo Studies

Antinociception Assay (Tail-Withdrawal Assay). The 55°C warm-water tail-withdrawal assay was performed as described earlier (McLaughlin et al., 1999, 2004) where the thermal nociceptive stimulus was 55°C water and the latency to tail withdrawal was taken as the endpoint. Baseline tail-withdrawal latencies (averaging 1.27 ± 0.07 seconds) were tested before drug administration as negative control. Mice that showed no response within 5 seconds in the initial control test were eliminated from the experiment. A cut-off time of 15 seconds was used; if the mouse failed to withdraw its tail in that time, the tail was removed from the water.

After determining baseline tail-withdrawal latencies, mice received saline or a single dose of an opioid receptor antagonist and were returned to their home cages. Treated mice were allowed to recover 80 minutes, 1, 3, 7, and/or 14 days, and then administered the KOR agonist U50,488 (10 mg/kg i.p. or 100 nmol i.c.v.). U50,488-induced antinociception was tested 40 minutes post-administration by measuring the tail-withdrawal latency of mice. In a separate experiment, mice were administered nor-BNI (30, 8, 2.7, and 1.4 nmol i.c.v.) 6 hours before the administration of U50,488 (30 nmol i.c.v.) and antinociception was tested as described above.
Ex Vivo Studies

**κ-Opioid Receptor Binding to Murine Brain Membranes.** Mice were pretreated with a single dose of saline (0.9%) or nor-BNI (10 mg/kg i.p.) 1, 7, or 14 days prior to brain dissection. Whole brains were dissected from C57BL/6J mice; the cerebellum was removed, and brain membranes were prepared as described previously (Toll et al., 1998). Membranes were used immediately in experiments to avoid changes resulting from freezing and thawing. Isolated membrane samples were washed twice by centrifugation (20,000g) with 50 mM Tris-HCl, pH 7.4, prior to the determination of the protein concentration. The protein concentration of the membranes after harvest was determined by the method of Bradford (1976) using bovine serum albumin as the standard. The effect of pretreatment on subsequent ligand binding to KOR was determined by incubating membrane protein (0.5 mg/ml) with the KOR-selective radioligand \(^{3}H\)U69,593 for 2 hours at 25°C in a final volume of 1 ml of 50 mM Tris-HCl, pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluoride and 5 mM MgCl\(_2\). Nonspecific binding was measured by inclusion of U50,488 (1 μM). Binding was terminated by filtering the samples through glass fiber filters, presoaked in 50 mM Tris-HCl (pH 7.4) containing 2 mg/ml bovine serum albumin, using a Tomtec cell harvester (Tomtec, Hamden, CT). After filtration, filters were washed with ice-cold 50 mM Tris-HCl, pH 7.4 (3 ml, 3×) and were counted in a Wallac beta-plate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA).

**Sample Preparation for LC-MS/MS Analysis**

Mice were administered nor-BNI or naloxone through either the intracerebroventricular or intraperitoneal routes and euthanized at various time points from 0 minutes to 21 days post-administration. Blood (200–250 μl) was collected from mice by cardiac puncture and was allowed to clot naturally at 37°C overnight. Serum was collected and transferred to a separate tube, and proteins were precipitated by adding 2 volumes of ice-cold acetonitrile containing the internal standard (IS) and centrifuged at 10,000 rpm for 5 minutes. The supernatants were collected and dried under vacuum using a Speed Vac (Thermo Fisher Scientific, Inc., Waltham, MA) and reconstituted in ammonium acetate (10 mM, 50 μl) buffer and again centrifuged. The supernatants (40 μl) were then transferred to the HPLC vials for the LC-MS/MS analysis. Mice were further transcardially perfused with ice-cold Dulbecco’s phosphate-buffered saline to remove traces of blood from the cerebrovasculature. Perfused brains were removed and placed on wet ice immediately until processed. The brains were weighed and homogenized in 500 μl of ice-cold Dulbecco's phosphate-buffered saline using an ergonomic homogenizer (Power Gen 125; Thermo Fisher Scientific). Immediately after the first homogenization, proteins were precipitated by addition of ice-cold acetonitrile (1 ml) containing IS (except in the receptor protection experiment in which acetonitrile without IS was used to precipitate proteins) and homogenized further. The homogenates were centrifuged at 10,000 rpm at 4°C for 10 minutes to form a pellet. The entire quantity of supernatant was collected and dried under vacuum as before. The residues were suspended in ammonium acetate (10 mM, 50 μl) buffer and centrifuged as described. The supernatants (25 μl) were then injected onto the HPLC column for the LC-MS/MS analysis.

**Receptor Protection Experiment**

Naloxone (100 nmol i.c.v.) was administered 15 minutes prior to the administration of nor-BNI (30 nmol i.c.v.). Mice were euthanized at various time points up to 24 hours, and brains were collected and processed as described above for the LC-MS/MS analysis.

**Instrumentation and Analytical Conditions**

The LC-MS system consisted of a 3200 Q TRAP triple-quadrupole linear ion trap mass spectrometer fitted with a TurboIonSpray interface (Applied Biosystems SCIEX, Framingham, MA) and a Shimadzu Prominance HPLC system (two LC-20Adp isocratic pumps, a CTO-20AC column oven, an SIL-20AC autosampler, a DGU-20A3 degasser, and a CBM-20A controller; Shimadzu, Kyoto, Japan). Separation was carried out on a C-18 reverse phase HPLC column (50 μm, 100 Å, 50 x 4.6 mm) fitted with a C-18 reverse phase guard cartridge (4 x 3.00 mm; Phenomenex, Torrance, CA) and eluted using a gradient of solvents A (10 mM ammonium acetate) and B (0.1% formic acid in acetonitrile) (adapted from Manfio et al., 2011) at 0.5 ml/min flow rate. The gradient was 2–40% B over 6 minutes, 40–95% B from 6 to 7 minutes, and kept for 1 minute, 95–2% B from 8 to 9 minutes, and restored to 2% B in 1 minute followed by re-equilibration for 3 minutes. The mass spectrometric (MS) method consisted of multiple reaction monitoring (MRM) scans for nor-BNI and naloxone (Supplemental Fig. 1). The ion transitions (m/z) monitored were 662.5/547.4, 662.5/256.2, 590.4/493.3, 590.4/226.3 for nor-BNI and 328.2/212.2, 328.2/253.3 for naloxone, with 15-ms dwell time and 5-ms pause time between the ion transitions (Supplemental Fig. 2). The peaks corresponding to each ion transition were summed to give a single peak for nor-BNI and naloxone, respectively, represented in a summed extracted ion chromatogram (see Supplemental Data and Fig. 2). The ion source parameters were spray voltage (5500 V), curtain gas (25 p.s.i.), source temperature (700°C), and ion source gas 1 (70 p.s.i.) and gas 2 (65 p.s.i.).

The samples were analyzed randomly with wash runs consisting of blank injections of solvents before and after every sample, and standards were run in between the sample runs. To correct for sample-to-sample variability due to potential autosampler injection error or variability in the biologic matrices, we included naloxone as an internal standard. Naloxone is physicochemically similar to the analyte nor-BNI, making it a suitable standard in the absence of a stable isotope derivative of nor-BNI. The chromatograms presenting signal intensities for either naloxone or nor-BNI are included as evidence for both the presence of nor-BNI in the samples and to confirm mass and retention times. The peak area for nor-BNI was calculated by measuring the signal intensity (ion counts per second) of nor-BNI and normalizing the data to the signal intensity of the internal standard. Graphs presenting these data (such as Fig. 3C) therefore use the area of the peaks corresponding to the respective analyte and/or internal standard. Note that for the receptor protection experiment (Fig. 4), the nor-BNI signal was not normalized to the internal standard because naloxone was also an analyte. Accordingly, the raw data were graphed as mean peak area (n = 3) for both naloxone and nor-BNI.

A nor-BNI concentration standard calibration curve was used to quantify nor-BNI in the brain samples. To generate the standard curve, known amounts of nor-BNI (nmol) were added directly to whole brain and then homogenized and processed as described above. The normalized peak area of nor-BNI in each sample was plotted as a response on the x-axis and the nor-BNI standard amounts on the y-axis to generate the calibration curve. Note that because we used the entire brain homogenate extract for the analysis, the peak area obtained for each standard amount represented all of the extractable nor-BNI from the brain homogenate. This calibration curve was used to calculate the amount (in nmol) of nor-BNI in the brain homogenate samples obtained from the mice treated with nor-BNI (10 mg/kg i.p.), using the normalized peak area of nor-BNI from each sample. Because the nor-BNI peak area represented the entire brain homogenate extract, the amount calculated was not within concentration units, but rather in mass units, as is commonly reported in the literature where only an aliquot of the brain homogenate extract is used for the analysis.

**Statistical Analysis**

Tail-withdrawal testing used a mixed factorial design, as the multiple factors are classified as both within-subject variables (repeated antinociceptive testing of the same group of mice over time)
and between-group variables (comparing results between treatment groups pretreated with saline, naloxone, or nor-BNI). Data comparing antinociceptive responses between sets were analyzed with one (factor: treatment) or two-way (factors: treatment × time) ANOVA using Prism 5.0 software (GraphPad Software, Inc., San Diego, CA), with significant effects further analyzed using Tukey’s, Dunnett’s, or Bonferroni multiple comparisons post hoc test as appropriate. Saturation $[^{3}H]$U69,593 binding data were analyzed by nonlinear regression analysis using Prism 5.0 software (GraphPad Software, Inc.), and the comparison of affinity and maximal binding values between sets was analyzed with one-way ANOVA, with significant effects further analyzed using Tukey’s multiple comparison post hoc test. Data comparing nor-BNI quantities in brain samples quantified by LC-MS/MS over time were analyzed by one-way ANOVA, with significant effects further analyzed using Tukey’s multiple comparison post hoc test. All data points shown are presented as mean responses with the S.E.M. represented by error bars.

**Results**

**In Vivo Time Course of KOR Antagonism Mediated by a Single Administration of Nor-BNI Lasts up to 14 Days in the Mouse 55°C Warm-Water Tail-Withdrawal Assay**

Mice pretreated with a single dose of naloxone or nor-BNI (10 mg/kg i.p.) produced a drug- and time-dependent antagonism of U50,488-induced antinociception $[P_{10,122} = 2.88, P = 0.0029$, two-way ANOVA with Bonferroni post hoc test; Fig. 1]. Each antagonist significantly blocked U50,488-mediated antinociception 2 hours after administration as compared with saline-pretreated animals ($P < 0.01$, Bonferroni post hoc test). As expected, naloxone-mediated antagonism of U50,488-induced antinociception was insignificant 24 hours after administration, whereas nor-BNI still produced significant inhibition of U50,488-induced antinociception compared with saline-pretreated mice ($P < 0.01$). The single intraperitoneal administration of nor-BNI produced significant KOR antagonism for at least 7 days ($P < 0.05$), with the return of significant U50,488-induced antinociception 14 days after initial administration. Notably, the prolonged antagonist effects cannot be attributed to antinociceptive tolerance from repeated administration of U50,488, because the saline-pretreated mice throughout the course of testing did not show significant changes in antinociception [$F_{4,39} = 0.18, P = 0.95$, not significant; one-way ANOVA with Tukey’s post hoc test].

To confirm the long lasting nor-BNI-induced antagonism of brain KOR, additional mice were pretreated once with nor-BNI (10 mg/kg i.p.). Mice were then administered intracerebroventricular U50,488 (100 nmol) once 1, 7, 14, or 21 days later, and the tail withdrawal latency measured. Vehicle-pretreated mice demonstrated significant U50,488-induced antinociception of 15.0 ± 0.0 seconds $[F_{5,79} = 385.9, P < 0.001$, one-way ANOVA followed by Tukey’s post hoc test]. Nor-BNI pretreatment significantly antagonized intracerebroventricular U50,488-induced antinociception up to 14 days later (1 day = 1.66 ± 0.12 seconds, 7 days = 1.28 ± 0.07 seconds, 14 days = 1.21 ± 0.09 seconds; all $P < 0.001$, Tukey’s). However, after a 21-day pretreatment with nor-BNI, mice demonstrated normal U50,488-induced antinociception (14.7 ± 0.34 seconds; not significant from U50,488 alone, Tukey’s post hoc test).

**Ex Vivo Studies**

**Ex Vivo Wash-Resistant Inhibition of Maximal $[^{3}H]$U69,593 Saturation Binding from Nor-BNI-Pretreated Murine Brain Membranes.** To determine whether nor-BNI produced wash-resistant inhibition of KOR binding, the brains of mice were harvested 1, 7, or 14 days after pretreatment with saline or nor-BNI (10 mg/kg i.p.), extensively washed, and used in $[^{3}H]$U69,593 saturation binding experiments. Scatchard analysis of $[^{3}H]$U69,593 binding to brain membranes isolated from saline- or antagonist-pretreated mice found no significant differences in the $K_{d}$ values at any time point [1, 7, or 14 days; $F_{2,12} = 0.16, P = 0.93$, not significant; Table 1]. However, membranes isolated from mice pretreated with nor-BNI showed significant, time-dependent decreases in the maximum value for $[^{3}H]$U69,593 binding $[F_{2,12} = 4.86, P < 0.05$; Table 1], with significant differences 1 and 7, but not 14 days after pretreatment.

**Detection of Nor-BNI and Naloxone in Mouse Blood and Brain Homogenate by LC-MS/MS**

A solvent system consisting of ammonium acetate, acetonitrile and formic acid was adapted from the previous LC-MS/MS.

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$K_{d}$</th>
<th>$B_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline control</td>
<td>0.94 ± 0.30</td>
<td>20.0 ± 2.25</td>
</tr>
<tr>
<td>nor-BNI pretreatment (10 mg/kg i.p.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Day post-treatment</td>
<td>1.01 ± 0.03</td>
<td>9.85 ± 0.38*</td>
</tr>
<tr>
<td>7 Days post-treatment</td>
<td>1.12 ± 0.28</td>
<td>11.5 ± 3.03*</td>
</tr>
<tr>
<td>14 Days post-treatment</td>
<td>0.90 ± 0.26</td>
<td>15.1 ± 1.48</td>
</tr>
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* $P < 0.05$ compared with saline pretreatment value.
analysis of morphine from human plasma (Manfio et al., 2011).

To optimize the brain extraction procedures and chromatographic and mass spectrometric parameters, we initially administered a single high dose of nor-BNI (100 nmol i.c.v. or 50 mg/kg i.p.) to C57BL/6J mice and detected the antagonist in the brain tissues collected up to 21 days later (see Supplemental Fig. 3). The identity of nor-BNI by this method was confirmed by comparing the mass transitions and retention times with those of the standard nor-BNI sample (Fig. 2, but see also Supplemental Data for details). These optimized LC-MS/MS protocol results were subsequently used to analyze the brain homogenate and serum samples specifically for the parent nor-BNI molecule.

Detection of Nor-BNI in Mouse Brain over 21 Days with LC-MS/MS

Detection of Nor-BNI in Mouse Brain after 30-nmol Intracerebroventricular Administration

After administering a single intracerebroventricular dose of 30 nmol, the presence of nor-BNI in the mouse brain was examined by LC-MS/MS in homogenates prepared from transtranically perfused brains harvested from animals over 21 days. Nor-BNI was found to be present in all mouse brain samples from 30 minutes (Fig. 3A) up to 21 days (Fig. 3B) and was significantly above control values up to 7 days post-injection [$F_{12,26} = 3.94$, $P = 0.0017$]. The peak intensity (ion intensity measured as ion counts per second) for nor-BNI in the sample on the 21st day was lower than that in the 30-minute sample, but remained above background noise. Overall, nor-BNI was slowly cleared from the brain over 21 days (Fig. 3C).

Receptor Protection

In a receptor protection experiment, naloxone (100 nmol i.c.v.) was administered 15 minutes prior to nor-BNI (30 nmol i.c.v.), and the levels of both compounds were monitored by LC-MS/MS analysis in brain homogenate extracts isolated from mice over the next 24 hours. Whereas naloxone was rapidly cleared from mouse brain, returning to control values in 2 hours, nor-BNI levels were significantly elevated (treatment: $F_{1,29} = 96.0$, $P < 0.0001$; time: $F_{7,29} = 2.90$, $P = 0.02$; two-way ANOVA with Bonferroni post hoc test) above control values over 24 hours in the same samples (Fig. 4A).

In a parallel experiment in vivo using the mouse 55°C warm-water tail-withdrawal assay, we attempted to protect the KOR from long-lasting nor-BNI antagonism with...
pretreatment of the short-acting antagonist naloxone. Mice were pretreated with vehicle or naloxone (30 mg/kg i.p.) 30 minutes prior to administering vehicle or nor-BNI (10 mg/kg i.p.). Twenty-four hours later, mice were administered U50,488 (10 mg/kg i.p.), and agonist-induced antinociception measured 40 minutes later (Fig. 4B). Mice pretreated with naloxone and saline demonstrated significant increases in tail-withdrawal latency \( [F_{1,47} = 45.85, P < 0.0001, \text{one-way ANOVA followed by Tukey’s post hoc test}] \) equivalent to the effect of U50,488 alone. As expected, mice pretreated with saline and nor-BNI demonstrated a significant antagonism of U50,488-induced antinociception \( (P < 0.001, \text{Tukey’s post hoc test}) \). However, naloxone pretreatment was unable to protect the KOR from nor-BNI-induced antagonism, as a statistically equivalent antagonism of U50,488-induced antinociception was observed in mice pretreated with naloxone and nor-BNI \( (P < 0.001 \text{ from U50,488 alone}, \text{but not significantly different from saline/nor-BNI pretreated mice; Tukey’s post hoc test}) \).

**Detection of Nor-BNI in Mouse Brain over 21 Days after a Single Intraperitoneal Injection**

As systemic administration is the common method of utilizing nor-BNI in behavioral studies, we administered nor-BNI (10 mg/kg i.p.) to mice and analyzed isolated serum and brain homogenate samples for the presence of the antagonist over the following 21 days. Ion chromatographic peaks used to measure nor-BNI were matched to ion transitions in the multiple reaction monitoring and chromatographic retention time for nor-BNI demonstrated with the standard samples. Nor-BNI was detectable in brain 30 minutes after intraperitoneal administration (Fig. 5A) and persisted up to 21 days (Fig. 5B). Although the peak intensity for nor-BNI in brain homogenate samples after a single intraperitoneal administration was lower than matching samples after direct intracerebroventricular administration, they remained well above the background noise demonstrated with blank control samples.

Although significantly peaking within 1 minute of intraperitoneal administration \( [F_{1,25} = 72.9, P < 0.0001] \), nor-BNI was rapidly cleared from blood within 90 minutes, becoming statistically insignificant from control values at 6 hours and undetectable after 24 hours (Fig. 6A). Nor-BNI levels in the brain were also statistically significant \( [F_{1,72} = 15.4, P < 0.0001] \), but reached their highest amounts 30 minutes post-administration (7.1 nmol), then falling rapidly over the next 60 minutes to reach a steady detectable level that gradually declined over a 21-day period (Fig. 6B). Quantification of nor-BNI in brain homogenate after a single intraperitoneal pretreatment detected amounts of nor-BNI (3.1 nmol) after 7 days that remained significantly greater than control \( (P < 0.01; \text{Fig. 6B}) \). Over the next 7 days, the amount of nor-BNI detected fell to levels not statistically different from control, 1.6 nmol, and remained consistent for the remainder of testing (21 days).

**KOR Antagonist Activity of Nor-BNI In Vivo Is Consistent with Doses Detected by LC-MS/MS**

To confirm if the concentration of nor-BNI found in brain was sufficient to antagonize the KOR, we directly administered mice intracerebroventricular doses of the antagonist equivalent to the nor-BNI initially tested (30 nmol) or detected by LC-MS/MS over time (2.7 or 1.4 nmol, approximating brain levels detected 7 and 14 days after intraperitoneal administration). Antagonism of U50,488 (30 nmol i.c.v.)-induced antinociception was measured 6 hours later. Nor-BNI
significantly antagonized the agonist effects of U50,488 in a dose-dependent manner \( F_{5,78} = 169.2, P < 0.001, \) one-way ANOVA followed by Tukey’s post hoc test; Fig. 7). Notably, the nor-BNI-mediated KOR antagonist activity in vivo was consistent with doses predicted from the concentrations of nor-BNI detected by LC-MS/MS. Pretreatment with a moderate dose of nor-BNI (2.7 nmol) reduced U50,488-induced antinociception significantly, but not completely. Moreover, pretreatment with the lowest dose of nor-BNI (1.4 nmol i.c.v.), approximating levels detected by LC-MS/MS in mouse brain 14 and 21 days after administration of nor-BNI (10 mg/kg i.p.), did not antagonize U50,488-induced antinociception, consistent with the absence of U50,488 antagonism observed behaviorally.

**Discussion**

The KOR antagonist nor-BNI was detected with LC-MS/MS analysis of mouse brain homogenates isolated up to 21 days after a single administration (30 nmol i.c.v. or 10 mg/kg i.p.). In a parallel experiment, the prolonged nor-BNI (10 mg/kg i.p.)-induced antagonism of KOR-mediated antinociception persisted for at least 1 week (and up to 14 days after direct central nervous system administration). The rate of recovery of maximal \(^3^H\)U69,593 binding measured in vitro in freshly isolated brain membranes after treatment in vivo correlated with the recovery of U50,488-induced antinociception following nor-BNI pretreatment in vivo. These pharmacological results are consistent with previous reports of long-lasting antagonism of KOR-mediated antinociception and receptor binding in ICR mice by nor-BNI administered intracerebroventicularly (Horan et al., 1992). Similar antagonism of KOR, but not \( \mu \)-opioid receptor, agonists lasting up to 21 days has been reported in both rats (Jones et al., 1998; Carroll et al., 2005) and rhesus monkeys (Butelman et al., 1993).
should be noted that nor-BNI has been reported to antagonize the KOR for much longer periods, up to 85 days (Potter et al., 2011), and Horan and colleagues (1992) reported the central administration of nor-BNI reduced the affinity of [3H]U69,593 binding in whole mouse brain for up to 56 days after treatment without changes in maximal binding, similar to findings reported by Bruchas et al. (2007). However, these differences in results could be methodological. As it has been speculated that nor-BNI may be released from degraded cell membranes (Horan et al., 1992), the present study avoided possible freeze-thaw deterioration of the membranes and proteins by analyzing the freshly harvested samples in binding assays immediately after isolation. Moreover, the dose and route of administration of nor-BNI used, the species treated, and the type of assay performed could all account for differences in the duration of KOR antagonism observed. Although the dose of nor-BNI (10 mg/kg i.p.) selected for this study was higher than used in some studies (Horan et al., 1992), it is typical for in vivo use (Beardsley et al., 2005; Bruchas et al., 2007) and has been previously demonstrated to be selective for the KOR after 4 hours (Portoghese et al., 1987; Endoh et al., 1992). Notably, an identical dose of naloxone produced a brief KOR antagonism lasting less than 1 day, consistent with previous reports (Akil et al., 1976). Together, these results suggest that the prolonged KOR antagonism cannot be explained simply by prolonged occupancy of KOR by a high dose of a reversible antagonist.

Liquid chromatography coupled with mass spectrometry presented us with an extraordinarily sensitive analytical technique to detect minute amounts of nor-BNI present in brain tissue for the first time. The present data confirmed that peripheral administration of nor-BNI resulted in rapid passage across the blood-brain barrier and a prolonged presence of the KOR antagonist in brain (but not blood) up to 21 days after administration. Although the nor-BNI levels measured in the brain were not very high, the amounts detected would be expected to produce pharmacological effects given the high (pM) affinity of nor-BNI for the KOR (Takemori et al., 1988). The duration of KOR antagonism measured behaviorally in the tail-withdrawal assay was shorter (7 days) than the number of days that nor-BNI was detected after intraperitoneal administration. However, the magnitude of KOR antagonism was mimicked by direct intracerebroventricular administration of doses of nor-BNI approximating those identified in the LC-MS/MS studies, confirming the present findings. These results suggest that the sensitivity of the present LC-MS/MS protocol was sufficient to detect nor-BNI at concentrations below that required to induce KOR antagonism. These results are in contrast to a recent report in which nor-BNI concentrations in rat brain homogenate were believed to be insignificant because of potential interference of nor-BNI from the plasma contaminant in the brain homogenate (Peters et al., 2011). To ensure that the detected nor-BNI in the mouse brain came from brain tissue and not from the cerebrovasculature, we performed transcardial perfusion on the treated mice. As the blood from the cerebrovasculature was thus removed before the brain extraction, we are confident that the detected nor-BNI came from brain tissue. These results could suggest a slow elimination of nor-BNI from the brain tissue, possibly resulting from a slow diffusion and subsequent interaction to produce prolonged inactivation of the KOR by direct or indirect mechanisms. It should be noted that the present analytical method detected only the parent nor-BNI molecule and not potential biotransformation products. Accordingly, it is possible that the observed U50,488 antagonism cannot wholly be attributed to the amount of nor-BNI detected in our assay, but may be produced over time by unmeasured metabolites. For example, the metabolite of morphine, morphine-6-glucuronide, is a potent opioid agonist, contributing to the effects of the parent compound (Stachulski and Meng, 2013). However, to the best of our knowledge, the existence and activity of nor-BNI metabolites have not been examined. Moreover, because nor-BNI was detected using a whole brain homogenate preparation in the LC-MS/MS analysis, it is difficult to comment on the distribution of nor-BNI in the mouse brain. Further determination of the distribution of nor-BNI in mouse brain and potential biotransformation products of nor-BNI may contribute important insights regarding the prolonged activity of nor-BNI.

In a receptor protection experiment, naloxone pretreatment did not prevent the prolonged presence of nor-BNI detected by LC-MS/MS in the brain samples. Consistent with these results, naloxone pretreatment did not prevent nor-BNI-mediated KOR antagonism 24 hours after nor-BNI administration in mice tested in the 55°C warm-water tail-withdrawal assay. This failure to protect the KOR where the nor-BNI is presumed to interact suggests that the observed pseudoirreversible activity of nor-BNI is not the result of high KOR affinity with slow dissociation rates. However, a report describing the binding of nor-BNI to a site on KOR separate from the agonist binding site (Takemori et al., 1988) raises the alternative suggestion that a physically distinct binding domain could exist for nor-BNI that accounts for a prolonged inhibition of binding and antagonism of KOR agonist activity (Chang et al., 1994). Alternatively, a single administration of the lipophilic morphinan derivatives BU72 [17-methyl-3-hydroxy-[5β,7β,3′,5′]-pyrrolidine-2′-[S]-phenyl-7α-methyl-6,14-endoetheno morphinan] and BU74 have been demonstrated to...
produce long-lasting agonist and antagonist effects, respectively, at the \( \mu \)-opioid receptor (Neilan et al., 2004; Husbands et al., 2005). The prolonged duration of action of these compounds has been attributed to their interactions with lipid membranes or lipophilic sites in target proteins, with a subsequent slow release of the compound from the resultant depot and binding to their respective targets over time (Neilan et al., 2004; Husbands et al., 2005). A similar lipid interaction and formation of depot may account for the prolonged pharmacological activity and reductions in maximal specific radioligand binding observed in the present study with nor-BNI. Given that the prolonged reduction in maximal binding by BU72 was reversed by treatments with high salt concentrations (Rothman et al., 1989; Xu et al., 1991), additional studies of antagonist-induced wash-resistant inhibition of binding of radiolabeled KOR ligands with treated receptors isolated ex vivo would be of value in comparing the in vivo antagonist activity profile and in vitro inhibition of binding. However, Munro et al. (2012) recently published a detailed pharmacokinetic analysis of long-acting KOR antagonists in mouse brain. They reported rapid elimination of nor-BNI as well as JDTic from plasma and observed prolonged retention of JDTic in the brain. The authors demonstrated that despite low lipophility (logP < 2), long acting KOR antagonists were prone to long retentions in the brain tissue. They speculated such retention could be due to the entrapment of the drugs in the cellular compartments such as lysosomes, although this was not directly tested.

Recent reports suggest the interaction of KOR antagonists with the KOR may be unique, where the KOR antagonists nor-BNI, JDTic, and 5'-guanidinoanaltrindole (as well as selective KOR agonists) activate the c-Jun-N-terminal kinase (JNK) mitogen-activated protein kinase cascade, an action the authors suggest is responsible for prolonged KOR antagonism (Bruchas et al., 2007; Melief et al., 2011). Although KOR-selective agonists activate JNK through a G\(_\text{\beta}\)\(_\gamma\)-protein-mediated mechanism (Kim et al., 2004), nor-BNI does not activate G\(_\text{\beta}\)\(_\gamma\)-proteins, leaving the mechanism mediating the observed JNK activation unknown. It has been suggested that a time-sensitive mechanism is required for the long-lasting antagonism, possibly involving ligand-specific compensatory changes in second messenger signaling (Bruchas and Chavkin, 2010). As the scope of the present study was limited to the detection of nor-BNI in the brain homogenate and does not suggest any novel mechanism of action, the possibility of signaling-mediated long-lasting KOR antagonism cannot be discounted from the present data, but need not be considered incompatible. On the other hand, the identified concentration of nor-BNI in the brain many days after a single administration was sufficient to antagonize KOR-antinociception independent of any potential nor-BNI-mediated changes in signal transduction. Comparing the signaling properties and the pharmacokinetic properties of KOR antagonists remain important topics for future examination to better understand the mechanism behind the long-lasting action of certain KOR antagonists.

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