JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086

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.

Kshitij A. Patkar, Jinhua Wu, Michelle L. Ganno, Harminder D. Singh, Nicolette C. Ross, Khampaseuth Rasakham, Lawrence Toll and Jay P. McLaughlin

Torrey Pines Institute for Molecular Studies, Port St. Lucie, Florida 34987 (K.A.P., J.W., M.L.G., H.D.S, N.C.R, L.T., J.P.M.); Department of Psychology, Northeastern University, Boston, MA, 02115 (K.R., J.P.M.)

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Detection of nor-BNI in mouse brain over 21 d with LC-MS/MS

Corresponding author: Dr. Jay P. McLaughlin

Torrey Pines Institute for Molecular Studies

11350 SW Village Parkway, Port St. Lucie, FL 34987

Tel: (772) 345-4715

Fax: (772) 345-3649

E-mail: jmclaughlin@tpims.org

Number of text pages: 31

Number of words: *Abstract (250 max)*: 245

Introduction (750 max): 681

Discussion (1500 max): 1498

Number of tables: 1

Number of figures: 7

Number of references: 42

Abbreviations: ANOVA, analysis of variance; cps; counts per second; GNTI, 5'-guanidinonaltrindole; i.c.v., intracerebroventricular; i.p., intraperitoneal; IS, internal standard; JDTic, (3*R*)-7-hydroxy-*N*-((1*S*)-1-[[3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl] methyl]-2-methylpropyl)-1,2,3,4-tetrathydro-3-isoquinolinecarboxamide; JNK, c-Jun-N-terminal kinase; KOR, kappa opioid receptor; LC-MS/MS, liquid chromatography-mass spectrometry/ mass spectrometry; MAPK, mitogen-activated protein kinase; nor-BNI, nor-binaltorphimine; PK, pharmacokinetics; U50,488, (±)-*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzenacetamide methane-sulfonate hydrate; [^{3}H]U69,593, [^{3}H](+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide

Recommended Section Assignment: Neuropharmacology

Abstract

In the mouse 55ºC warm-water tail-withdrawal assay, a single administration of 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 a 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 [^{3}H]U69,593 binding. To determine whether brain concentrations of nor-BNI were sufficient to explain the antagonism of KOR-mediated antinociception, mouse blood and perfused brain were harvested at time points ranging from 30 min to 21 days after a single administration and analyzed for the presence of nor-BNI using 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 antagonism observed *in vivo* at this time point after pre-treatment. Overall, the physical presence of nor-BNI in the mouse brain paralleled it's *in vivo* pharmacological profile, suggesting physico-chemical 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., 2000), and the ability to block stress-induced reinstatement of cocaine-seeking behavior (Beardsley et al., 2005; Carey et al., 2007). Nor-binaltorphimine (nor-BNI) was the first KOR antagonist developed (Portoghese et al., 1987) that selectively antagonized KOR at nanomole doses (Takemori et al., 1988). Horan and co-workers showed that a single intracerebroventricular (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 [^{3}H]U69,593 binding to isolated brain membranes for over 28 days (Horan et al., 1992). 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 (GNTI; 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).

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

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 to produce 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 (MAPK), leading to prolonged inactivation of KOR signaling persisting for weeks after a single exposure (Bruchas et al., 2007; Melief et al., 2010; Melief et al., 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 (PK) studies of nor-BNI *in vivo* are available. One of the important PK 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

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

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 if a single administration of nor-BNI to C57BL/6J mice, through either the intracerebroventricular (i.c.v.) or intraperitoneal (i.p.) 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 55ºC 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 [^{3}H]U69,593 to murine brain membranes containing KOR.

Methods

Animals

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

Chemicals and reagents

Nor-binaltorphimine (nor-BNI)-dihydrochloride, naloxone-hydrochloride, (±)-*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzenacetamide methane-sulfonate hydrate (U50,488), ammonium acetate and analytical grade acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO). [^{3}H](+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-

oxaspiro[4.5]dec-8-yl] benzeneacetamide ([^{3}H]U69,593, (55 Ci/mmol)) was purchased from GE Healthcare (Pittsburgh, PA).

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 concentration in saline (0.9%) before administration. Nor-BNI or naloxone was administered *via* intracerebroventricular (i.c.v.) or an intraperitoneal (i.p.) 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 naïve 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 (i.c.v.) injections were made directly into the lateral ventricle according to the modified method of Haley and McCormick (1957). The volume of all i.c.v. injections was 5 μL, using a 10 μL Hamilton microliter syringe. The mouse was lightly anesthetized with isoflurane, an incision made in the scalp, and the injection made free-hand 2 mm lateral and 2 mm caudal to bregma at a depth of 3 mm.

Mice were administered nor-BNI or naloxone through the i.c.v. or i.p. routes 80 min or 6, 23.3, 47.3, 167.3, 335.3 or 503.3 h prior to administration of U50,488 (see below) or euthanasia for serum and brain harvest post injection (see below).

***In vivo* studies**

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

Antinociception assay (Tail-flick assay)

The 55^{o}C warm-water tail-withdrawal assay was performed as described earlier (McLaughlin et al., 1999; McLaughlin et al., 2004) where the thermal nociceptive stimulus was 55°C water and the latency to tail withdrawal was taken as the endpoint. Baseline tail-flick latencies (averaging 1.27±0.07 s) were tested prior to drug administration as negative control. Mice that showed no response within 5 s in the initial control test were eliminated from the experiment. A cut-off time of 15 s 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 min, 1, 3, 7, and/or 14 d, 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 min 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 h prior to the administration of U50,488 (30 nmol, i.c.v.) and antinociception was tested as described above.

***Ex vivo* studies**

Kappa opioid receptor binding to murine brain membranes

Mice were pretreated with a single dose of saline (0.9%), naloxone, or nor-BNI (10 mg/kg, i.p., each) 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

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

centrifugation (20,000 x g) 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 PMSF 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 2mg/ml bovine serum albumin using a Tomtec cell harvester (Hamden, CT). After filtration, filters were washed with cold 50 mM Tris-HCl, pH 7.4 (3 mL, 3X), and were counted in a Wallac beta-plate reader (Perkin Elmer, Waltham, MA).

Sample preparation for LC-MS/MSMS analysis

Mice were administered nor-BNI or naloxone through either the i.c.v. or i.p. routes and euthanized at various time points from 0 min to 21 days post administration. Blood (200-250 μL) was collected from mice by cardiac puncture and was allowed to clot naturally at 37ºC 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 min. The supernatants were collected and dried under vacuum using a SpeedVac (Thermo Fisher Scientific, Inc., Waltham, MA) and reconstituted in ammonium acetate (10 mM, 50 μL) buffer and again centrifuged. The supernatants (40 μL) were transferred to the HPLC vials for the LC-MS/MS analysis.

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

Mice were further transcardially perfused with ice cold Dulbecco's phosphate buffered saline (DPBS) 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 DPBS using an ergonomic homogenizer (Power Gen 125, Thermo Fisher Scientific). Immediately following the first homogenization, proteins were precipitated by addition of ice cold acetonitrile (1 mL) containing IS (except in the receptor protection experiment where acetonitrile without IS was used to precipitate proteins) and homogenized further. The homogenates were centrifuged at 10,000 rpm at 4 ºC for 10 min 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 min prior to the administration of nor-BNI (30 nmol, i.c.v.). Mice were euthanized at various time points up to 24 h 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/MDS Sciex, Darmstadt, Germany) and a Shimadzu Prominence HPLC system (two LC-20ADsp isocratic pumps, a CTO-20AC column oven, an SIL-20AC autosampler, a DGU-20A3 degasser, and a CBM-20A

controller). Separation was carried out on a C-18 reverse phase column (Luna 50 μ, 100 Å, 50 X 4.6 mm) fitted with a C-18 reverse phase guard cartridge (Phenomenex, 4 X 3.00 mm) 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 min, 40-95% B from 6-7 min, and kept for 1 min, 95-2% B from 8-9 min and restored to 2% B in 1 min followed by re-equilibration for 3 min. The mass spectrometric (MS) method consisted of multiple reaction monitoring (MRM) scans for nor-BNI and naloxone (Supplemental Figure 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 Figure 2). The peaks corresponding to each ion transition were summed to give a single peak for nor-BNI and naloxone respectively represented in the summed extracted ion chromatogram (see Supplemental Data and Figure 2). The ion source parameters were spray voltage (5500 V), curtain gas (25 psi), source temperature (700 ºC), ion source gas 1 (70 psi) and gas 2 (65 psi).

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 biological matrices, we included naloxone as an internal standard. Naloxone is physico-chemically similar to the analyte nor-BNI, making it a suitable standard it in absence of a stable isotope derivative of nor-BNI. The chromatograms presenting signal intensities for either naloxone or nor-BNI are included as evidence both for the presence of nor-BNI in the samples and also to confirm mass and retention times. The peak area for nor-BNI was calculated by measuring the signal intensity (ion counts per second, or cps) of nor-BNI and normalizing the

data to the signal intensity of the internal standard. Graphs presenting these data (such as Figure 3C) therefore utilize the area of the peaks corresponding to the respective analyte and/ or internal standard. Note that for the receptor protection experiment (Figure 4), the nor-BNI signal was not normalized to the internal standard since naloxone was also an analyte. Accordingly, the raw data was graphed as mean peak area (n =3) for both naloxone and nor-BNI.

A nor-BNI concentration standard calibration curve was used to quantitate nor-BNI in the brain samples. To generate the standard curve, known amounts of nor-BNI (nmols) 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 Y-axis and the nor-BNI standard amounts on the X-axis to generate the calibration curve. Note that since 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 i.p. nor-BNI (10 mg/kg), using the normalized peak area of nor-BNI from each sample. Since the nor-BNI peak area represented the entire brain homogenate extract, the amount calculated was not in 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 utilized 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

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

between sets were analyzed with one- (factor: treatment) or two-way (factors: treatment x time) ANOVA using Prism 5.0 software (GraphPad Software, 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, San Diego, CA), and the comparison of affinity and maximal binding values between sets were analyzed with one-way ANOVA, with significant effects further analyzed using Tukey's 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^{o}C warm-water tail-withdrawal assay.

Mice pretreated with a single dose of naloxone or nor-BNI (10 mg/kg i.p., each), produced a drug- and time-dependent antagonism of U50,488-induced antinociception ($F_{(10,122)}$ = 2.88, P = 0.0029, two-way ANOVA with Bonferroni post hoc test; Figure 1). Each antagonist significantly blocked U50,488-mediated antinociception 2 h after administration as compared to saline-pretreated animals ($P < 0.01$, Bonferroni post hoc test). As expected, naloxone-mediated antagonism of U50,488-induced antinociception was insignificant 24 h after administration, while nor-BNI still produced significant inhibition of U50,488-induced antinociception compared to saline-pretreated mice ($P < 0.01$). The single intraperitoneal administration of nor-

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

BNI produced significant KOR antagonism 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, as the saline pretreated mice throughout the course of testing did not show significant changes in antinociception ($F_{(4,39)} = 0.18$, $P = 0.95$, n.s.; 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 d later and the tail withdrawal latency measured. Vehicle-pretreated mice demonstrated significant U50,488-induced antinociception of 15.0±0.0 s ($F_{(5,79)} = 385.9$, $p < 0.001$, one-way ANOVA followed by Tukey's post hoc test). Nor BNI pretreatment significantly antagonized i.c.v. U50,488-induced antinociception up to 14 days later (1 d = 1.66±0.12 s, 7 d = 1.28±0.07 s, 14 d = 1.21±0.09 s; all $P<0.001$, Tukey's). However, after a 21 d pretreatment with nor-BNI, mice demonstrated normal U50,488-induced antinociception (14.7±0.34 s; n.s. from U50,488 alone, Tukey's).

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 if nor-BNI produced wash-resistant inhibition of KOR binding, the brains of mice were harvested 1, 7 or 14 d 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-

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

pretreated mice found no significant differences in the K_d values at any time point (1, 7 or 14 days; $F_{(3,12)} = 0.16$, $p = 0.93$, n.s.; 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 d 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 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 Figure 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 (Figure 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 following 30 nmol, i.c.v. administration

After administering a single i.c.v. dose of 30 nmol, the presence of nor-BNI in the mouse brain was examined by LC-MS/MS in homogenates prepared from transcardially perfused brains harvested from animals over 21 days. Nor-BNI was found to be present in all mouse brain samples from 30 min (Figure 3A) up to 21 days (Figure 3B), and significantly above control values up to 7 d post injection ($F_{(12,26)}$=3.94, p=0.0017). The peak intensity (ion intensity

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

measured as ion counts per second (cps)) for nor-BNI in the sample on the 21st day was lower than that in the 30 min sample, but remained above background noise. Overall, nor-BNI was slowly cleared from the brain over 21 days (Figure 3C).

Receptor protection

In a receptor protection experiment, naloxone (100 nmol, i.c.v.) was administered 15 min 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 h, 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 h in the same samples (Figure 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 min prior to administering vehicle or nor-BNI (10 mg/kg, i.p.). Twenty-four hours later, mice were administered U50,588 (10 mg/kg, i.p.) and agonist-induced antinociception measured 40 min later (Figure 4B). Mice pretreated with naloxone and saline demonstrated significant increases in tail-withdrawal latency ($F_{(4,67)} = 45.85$, $P < 0.0001$, 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 ($P < 0.001$, Tukey's post hoc test). However, naloxone pretreatment was unable to protect the KOR from nor-BNI-induced antagonism, as a statistically equivalent antagonism

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

of U50,488-induced antinociception was observed in mice pretreated with naloxone and nor-BNI ($P < 0.001$ from U50,488 alone, 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 following a single i.p. 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 MRM and chromatographic retention time for nor-BNI demonstrated with the standard samples. Nor-BNI was detectable in brain 30 min following i.p. administration (Figure 5A), and persisted up to 21 days (Figure 5B). While the peak intensities for nor-BNI in brain homogenate samples after a single i.p. administration was lower than matching samples following direct i.c.v. administration, they remained well above the background noise demonstrated with blank control samples.

Although significantly peaking within 1 min of i.p. administration ($F_{(8,25)}=72.9$, $p<0.0001$), nor-BNI was rapidly cleared from blood within 90 min, becoming statistically insignificant from control values at 6h, and undetectable after 24 h (Figure 6A). Nor-BNI levels in the brain were also statistically significant ($F_{(8,25)}=15.4$, $p<0.0001$), but reached their highest amounts 30 min post administration (7.1 nmol), then falling rapidly over the next 60 min to reach a steady detectable level that gradually declined over a 21 d period (Figure 6B). Quantification of nor-BNI in brain homogenate following a single i.p. pretreatment detected amounts of nor-BNI (3.1 nmol) after 7 days that remained significantly greater than control ($p<0.01$; Figure 6B).

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

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 d).

KOR antagonist activity of nor-BNI *in vivo* is consistent with doses detected by LCMS/MS.

To confirm if the concentration of nor-BNI found in brain was sufficient to antagonize the KOR, we directly administered mice i.c.v. doses of the antagonist equivalent to the nor-BNI initially tested (30 nmol) or detected by LC-MS/MS over time (2.7 nmol or 1.4 nmol, approximating brain levels detected at 7 and 14 days post i.p. administration). Antagonism of U50,488 (30 nmol, i.c.v.)-induced antinociception was measured 6 h 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; Figure 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 d 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 CNS administration). The rate of recovery of maximal [^{3}H]U69,593 binding measured *in vitro* in freshly isolated brain membranes following 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 i.c.v. (Horan et al., 1992). Similar antagonism of KOR, but not mu opioid receptor (MOR), 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). It should be noted that nor-BNI has been reported to antagonize the KOR for much longer periods, up to 85 d (Potter et al., 2011), and Horan and colleagues reported the central-administration of nor-BNI reduced the affinity of [^{3}H]U69,593 binding in whole mouse brain for up to 56 days after treatment without changes in maximal binding (Horan et al., 1992), 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 following 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 h (Portoghese et al., 1987; Endoh et al., 1992). Notably, an identical dose of naloxone produced a brief KOR

antagonism lasting less than a day, consistent with previous reports (Akil et al., 1976). Together, these results suggest that the prolonged KOR antagonism cannot be simply explained 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 d following 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 d) than the number of days that nor-BNI was detected after i.p. administration. However, the magnitude of KOR antagonism was mimicked by direct i.c.v. 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 where nor-BNI concentrations in rat brain homogenate were believed to be insignificant due to the 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 the detected nor-BNI came from brain tissue. These results could suggest a slow elimination of nor-

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

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 be wholly 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, since 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 h after nor-BNI administration in mice tested in the 55^{o}C warm-water tail-withdrawal assay. This failure to protect the KOR where the nor-BNI is presumed to interact suggests that the observed pseudo-irreversible 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 and BU74 have been demonstrated to produce long-lasting agonist and antagonist effects, respectively, at the MOR (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 and co-workers (Munro et al., 2012) recently published a detailed pharmacokinetic analysis of long-acting KOR antagonists in mice 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 in spite of low lipophilicity (log D < 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 GNTI (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 $G_{\beta\gamma}$-protein mediated mechanism (Kam et al., 2004), nor-BNI does not activate G-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.

Acknowledgements: The authors would like to thank Dr. Brian Reed, Dr. Michelle Hoot and Dr. Jason Paris for their valuable suggestions in the preparation of this manuscript.

Authorship Contributions:

Participated in research design: Patkar, Ganno, Ross, Rasakham, Wu, Toll, and McLaughlin

Conducted experiments: Patkar, Ganno, Singh, Ross, Rasakham, and Wu

Contributed new reagents or analytic tools: Patkar

Performed data analysis: Patkar and McLaughlin

Wrote and contributed to the writing of manuscript: Patkar, McLaughlin, and Toll

References:

Akil H, Mayer DJ and Liebeskind JC (1976) Antagonism of Stimulation-produced Analgesia by Naloxone, A Narcotic Antagonist. *Science* **191**:961-962.

Aldrich JV and McLaughlin JP (2009) Peptide kappa opioid receptor ligands: potential for drug development. *AAPS J* **11**:312-322.

Aldrich JV and Vigil-Cruz S (2002) Narcotic Analgesics, in *Burger's Medicinal Chemistry and Drug Discovery* (Abraham D ed), John Wiley & Sons, Inc., New York.

Beardsley PM, Howard JL, Shelton KL and Carroll FI (2005) Differential effects of the novel kappa opioid receptor antagonist, JDTic, on reinstatement of cocaine-seeking induced by footshock stressors vs cocaine primes and its antidepressant-like effects in rats. *Psychopharmacology* **183**:118-126.

Broadbear JH, Negus SS, Butelman ER, de Costa BR and Woods JH (1994) Differential effects of systemically administered nor-binaltorphimine (nor-BNI) on kappa-opioid agonists in the mouse writhing assay. *Psychopharmacology* **115**:311-319.

Bruchas MR and Chavkin C (2010) Kinase cascades and ligand-directed signaling at the kappa opioid receptor. *Psychopharmacology* **210**:137-147.

Bruchas MR, Yang T, Schreiber S, Defino M, Kwan SC, Li S and Chavkin C (2007) Long-acting kappa opioid antagonists disrupt receptor signaling and produce noncompetitive effects by activating c-Jun N-terminal kinase. *J Biol Chem* **282**:29803-29811.

Butelman ER, Negus SS, Ai Y, de Costa BR and Woods JH (1993) Kappa opioid antagonist effects of systemically administered nor-binaltorphimine in a thermal antinociception assay in rhesus monkeys. *J Pharmacol Exp Ther* **267**:1269-1276.

Carey AN, Borozny K, Aldrich JV and McLaughlin JP (2007) Reinstatement of cocaine place-conditioning prevented by the peptide kappa-opioid receptor antagonist arodyn. *Eur J Pharmacol* **569**:84-89.

Carroll FI, Harris LS and Aceto MD (2005) Effects of JDTic, a selective kappa-opioid receptor antagonist, on the development and expression of physical dependence on morphine using a rat continuous-infusion model. *Eur J Pharmacol* **524**:89-94.

Chang AC, Takemori AE, Ojala WH, Gleason WB and Portoghese PS (1994) kappa Opioid receptor selective affinity labels: electrophilic benzeneacetamides as kappa-selective opioid antagonists. *J Med Chem* **37**:4490-4498.

Chen C, Yin J, Riel JK, DesJarlais RL, Raveglia LF, Zhu J and Liu-Chen LY (1996) Determination of the amino acid residue involved in [3H]beta-funaltrexamine covalent binding in the cloned rat mu-opioid receptor. *J Biol Chem* **271**:21422-21429.

Endoh T, Matsuura H, Tanaka C and Nagase H (1992) Nor-binaltorphimine: a potent and selective kappa-opioid receptor antagonist with long-lasting activity in vivo. *Arch Int Pharmacodyn Ther* **316:**30-42.

Haley TJ and McCormick WG (1957) Pharmacological effects produced by intracerebral injections of drugs in the conscious mouse. *Br J Pharmacol Chemother* **12:**12-15.

Horan P, Taylor J, Yamamura HI and Porreca F (1992) Extremely long-lasting antagonistic actions of nor-binaltorphimine (nor-BNI) in the mouse tail-flick test. *J Pharmacol Exp Ther* **260**:1237-1243.

Husbands SM, Neilan CL, Broadbear J, Grundt P, Breeden S, Aceto MD, Woods JH, Lewis JW and Traynor JR (2005) BU74, a complex oripavine derivative with potent kappa opioid receptor agonism and delayed opioid antagonism. *Eur J Pharmacol* **509**:117-125.

Jones DN and Holtzman SG (1992) Long term kappa-opioid receptor blockade following nor-binaltorphimine. *Eur J Pharmacol* **215**:345-348.

Jones RM, Hjorth SA, Schwartz TW and Portoghese PS (1998) Mutational evidence for a common kappa antagonist binding pocket in the wild-type kappa and mutant mu[K303E] opioid receptors. *J Med Chem* **41**:4911-4914.

Kam AY, Chan AS and Wong YH (2004) Kappa-opioid receptor signals through Src and focal adhesion kinase to stimulate c-Jun N-terminal kinases in transfected COS-7 cells and human monocytic THP-1 cells. *J Pharmacol Exp Ther* **310**:301-310.

Knoll AT, Meloni EG, Thomas JB, Carroll FI and Carlezon WA, Jr. (2007) Anxiolytic-like effects of kappa-opioid receptor antagonists in models of unlearned and learned fear in rats. *J Pharmacol Exp Ther* **323**:838-845.

Mague SD, Pliakas AM, Todtenkopf MS, Tomasiewicz HC, Zhang Y, Stevens WC, Jr., Jones RM, Portoghese PS and Carlezon WA, Jr. (2003) Antidepressant-like effects of kappa-opioid receptor antagonists in the forced swim test in rats. *J Pharmacol Exp Ther* **305**:323-330.

Manfio JL, Santos VJ, Lanchote VL, Santos LM, Carmona MJ, Auler JO, Brum LJ, Donaduzzi CM and Santos SR (2011) Development and validation of an HPLC/MS/MS method for the determination of sufentanil and morphine in human plasma. *J AOAC Int* **94**:136-142.

McLaughlin JP, Hill KP, Jiang Q, Sebastian A, Archer S and Bidlack JM (1999) Nitrocinnamoyl and chlorocinnamoyl derivatives of dihydrocodeinone: in vivo and in vitro characterization of mu-selective agonist and antagonist activity. *J Pharmacol Exp Ther* **289**:304-311.

McLaughlin JP, Myers LC, Zarek PE, Caron MG, Lefkowitz RJ, Czyzyk TA, Pintar JE and Chavkin C (2004) Prolonged kappa opioid receptor phosphorylation mediated by G-protein receptor kinase underlies sustained analgesic tolerance. *J Biol Chem* **279**:1810-1818.

Melief EJ, Miyatake M, Bruchas MR and Chavkin C (2010) Ligand-directed c-Jun N-terminal kinase activation disrupts opioid receptor signaling. *Proc Natl Acad Sci U S A* **107**:11608-11613.

Melief EJ, Miyatake M, Carroll FI, Beguin C, Carlezon WA, Jr., Cohen BM, Grimwood S, Mitch CH, Rorick-Kehn L and Chavkin C (2011) Duration of action of a broad range of selective kappa-opioid receptor antagonists is positively correlated with c-Jun N-terminal kinase-1 activation. *Mol Pharmacol* **80**:920-929.

Metcalf MD and Coop A (2005) Kappa opioid antagonists: past successes and future prospects. *AAPS J* **7**:E704-722.

Munro TA, Berry LM, Van't Veer A, Beguin C, Carroll FI, Zhao Z, Carlezon WA, Jr. and Cohen BM (2012) Long-acting kappa opioid antagonists nor-BNI, GNTI and JDTic: pharmacokinetics in mice and lipophilicity. *BMC Pharmacol* **12**:5.

Neilan CL, Husbands SM, Breeden S, Ko MC, Aceto MD, Lewis JW, Woods JH and Traynor JR (2004) Characterization of the complex morphinan derivative BU72 as a high efficacy, long-lasting mu-opioid receptor agonist. *Eur J Pharmacol* **499**:107-116.

Peters MF, Zacco A, Gordon J, Maciag CM, Litwin LC, Thompson C, Schroeder P, Sygowski LA, Piser TM and Brugel TA (2011) Identification of short-acting kappa-opioid receptor antagonists with anxiolytic-like activity. *Eur J Pharmacol* **661**:27-34.

Portoghese PS, Lipkowski AW and Takemori AE (1987) Binaltorphimine and nor-binaltorphimine, potent and selective kappa-opioid receptor antagonists. *Life Sci* **40**:1287-1292.

Potter DN, Damez-Werno D, Carlezon WA Jr., Cohen BM and Chartoff EH (2011) Repeated exposure to the kappa-opioid receptor agonist salvinorin A modulates extracellular signal-regulated kinase and reward sensitivity. *Biological Psychiatry* **70:** 744-753.

Rothman RB, Gorelick DA, Heishman SJ, Eichmiller PR, Hill BH, Norbeck J and Liberto JG (2000) An open-label study of a functional opioid kappa antagonist in the treatment of opioid dependence. *J Subst Abuse Treat* **18**:277-281.

Rothman RB, Long JB, Holaday JW, Bykov V, de Costa BR, Kim CH, Jacobson AE and Rice KC (1989) A study of the interaction of the alkylating agent, NIH10236, with opioid receptors in vitro and in vivo. *Neuropharmacology* **28**:1349-1356.

Stachulski AV and Meng X (2013) Glucuronides from metabolites to medicines: a survey of the in vivo generation, chemical synthesis and properties of glucuronides. *Nat Prod Rep* **30:**806-848.

Takemori AE, Ho BY, Naeseth JS and Portoghese PS (1988) Nor-binaltorphimine, a highly selective kappa-opioid antagonist in analgesic and receptor binding assays. *J Pharmacol Exp Ther* **246**:255-258.

Thomas JB, Atkinson RN, Rothman RB, Fix SE, Mascarella SW, Vinson NA, Xu H, Dersch CM, Lu Y, Cantrell BE, Zimmerman DM and Carroll FI (2001) Identification of the first trans-(3R,4R)- dimethyl-4-(3-hydroxyphenyl)piperidine derivative to possess highly potent and selective opioid kappa receptor antagonist activity. *J Med Chem* **44**:2687-2690.

Toll L, Berzetei-Gurske IP, Polgar WE, Brandt SR, Adapa ID, Rodriguez L, Schwartz RW, Haggart D, O'Brien A, White A, Kennedy JM, Craymer K, Farrington L and Auh JS (1998) Standard binding and functional assays related to medications development division testing for potential cocaine and opiate narcotic treatment medications. *NIDA Res Monogr* **178:**440-446.

Ward SJ, Portoghese PS and Takemori AE (1982) Pharmacological profiles of beta-funaltrexamine (beta-FNA) and beta-chlornaltrexamine (beta-CNA) on the mouse vas deferens preparation. *Eur J Pharmacol* **80**:377-384.

Wittmann W, Schunk E, Rosskothen I, Gaburro S, Singewald N, Herzog H and Schwarzer C (2009) Prodynorphin-derived peptides are critical modulators of anxiety and regulate neurochemistry and corticosterone. *Neuropsychopharmacology* **34**:775-785.

Xu H, Kim CH, Zhu YC, Weber RJ, Jacobson AE, Rice KC and Rothman RB (1991) (+)-cis-3-methylfentanyl and its analogs bind pseudoirreversibly to the mu opioid binding site: evidence for pseudoallosteric modulation. *Neuropharmacology* **30**:455-462.

Zhang H, Shi YG, Woods JH, Watson SJ and Ko MC (2007) Central kappa-opioid receptor-mediated antidepressant-like effects of nor-Binaltorphimine: behavioral and BDNF mRNA expression studies. *Eur J Pharmacol* **570**:89-96.

Footnotes

This research was supported by funds from Northeastern University [Provost RSDF award to JPM] and the State of Florida, Executive Office of the Governor's Office of Tourism, Trade and Economic Development to KAP, LT and JPM.

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1. Duration of nor-BNI- or naloxone-induced antagonism of U50,488- induced antinociception. Mice were administered a single dose of saline (circles), naloxone (inverted triangles) or nor-BNI (squares). Mice were then administered the KOR agonist U50,488 (10 mg/kg, i.p.) 80 min, 1, 3, 7 and 14 d after pretreatment and tested 40 min later for antinociceptive effect by measuring the latency to withdraw their tail from 55^{o}C warm water. Points represent mean ± S.E.M. % antinociception of 6-8 mice. * = $p<0.05$, significantly different from daily baseline latency response, two-way ANOVA followed by Bonferroni post hoc test.

Figure 2. Ion chromatographic peaks of nor-BNI and naloxone standards from brain homogenate.

Figure 3. LC-MS/MS detection of nor-BNI extracted from mouse brains: Ion chromatographic peaks of nor-BNI in the brain homogenate harvested from mice **A.)** 30 min and **B.)** 21 days after a single administration (30 nmol i.c.v.) C. nor-BNI detected by LC-MS/MS in the brain homogenates from perfused mice over 21 days. Peak area of nor-BNI is normalized to that of the internal standard to correct for processing and injection variability. All data points represent mean ± S.E.M. (qualitative) of peak area of nor-BNI from the brains of 3 mice. * = $p<0.05$, significantly different from control value; one-way ANOVA followed by Dunnett's multiple comparisons post hoc test.

Figure 4. Naloxone pretreatment does not prevent nor-BNI accumulation in brain or antagonism of U50,488-induced antinociception 24 h later. **A.)** LC-MS/MS analysis. Pretreatment with naloxone (100 nmol, i.c.v.) did not prevent the accumulation of a subsequent administration of nor-BNI (30 nmol, i.c.v.) over 24 h period. Levels of naloxone and nor-BNI detected by LC-

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

MS/MS in brain homogenates were obtained from processed brains of treated mice. All data points represent mean ± S.E.M. (qualitative) of peak area of nor-BNI or naloxone from the brains of 3 mice, except the 6 h nor-BNI point, where n=2. * = $p<0.05$, significantly different from control value; two-way ANOVA followed by Bonferroni multiple comparisons post hoc test. **B.)** Behavioral testing. After collecting baseline latencies (open bar, left), mice were pretreated with vehicle or naloxone (30 mg/kg, i.p.) followed 30 min later by vehicle or nor-BNI (10 mg/kg, i.p.). The next day, all mice were administered U50,488 (10 mg/kg, i.p.) and tested 40 min later in the 55^{o}C warm-water tail-withdrawal test. Naloxone pretreatment did not prevent the nor-BNI mediated antagonism of U50,488-induced antinociception 24 h later. Bars represent mean ± S.E.M. % antinociception of 8-10 mice, except baseline, which represents all mice (n=34). * = $P<0.05$, significantly different from baseline latency response; †= $p<0.05$, significantly different from latency response after U50,488 administration; n.s. = not significantly different; one-way ANOVA followed by Tukey's post hoc test.

Figure 5. LC-MS/MS detection of nor-BNI in brains of mice treated with a single peripheral administration (10 mg/kg, i.p.). Ion chromatographic peaks of nor-BNI in the brain homogenate harvested from mice **A.)** 30 min and **B.)** 21 days following antagonist administration.

Figure 6. Amount of nor-BNI detected by LC-MS/MS over 21 days following administration of a single dose (10 mg/kg, i.p.) of the KOR antagonist. **A.)** Levels of nor-BNI in mouse serum. All data points represent mean peak area ± SEM (qualitative) of nor-BNI normalized to the internal standard from serum collected from 3 mice/ pretreatment duration, except the 1 min nor-BNI point, where n=2. **B.)** Concentration of nor-BNI detected in mouse brain over 21 days. All data points represent mean nmol ± SEM (quantitated) of nor-BNI in the brain homogenate from 3 mice/pretreatment duration, as quantitated from the nor-BNI standard curve (inset graph). * =

$p<0.05$, significantly different from control value; one-way ANOVA followed by Dunnett's multiple comparisons post hoc test.

Figure 7. Dose-dependent KOR antagonist activity of nor-BNI is consistent with concentrations of the antagonist in detected in brain by LC-MS/MS. Mice were pretreated 6 h with vehicle or nor-BNI (1.4, 2.7 or 30 nmol), i.c.v. Doses or nor-BNI were selected to approximate brain levels as initially tested (30 nmol) or as determined by LC-MS/MS 7 d (2.7 nmol) or 14 d (1.4 nmol) after i.p. administration of nor-BNI. Tail-withdrawal latencies were measured in the mouse 55°C warm-water tail-withdrawal test 40 min after administration of U50,488 (30 nmol, i.c.v.). Bars represent mean latency to withdraw tail ± S.E.M. of 8 mice. * = $p<0.05$, significantly different from response of mice treated with U50,488 alone; one-way ANOVA followed by Tukey's post hoc test.

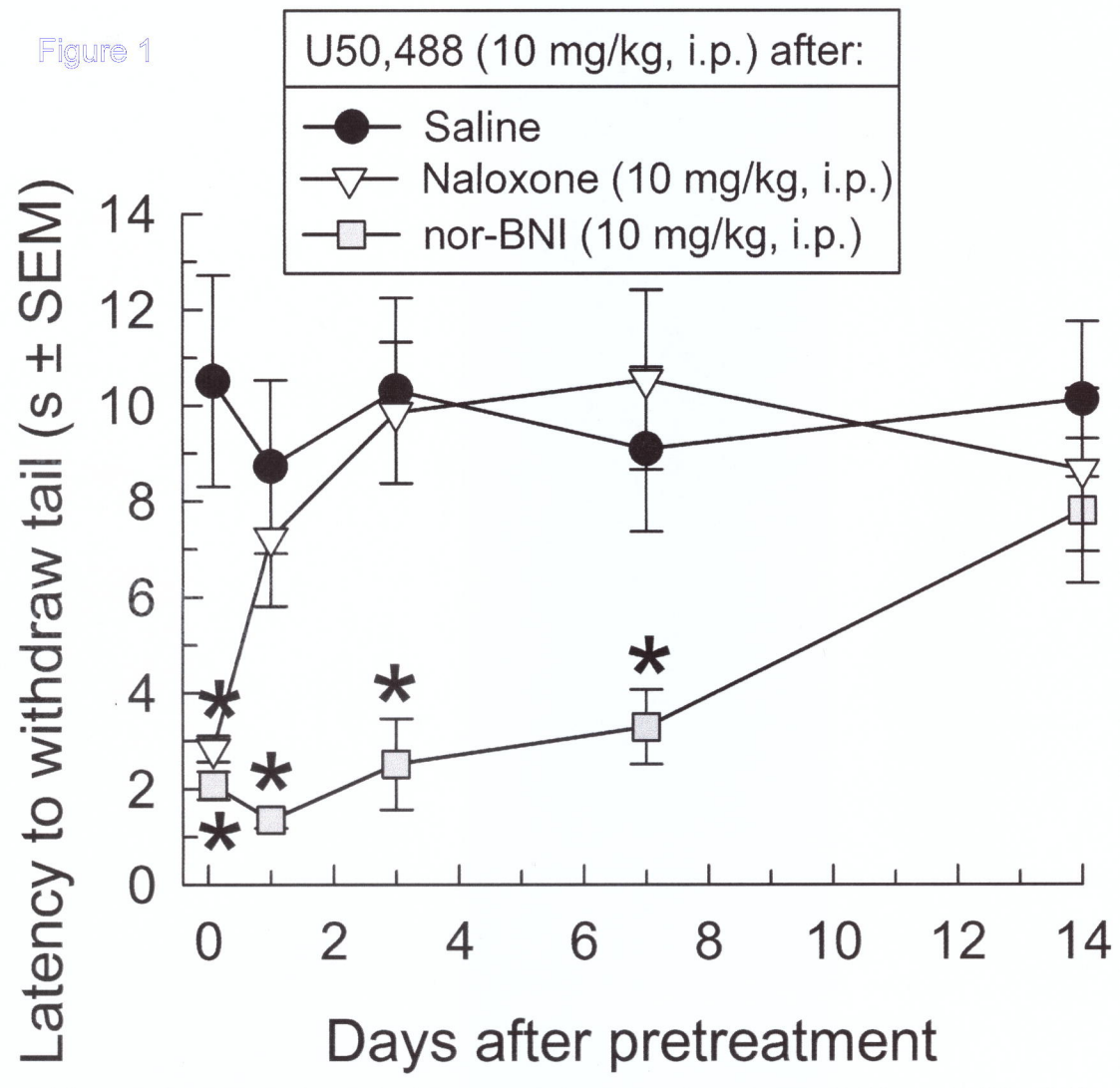
JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1.

[3H]U69,593 saturation binding results *ex vivo* with mouse brain membranes pretreated *in vivo* with saline or nor-BNI: Mice were pretreated once with saline or the opioid receptor antagonist nor-BNI (10 mg/kg, i.p.). Brain membranes were harvested 1, 7 or 14 days post-treatment, followed by two centrifugal washes. Membranes were finally resuspended in 50 mM Tris-HCl, pH 7.4, and [^{3}H]U69,593 saturation binding at concentrations of 0.05-6.4 nM was measured as described in Methods. Data are expressed as mean value ± S.E.M. for 3 determinations performed in triplicate. * $p < 0.05$ compared to saline pretreatment value.

Treatment	K_d *(nM)*	B_{max} *(fmol/mg of protein)*
Saline Control	0.94±0.30	20.0±2.25
nor-BNI Treatment (10 mg, kg, i.p.):		
1 Days Post-Treatment	1.01±0.03	9.85±0.38*
7 Days Post-Treatment	1.12±0.28	11.5±3.03*
14 Days Post-Treatment	0.90±0.26	15.1±1.48

Figure 1


U50,488 (10 mg/kg, i.p.) after:
Saline
Naloxone (10 mg/kg, i.p.)
nor-BNI (10 mg/kg, i.p.)
Latency to withdraw tail (s ± SEM)
0
2
4
6
8
10
12
14
Days after pretreatment
*
*
*
*

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2

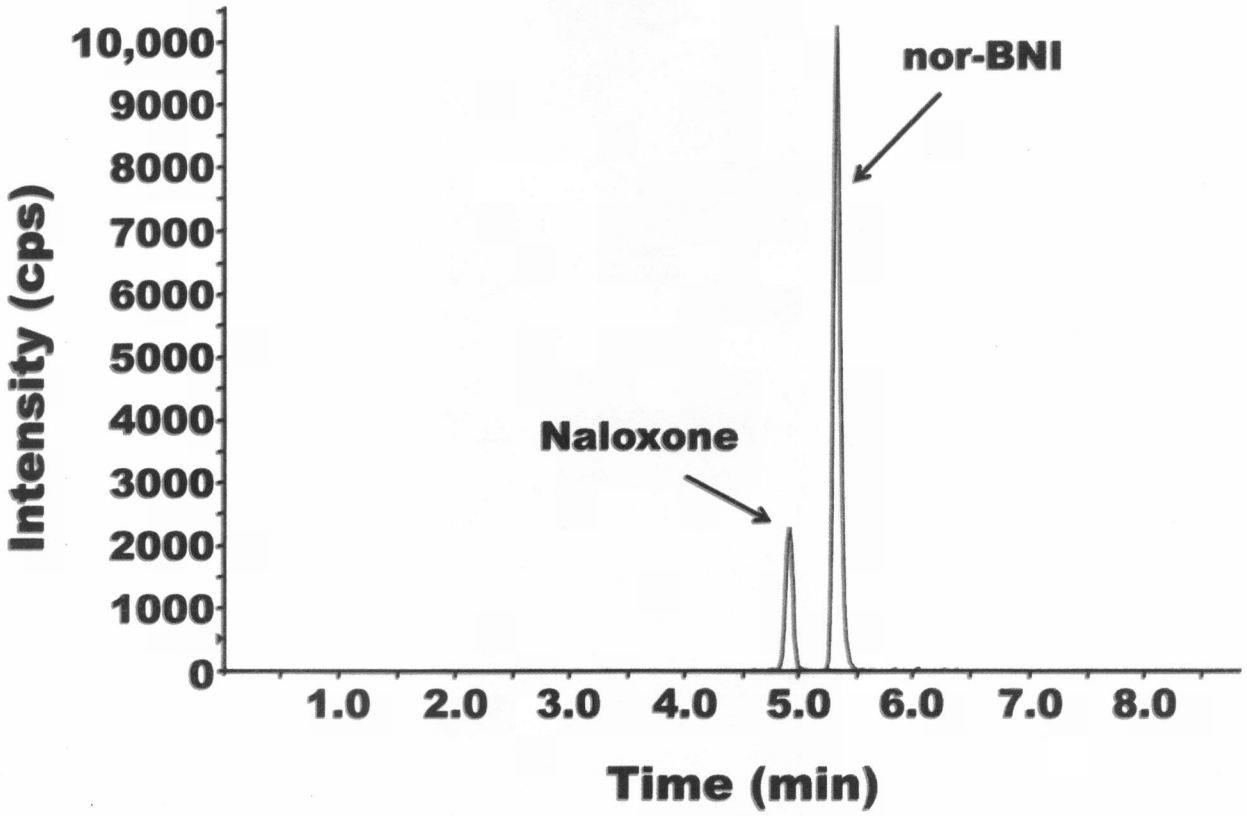


Figure 3

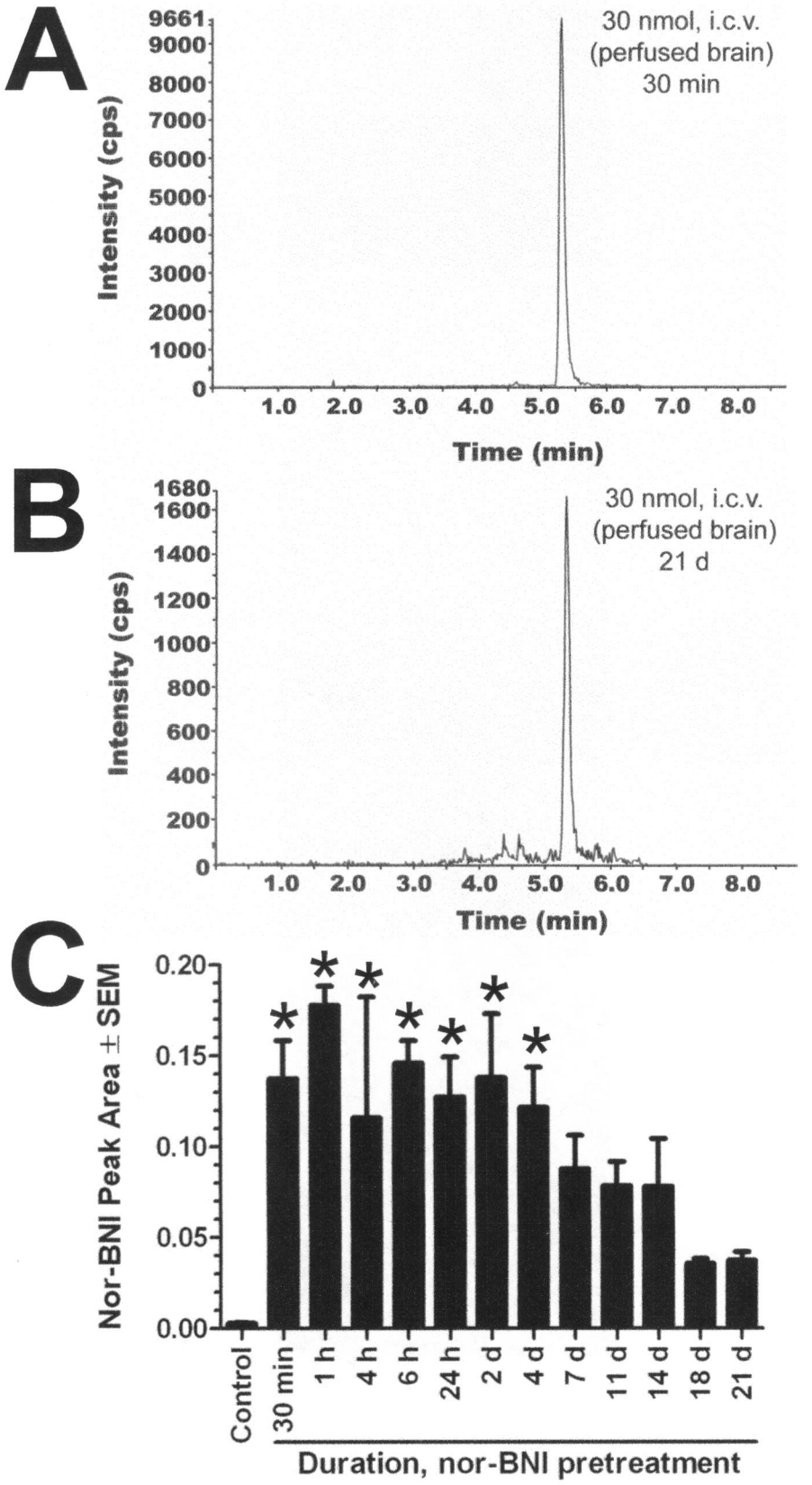
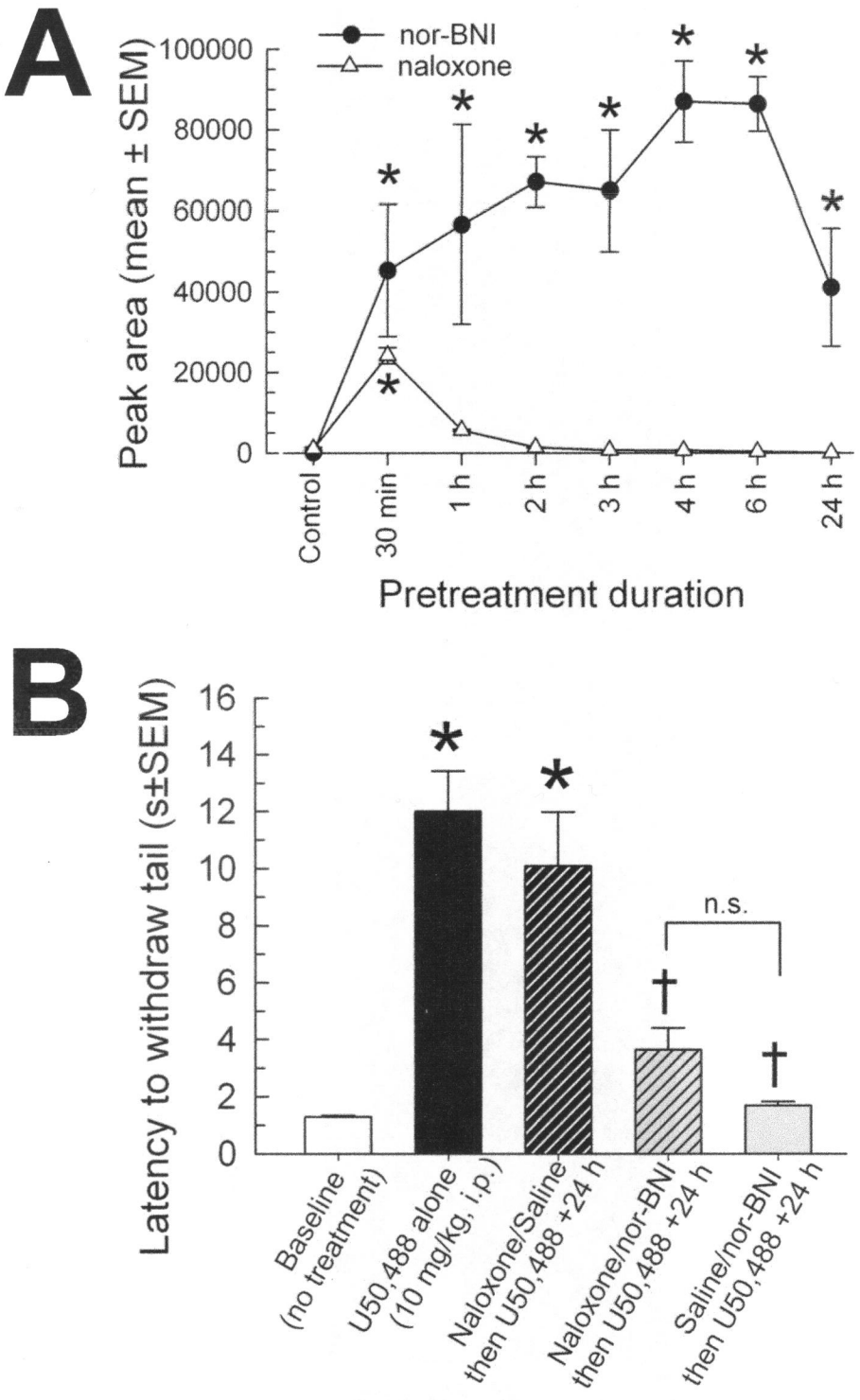

A
9661
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Intensity (cps)
30 nmol, i.c.v.
(perfused brain)
30 min
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Time (min)
B
1680
1600
1400
1200
1000
800
600
400
200
0
Intensity (cps)
30 nmol, i.c.v.
(perfused brain)
21 d
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Time (min)
C
0.20
0.15
0.10
0.05
0.00
Nor-BNI Peak Area ± SEM
Control
30 min
1 h
4 h
6 h
24 h
2 d
4 d
7 d
11 d
14 d
18 d
21 d
Duration, nor-BNI pretreatment

Figure 4


A
nor-BNI
naloxone
Peak area (mean ± SEM)
100000
80000
60000
40000
20000
0
Control
30 min
1 h
2 h
3 h
4 h
6 h
24 h
Pretreatment duration
B
Latency to withdraw tail (s±SEM)
16
14
12
10
8
6
4
2
0
n.s.
Baseline (no treatment)
U50,488 alone (10 mg/kg, i.p.)
Naloxone/Saline then U50,488 +24 h
Naloxone/nor-BNI then U50,488 +24 h
Saline/nor-BNI then U50,488 +24 h

JPET Fast Forward. Published on July 12, 2013 as DOI: 10.1124/jpet.113.206086
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5

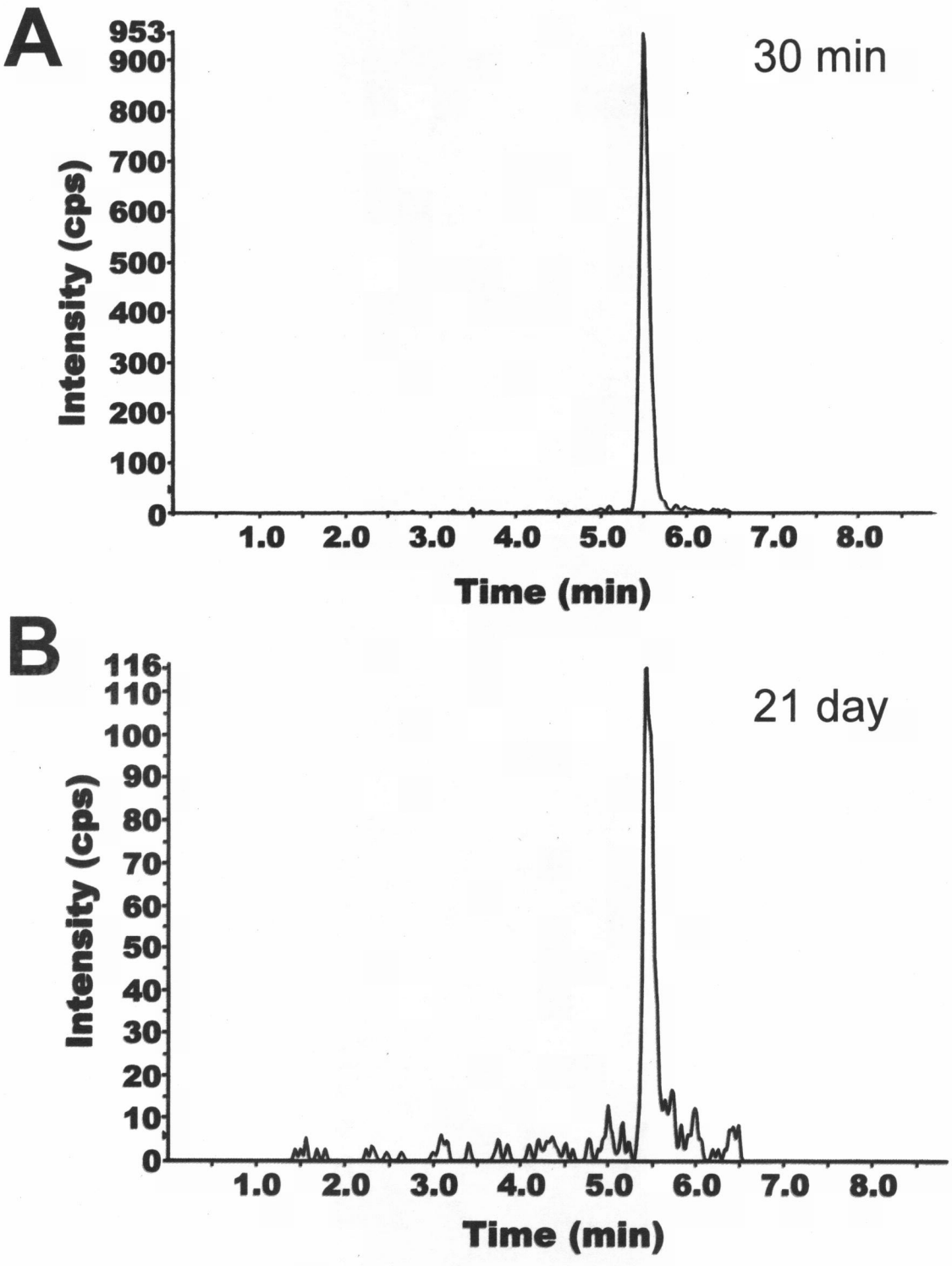


Figure 6

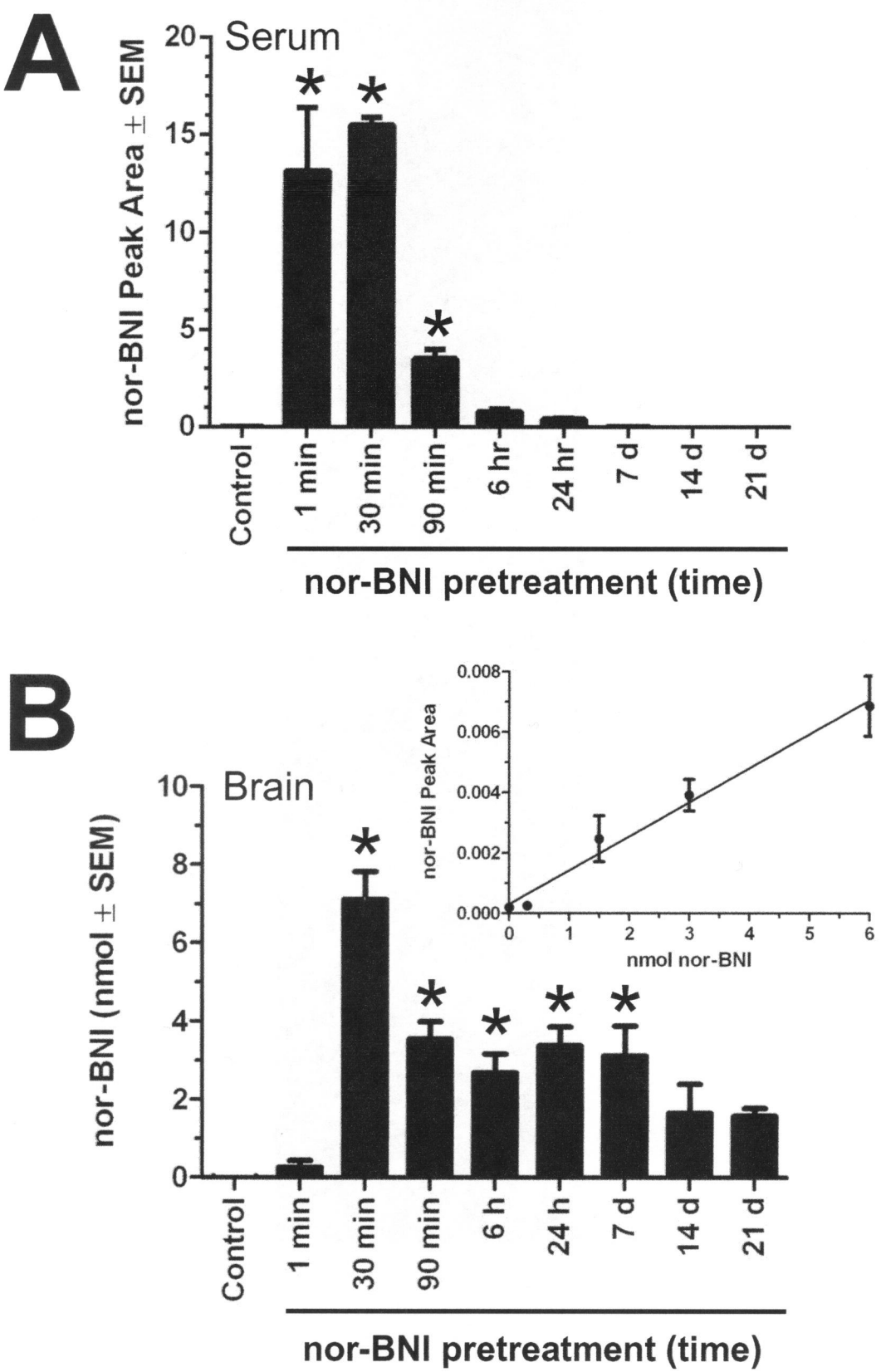
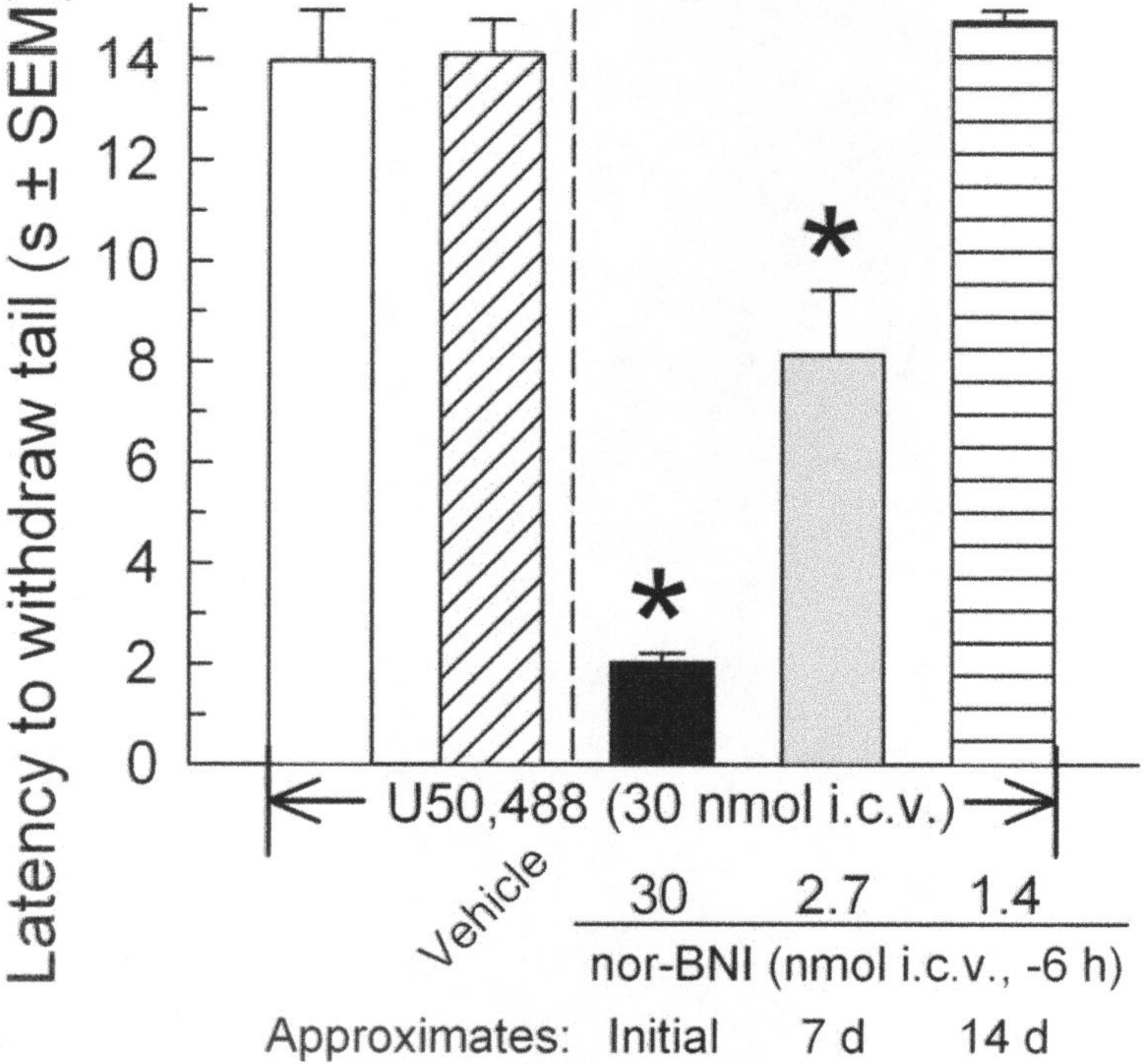

A
Serum
nor-BNI Peak Area ± SEM
20
15
10
5
0
*
*
*
Control
1 min
30 min
90 min
6 hr
24 hr
7 d
14 d
21 d
nor-BNI pretreatment (time)
B
Brain
nor-BNI (nmol ± SEM)
10
8
6
4
2
0
*
*
*
*
*
Control
1 min
30 min
90 min
6 h
24 h
7 d
14 d
21 d
nor-BNI pretreatment (time)
nor-BNI Peak Area
0.008
0.006
0.004
0.002
0.000
0
1
2
3
4
5
6
nmol nor-BNI

Figure 7


Latency to withdraw tail (s ± SEM)
0
2
4
6
8
10
12
14
*
*
U50,488 (30 nmol i.c.v.)
Vehicle
30
2.7
1.4
nor-BNI (nmol i.c.v., -6 h)
Approximates: Initial
7 d
14 d

Physical presence of nor-binaltorphimine in mouse brain over 21 days after a single administration corresponds to its long-lasting antagonist effect on kappa opioid receptors.

Kshitij. A. Patkar, Jinhua Wu, Michelle. L. Ganno, Harminder. D. Singh, Nicolette C. Ross Khampaseuth Rasakham, Lawrence Toll and Jay P. McLaughlin*

Torrey Pines Institute for Molecular Studies, Port St. Lucie, Florida

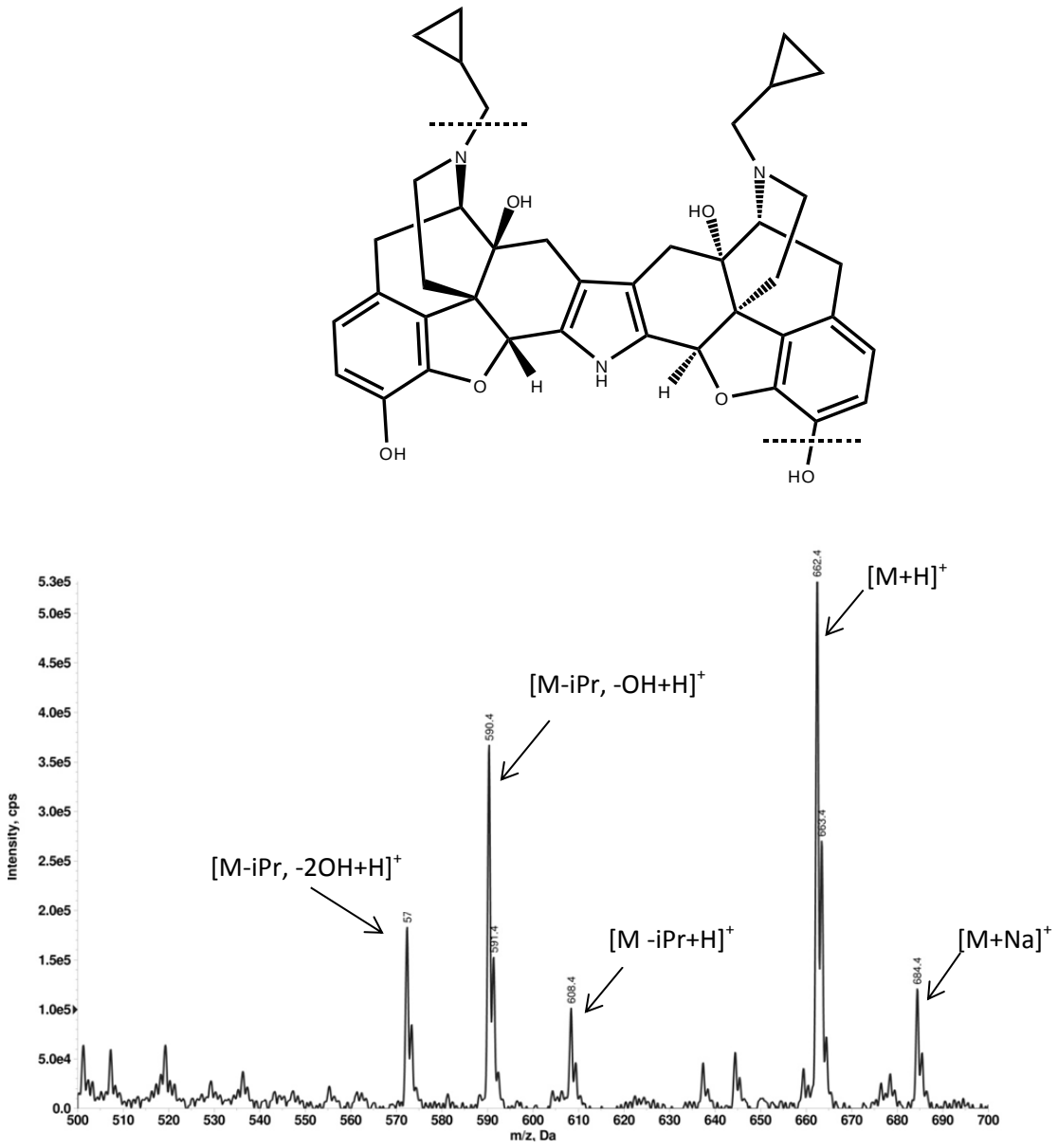
Journal of Pharmacology and Experimental Therapeutics

Supplemental Data

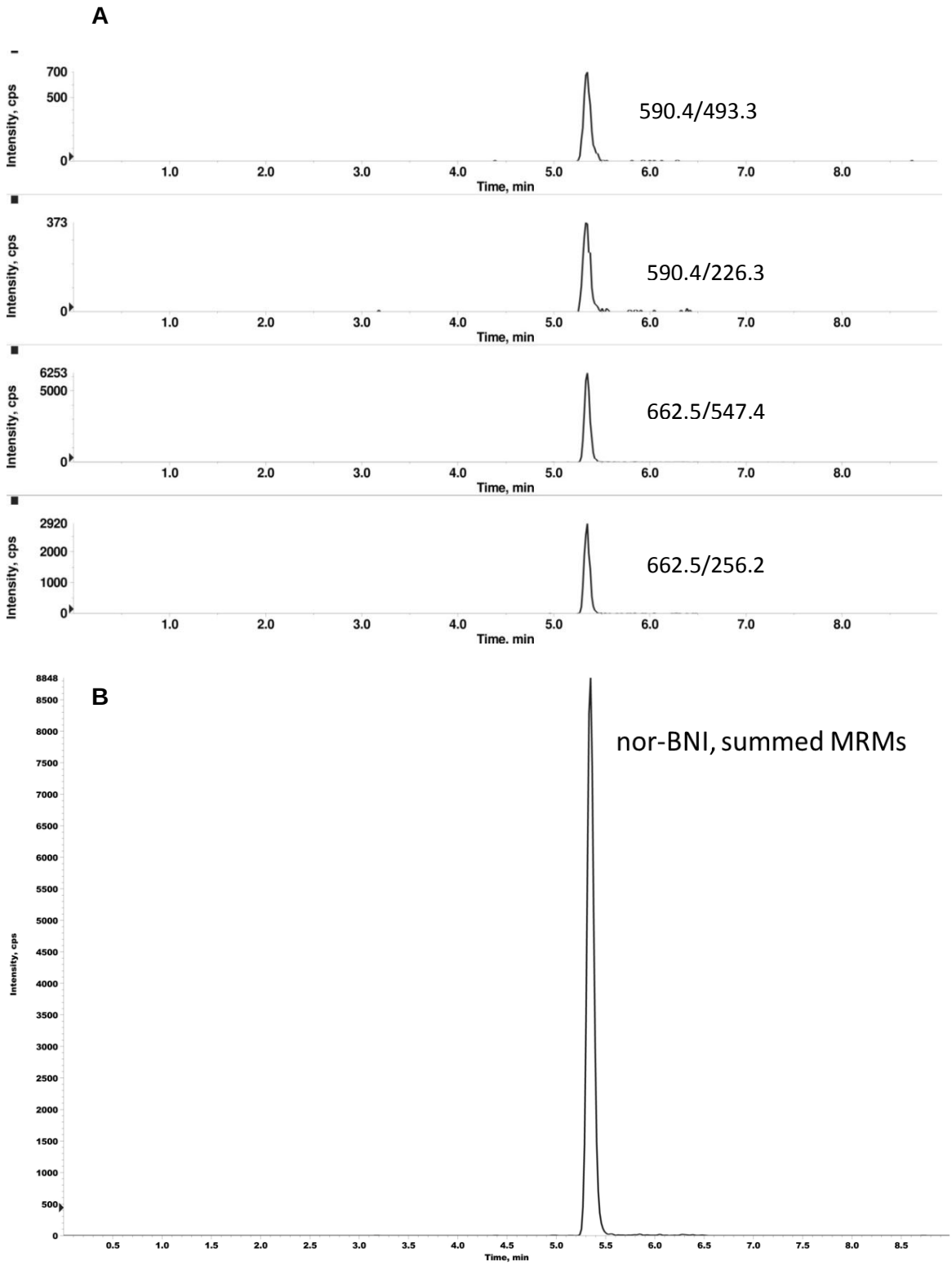
Nor-BNI underwent ionization to give an ion of the parent nor-BNI molecule (*m*/*z* 662.5). Some of the parent nor-BNI ions spontaneously underwent fragmentation in the electrospray source ((“in source decay” (ISD)) by losing one or more of the methylisopropyl and the hydroxyl (-OH) groups (Supplemental Figure 1), the most prominent being a fragment of *m*/*z* 590.4. It is difficult to say if the loss of –OH is from the phenols or the secondary alcohols in the nor-BNI structure; however, resonance stabilization offered by the benzene ring to the radical cation may prefer the loss of –OH from the phenol. In addition to the nor-BNI parent ion (*m*/*z* 662.5) we also chose the most prominent ISD fragment of nor-BNI (*m*/*z* 590.4) for the multiple reaction monitoring (MRM) analysis to detect nor-BNI in the samples. In this mode of analysis, the parent ion of an analyte after entering the mass analyzer is forced to undergo fragmentation (not to be mistaken for the spontaneous fragmentation mentioned before) to produce “daughter” ions inside a collision cell of the mass analyzer which are unique to the parent ion. The most prominent daughter ions are selected and a signal corresponding to each such transition of the

parent ion to the daughter ion is converted into an ion chromatogram peak. Presence of peaks corresponding to the ion transitions of *m*/*z* 590.4 not only increased the overall ion intensity but also provided additional confirmation for nor-BNI in the samples, since this ISD fragment is unique for nor-BNI and cannot come from any isobaric impurities in the samples from brain or serum, especially at very low levels anticipated in the brain samples from the mice that received nor-BNI dose weeks prior to the analysis. The areas corresponding to the peak of the individual transitions of both the nor-BNI ((*m*/*z* 662.5) to its daughter ions and the ISD fragment (*m*/*z* 590.4) to its daughter ions were summed to give a single peak for nor-BNI (Supplemental Figure 2).

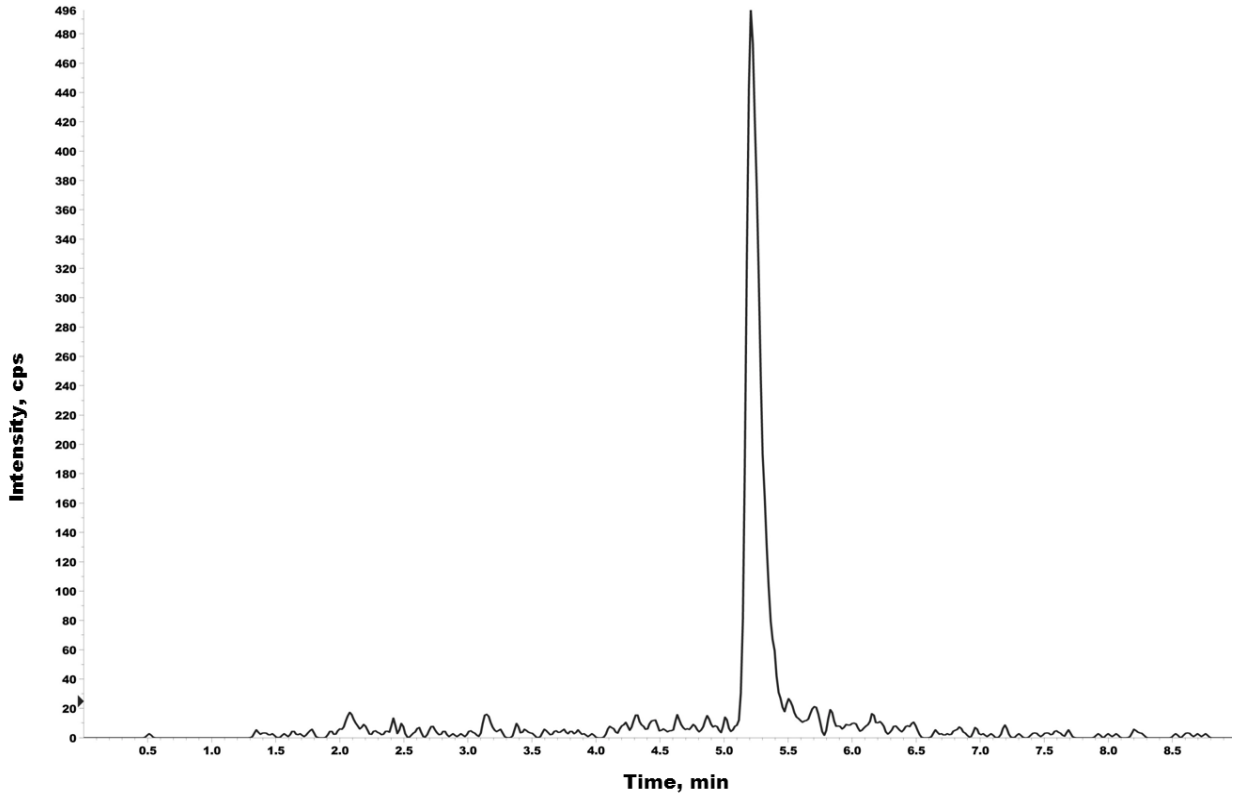
After optimizing the mass spectrometric parameters for nor-BNI, samples of mouse brain homogenates were obtained on the 21st day from mice pretreated with a single dose of nor-BNI (50 mg/kg, i.p.). These samples were utilized to develop the most sensitive chromatographic method consisting of 10 mM ammonium acetate and acetonitrile containing 0.1% formic acid to detect nor-BNI (Supplemental Figure 3).



Supplemental Figure 1. Structure of nor-BNI and mass spectrum showing parent ion and it's in source decay (ISD) fragments in the electrospray ionization source.



Supplemental Figure 2. A. Chromatograms showing individual ion transitions of nor-BNI (m/z 662.5) and its ISD fragment (m/z 590/4), and B. the summed ion chromatogram for nor-BNI generated by summing all the peaks for individual ion transitions for nor-BNI and its ISD fragment.



Supplemental Figure 3. nor-BNI in mouse brain homogenate on the 21st day after a single treatment (50 mg/kg, i.p.)