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Characterization of the Antinociceptive Actions of Bicifadine in Models of Acute, Persistent, and Chronic Pain.

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Running Title: The Antinociceptive Effects of Bicifadine

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Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; CFA, complete Freund's adjuvant; CI, confidence interval; C_{max}, maximum plasma concentration; COX, cyclooxygenase; DA, dopamine; DAT, dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; $ED_{50,}$ dose yielding a 50% of maximal response; EDTA, Ethylenediamine-tetraacetic acid; 5-HT, serotonin; IC_{50}, concentration yielding 50% of maximal inhibition; GABA, γ-aminobutyric acid; Gbp, gabapentin; h, human; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; 5-HIAA, 5-hydroxyindoleacetic acid; HPLC, high pressure liquid chromatography; HVA, homovanillic acid; IP, intraperitoneal; LC, locus coeruleus; LC-MS/MS, liquid chromatography, mass-spectrometry/mass spectrometry; LD_{50}, dose lethal to 50% of subjects; MED, minimum effective dose; MPE%, percentage of maximum possible effect; MOR, morphine; NE, norepinephrine; NET, norepinephrine transporter, NMDA, N-methyl-D-aspartate; NSAID, nonsteroidal anti-inflammatory; PBS, phosphate buffered saline; pfCTX, prefrontal cortex; PO, per os; PPQ, phenylparaquinone; SC, subcutaneous; SD, standard deviation; SEM, standard error of the mean; SERT, serotonin transporter; SNL, spinal nerve ligation; STZ, streptozotocin, Str, striatum; TCA,

tricyclic antidepressant; t_{max}, time of maximum plasma concentration; Veh, vehicle.

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ABSTRACT

Bicifadine (1-p-tolyl-3-azabicyclo[3.1.0]hexane) inhibits monoamine neurotransmitter uptake by recombinant human transporters *in vitro* with a relative potency of NE>5-HT>DA (≈1:2:17). This *in vitro* profile is supported by microdialysis studies in freely moving rats, where bicifadine (20 mg/kg IP) increased extrasynaptic norepinephrine and serotonin levels in the prefrontal cortex, norepinephrine levels in the locus coeruleus, and dopamine levels in the striatum. Orally administered bicifadine is an effective antinociceptive in several models of acute, persistent and chronic pain. Bicifadine potently suppressed pain responses in both the Randall-Selitto and kaolin models of acute inflammatory pain, and in the phenyl-p-quinone-induced and colonic distension models of persistent visceral pain. Unlike many transport inhibitors, bicifadine was potent and completely efficacious in both phases of the formalin test in both rats and mice. Bicifadine also normalized the nociceptive threshold in the complete Freund's adjuvant model of persistent inflammatory pain and suppressed mechanical and thermal hyperalgesia, and mechanical allodynia in the spinal nerve ligation model of chronic neuropathic pain. Mechanical hyperalgesia was also reduced by bicifadine in the streptozotocin model of neuropathic pain. Administration of the D_2 receptor antagonist (-)-sulpiride reduced the effects of bicifadine in the mechanical hyperalgesia assessment in rats with spinal nerve ligations. These results indicate that bicifadine is a functional triple reuptake inhibitor with antinociceptive and anti-allodynic activity

in acute, persistent and chronic pain models, with activation of dopaminergic pathways contributing to its antihyperalgesic actions.

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INTRODUCTION

Multiple neurotransmitters have been implicated in the modulation of nociceptive signaling at both the spinal and supraspinal levels of central nervous system processing (Millan, 2002). Among these, norepinephrine (NE) and serotonin (5-HT) play important roles in modulating nociceptive information via pathways descending from the brainstem to the level of the dorsal horn. Noradrenergic fibers from the locus coeruleus and subcoeruleus descend through the pons and synapse onto neurons in laminae I/II, IV/V and X of the dorsal horn (Westlund, 1992). Stimulation of the locus coeruleus or application of norepinephrine onto dorsal horn neurons reduces their excitability in response to nociceptive stimuli, an effect mediated by postsynaptic α_2 adrenoceptors (Millan, 2002). Serotonergic pathways arising from the nucleus raphe magnus in the rostroventral medulla and terminating in spinal laminae I/II and IV-VI can facilitate and/or suppress neuronal activity in spinal pain processing pathways (Millan, 2002). The identity and locations of the 5-HT receptors mediating these disparate actions of 5-HT remain under investigation, with 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2C} and 5-HT_3 receptors producing both pro- and anti-nociceptive activity upon activation, depending on the depending on the nature and location of the stimulus and the pain model (Millan 2002). While there has been a focus on the roles of NE and 5-HT in modulating nociceptive information, dopaminergic pathways are also involved in processing noxious stimuli at both the spinal and supraspinal levels. Activation of striatal dopaminergic pathways suppresses (Lin et al., 1981), and depletion of dopaminergic nigrostriatal neurons enhances pain

sensitivity (Chudler and Dong, 1995). In addition, dopaminergic pathways arising from the posterior paraventricular nucleus of the hypothalamus descend to the dorsal horn of the spinal cord (Skagerberg et al., 1982), where they may modulate nociceptive input (Millan, 2002). At both the spinal (Tamae et al., 2005) and supraspinal levels (Magnusson and Fisher, 2000), the influence of DA on nociceptive processing is mediated predominantly through D_2 receptors.

Activation of the noradrenergic and serotonergic receptors subserving pain-modulating pathways can be achieved through blockade of the NE (NET) and 5-HT (SERT) transporters. Dual NET and SERT inhibitors such as the tricyclic antidepressants (TCAs) amitriptyline and desipramine (Owens et al., 1997) possess greater analgesic efficacy, particularly in painful neuropathic states such as herpes neuralgia, diabetic neuropathy and nerve crush syndromes (Sindrup et al., 2005), than either selective NET or SERT inhibitors (Fishbain et al., 2000). Similarly, the contribution of dopaminergic pathways to analgesic processes is supported by observations that dopamine transport (DAT) inhibitors (Pedersen et al., 2005), and D_2 agonists (Magnusson and Fisher, 2000) are antinociceptive in models of acute and chronic pain elicited by a number of modalities. Clinical evidence of a role for DA in modulating nociceptive inputs is provided by the hyperalgesia associated with dopaminergic hypofunction, such as in Parkinson's disease (Drake et al., 2005), while the DAT inhibitor bupropion (Semenchuk et al., 2001) and DA precursor levodopa (Ertas et al., 1998) are analgesic in neuropathic pain syndromes (Hagelberg et al., 2004).

While the analgesic efficacy of TCAs has been established, they exhibit a significant and dose-limiting side-effect profile, including xerostomia, nausea and intractable arrythmias, leading to problems of compliance (Sindrup et al., 2005). Dual uptake inhibitors such as venlafaxine and duloxetine, which are clinically effective in reducing neuropathic pain (Iyengar et al., 2004, Lang et al., 1996), have mitigated many of these limiting side effects. However, enhancement of dopaminergic neurotransmission may further expand the favorable analgesic profile of dual NET and SERT inhibitors (Pedersen et al., 2005). To this end, we have characterized the antinociceptive properties of bicifadine (1-p-tolyl-3-azabicyclo[3.1.0]hexane), an inhibitor of biogenic amine transporters, in animal models of acute and chronic pain.

METHODS

Animals

Male Sprague-Dawley rats (175-260 g, Janvier, France: Randall-Selitto, kaolin tests; Harlan, Indianapolis IN, USA: CFA, SNL models; Prokocim, Krakow, Poland: open field; rotarod), male Wistar rats (150-160 g, Elevage Janvier, France, colonic distension; Inst of Pharmacology, Krakow Poland: microdialysis), male albino Swiss mice (18-30 g, Royalhart Farms, PPQ, Inst Pharmacology, Krakow, Poland, tail flick test) and male CD-1 mice (22-26 g, BioLasco, Taiwan, formalin test) were used in the study. The investigators adhered to experimental procedures and housing conditions in accordance with the Animal Protection Act of August 21, 1997 (Poland's Government Regulations and Laws Gazette), the French Ministry for Agriculture and Fisheries, the International Associations for the Study of Pain guidelines and the University of Minnesota Animal Care and Use Committee.

Upon receipt, the rats and mice were allowed at least 5 days to acclimate to their surroundings prior to testing. These animals were housed and fed in accredited facilities maintained on a standard 12-h light/dark cycle (lights on 6:00 AM; lights off, 6:00 PM) at a room temperature of 19.5-24.5 °C and relative humidity of 45-65%. Free access to food and water was maintained throughout the study period. Animals were assigned randomly to treatment groups.

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Biochemistry and Physiology

Recombinant Human Monoamine Transporters: Binding and Uptake Studies.

[^{125}I]RTI-55 (3 beta-(4-iodophenyl)tropan-2 beta-carboxylic acid methyl ester) was used in competition binding assays characterizing bicifadine affinity for the three monoamine neurotransmitter transporters using previously described techniques (Eshleman et al., 1999). Briefly, to an aliquot of recombinant human transporter preparation (≈ 12-30 μg protein) were added bicifadine, [^{125}I]RTI-55 (40-80 pM final concentration, Perkin-Elmer, MA, USA) and Krebs-HEPES assay buffer to yield a final volume of 250 μL. Specific binding was defined using either 5 μM mazindol (hDAT, hNET) or 5 μM imipramine (hSERT). The reaction was incubated for 90 min at room temperature in the dark and was terminated by vacuum filtration.

The potency of bicifadine in suppressing monoamine neurotransmitter uptake was determined using suspensions of cell lines recombinantly expressing human transporters. These suspensions were prepared by removing the medium from cells grown on 150 mm diameter tissue culture dishes, then washing the plates twice with Ca^{2+}, Mg^{2+}-free phosphate buffered saline (PBS). Fresh Ca^{2+}, Mg^{2+}-free PBS (2.5 mL) was then added to each plate and the plates placed into a 25°C water bath for 5 min. The cells were gently scraped from the plates and cell clusters separated by trituration with a pipette for 5-10 aspiration/ejection cycles (Eshleman, et al., 1999). To these suspensions were added bicifadine, Krebs-HEPES assay buffer, and, after a 10 minute pre-

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incubation of the isolated cells at 25°C, either [^{3}H]DA, [^{3}H]5-HT, or [^{3}H]NE, (56, 26.9, 60 Ci/mmol, respectively, 20 nM final concentration). The assay was incubated an additional 10 minutes, and the radiolabelled neurotransmitter uptake terminated by vacuum filtration. Specific uptake was defined as the difference in uptake observed in the absence and presence of 5 μM mazindol (hDAT and hNET) or 5 μM imipramine (hSERT).

Characterization of Bicifadine Interactions with Other Receptor Systems.

The affinity of bicifadine for a number of receptor systems was assessed using validated radioligand competition binding assays under conditions defined by the contractor (MDS, WA, USA [http://www.discovery.mdsps.com/Catalog/Assays], or CEREP, Rueil-Malmaison, France [http:// www.cerep.fr/Cerep/Users/pages/catalog/assay]) (Table 1). For those receptors where the K_i for bicifadine was < 10 μM, additional investigations were performed to identify the pharmacological nature of the interaction (i.e., agonist or antagonist) using previously described biochemical and physiological assays performed under conditions validated by the contractor (MDS, WA, USA or CEREP, Rueil-Malmaison, France).

N-Methyl-D-Aspartate (NMDA) Receptor Electrophysiology:

The effect of bicifadine was investigated on NMDA-gated currents in primary cultures of rat hippocampal pyramidal neurons (Nahum-Levy et al., 2002). Whole cell patch clamp experiments were conducted at room

temperature between 1 to 2 weeks after plating the neurons, with a holding potential for the voltage-clamp experiments of -60 mV.

Microdialysis

Rats were anaesthetized with ketamine (75 mg/kg IM) and xylazine (10 mg/kg IM) and placed into a stereotaxic apparatus (David Kopf Instruments, CA, USA). The skulls were exposed and small holes drilled for insertion of the microdialysis probes using the following coordinates:1.8 mm anterior from the bregma; 2.7 mm lateral from the sagittal suture;–7.0 mm ventral from the dural surface (striatum); 2.9 mm anterior from the bregma; 0.8 mm lateral from sagittal suture; -4.5 mm ventral from the dural surface (prefrontal cortex);-9.8 mm posterior from the bregma; 1.3 mm lateral from the sagittal suture; –5.7 mm ventral from the dural surface (locus coeruleus). For striatal and prefrontal cortex recording, microdialysis probes were constructed by inserting two fused silica tubes (30 and 35 mm long, 150 µm outside diameter (o.d.; Polymicro Technologies Inc., Phoenix, AZ, USA) into a microdialysis fiber (220 µm o.d.; AN69, Hospal, Bologna, Italy). The tube assembly was placed in a stainless steel cannula (22G, 10 mm) forming the shaft of the probe. Portions of the inlet and outlet tubes were individually placed inside polyethylene PE-10 tubing and glued. The free end of the dialysis fibre was sealed, and 4 or 3 mm of the exposed length used for dialysis in the striatum or prefrontal cortex, respectively. For the locus coeruleus, microdialysis probes (CMA/11, Carnegie Medicin, Stockholm, Sweden) were used. All probes were connected to a syringe pump (BAS, IN,

USA) which delivered an artificial cerebrospinal fluid composed of [mM]: NaCl 145, KCl 2.7, $MgCl_2$ 1.0, $CaCl_2$ 1.2; pH = 7.4 at a flow rate of 1.5 µl/minute. Baseline samples were collected every 20 minutes after the washout period to obtain a stable extracellular neurotransmitter level. Bicifadine was then administered and dialysate fractions collected for 240 minutes. At the end of the experiment the rats were sacrificed and their brains histologically examined to validate probe placement.

Neurotransmitter analysis

DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), as well as 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were analysed by HPLC with electrochemical detection. Chromatography was performed using an LC-10 AD pump (Shimadzu Europa GmbH, Warsaw, Poland), an LC-4B amperometric detector with a cross-flow detector cell (BAS, IN, USA) and BDS-Hypersil C_{18} analytical column (3 x 100 mm). The mobile phase was composed of 0.1 M monochloroacetic acid adjusted to pH = 3.7 with 3 M sodium hydroxide, 0.5 mM EDTA, 25 mg/L 1-octanesulfonic acid sodium salt, a 5.7 % methanol and a 2.5 % acetonitrile. The flow rate was 0.5 ml/min, and the applied potential of a 3 mm glassy carbon electrode was +600 mV with a sensitivity of 2 nA/V. NE was measured using an HPLC system equipped with a P580 pump (Dionex, CA, USA) connected to an injection valve with a 10 µl loop and a BDS-Hypersil analytical column (2.0 x 100 mm). The mobile phase was composed of 0.05 M KH_2PO_4 (adjusted to pH = 3.7 with ortho-phosphoric acid),

0.5 mM EDTA, 150 mg/L 1-octanesulfonic acid sodium salt, 10 mM NaCl and 1.2% acetonitrile. The flow rate was 180 µL/minute. NE was detected in dialysates with a radial flow detector cell coupled to a LC-4B amperometric detector (BAS, IN, USA). The applied potential of a 3-mm glassy carbon electrode was +600 mV with a sensitivity of 2 nA/V. The chromatographic data were processed by Chromax 2001 (Pol-Lab, Warsaw, Poland) software run on a PC computer. The values were not corrected for *in vitro* probe recovery, which was approximately 15%.

Pharmacokinetic Analysis

Male rats were orally administered 6, 20 or 60 mg/kg (n =3/dose) of bicifadine, and blood samples (approximately 1 mL) were taken 1, 2, 4, 8 and 24 hours post dosing. Plasma levels of bicifadine were determined using a validated LC MS/MS assay (WIL Research Laboratories, OH, USA). Briefly, plasma aliquots (0.2 mL) were transferred to tubes containing 50 µL 0.5 M KOH, 25 µL internal standard (8 µg/mL (1R,5S)-(+)-1-(3,4-dichlorophenyl)-3-azabicyclo[3.1.0]hexane), and 300 µL blank rat plasma, mixed and allowed to stand for 15 minutes. Diethyl ether (8 mL) was then added to each tube, which were shaken for 15 minutes, then centrifuged at 3500 rpm for 5 minutes. The organic fraction was transferred to clean tubes and the samples were evaporated to dryness under nitrogen. This step was repeated, and the samples finally reconstituted in 200 µL of methanol. Aliquots of the fully reconstituted samples were then transferred to autosampler tubes.

The extracted samples (40 µL) were then injected onto an HPLC (2695, Waters, MA, USA) with a Hypersil C18-BDS column (50 x 4.6 mm, 3 µm particle size) and a C18 guard column (Phenomenex Security Guard). The sample was isocratically eluted with a mobile phase (30% acetonitrile, 35% methanol, 0.5% formic acid and 34.5% 5 mM ammonium acetate) at a flow rate of 0.4 mL/min. The retention time for bicifadine was approximately 2.3 min, with a retention time of 3.0 minutes for the internal standard. Total run time was approximately 6 minutes.

Bicifadine was detected with a tandem mass spectrometer (Micromass Quattro Micro) equipped with an atmospheric pressure chemical ionization interface in positive ionization mode. Detection settings used were: Corona 20 µA; Cone (bicifadine) 35 V; Extractor 2V; RF lens 0.2 V; Source temperature 130 °C; Desolvation temperature 400°C; Cone gas flow 100 L/hr nitrogen; Desolvation gas flow 700 L/hr nitrogen. Bicifadine was monitored using an m/z = 174/133 (parent/product ion), with using argon as the collision gas at a pressure of 2.5×10^{-3} mbar and an energy of 18 eV. Data acquisition and analysis were performed using MassLynx software ver. 3.5.

In addition to the plasma samples taken from the dosed rats, each set of analyses consisted of: calibration samples (8-9 concentrations) in duplicate; one solvent blank; one blank rat plasma sample; one blank rat plasma sample doped with internal standard; quality control samples (3 concentrations in triplicate). Valid sample runs required that 2/3 of quality control samples not deviate more

than ± 15% from target concentration values. The lower limit of detection of this assay was 8.26 ng bicifadine/mL, and the upper limit was 1652 ng/mL.

Models of Acute Inflammatory Pain

Randall-Selitto Test

These studies were performed by Calvert Laboratories, Inc. (PA, USA). Inflammation was induced by the subplantar injection of 0.1 mL of 20% brewer's yeast suspension into the plantar surface of the rat right hind paw (LeBars et al., 2001). Test agents or vehicle were orally (PO) administered one hour after the yeast injection. Two hours after the yeast injection, the mechanical nociceptive threshold was measured using an analgesiometer (Ugo Basile, Milan, Italy). The nociceptive threshold was defined as the force (g) at which the rat vocalized or withdrew its paw. The upper limit for pressure administration was 250 g.

Kaolin-Induced Arthritis Model

Studies using this model were performed by Cerep. Arthritis was induced by injection of 100 µL of 10% (w/v) kaolin suspension in saline into the knee joint of the rat right hind paw (Hertz et al., 1980). The vehicle and test substances were orally administered 30 minutes after kaolin injection. Behavioral assessments of gait behavior were conducted every hour from 1 to 5 hours following drug dosing using the following indices: 0 = Normal gait; 1 = Mild disability; 2 = Intermittent raising of paw; 3 = Elevated paw.

Models of Acute Nociceptive Pain

Tail-Flick Test

The tail flick latency is the time taken by a mouse to withdraw its tail from a radiant heat source as measured using a semi-automated device (H. Sachs Elektronik # 812, Hugstetten, Germany) (Le Bars et al., 2001). The latency data was converted to the maximum possible effect using the equation: MPE = 100 x ([$Latency_{Drug}$ – $Latency_{Baseline}$]/[$Latency_{cutoff}$-$Latency_{Baseline}$]. The heat source was adjusted for testing subjects under high intensity (12 second cut-off latency) or low intensity (25 second cut-off latency) stimuli. Vehicle-treated mice responded to the high and low intensity stimuli with an average latency of 6.3 ± 0.18 and 16.8 ± 0.41 seconds, respectively. Subjects were tested before and 60 minutes after PO or subcutaneous (SC) administration of test compounds under blinded conditions.

Hot Plate Test

Mice were placed on a heated surface maintained at either 55 or 59 ± 0.5 °C. The time between placement on the hot plate and the occurrence of licking of the hind paws, shaking or jumping off the surface was recorded as the response latency (Le Bars et al., 2001). Animals with baseline latencies of less than 12 seconds or more than 18 seconds were excluded from the study. A maximum cutoff time of 30 seconds was used to prevent tissue damage, with a control rat response time of approximately 6-8 seconds.

Models of Persistent Pain

Phenyl-p-quinone (PPQ)-Induced Abdominal Contractions

Studies using this model were performed by Cerep. Test compounds were administered 30-60 minutes before PPQ administration. Abdominal contractions were induced by intraperitoneal (IP) injection of PPQ (1 mg/kg) (LeBars et al., 2001). Mice were placed individually into observation boxes15 minutes after PPQ injection, and the number of writhes (i.e., stretching, twisting a hind leg inward, or contracting the abdomen) recorded over a 3 minute period. Control (vehicle-treated) mice produce approximately 30 ± 5 (mean $\pm$ SD) incidences of these behaviors during this period. ED_{50} values were calculated as the dose required to reduce the number of writhes to <18 in 50% of the treated mice.

Colonic Distension Model

Rats received an intra-colonic application of 1.5 mL of 1% acetic acid, followed 2.5 hours later by catheter-induced colonic distension lasting for 10 minutes. Pain was scored by visual counting of the abdominal contractions over a 10 minute period. Vehicle and test substances were administered 60 minutes before initiation of colonic distension. This investigation was performed by Porsolt & Partners (Le Genest-Saint-Isle, France).

Formalin test

Vehicle or test substances were orally administered 60 minutes before formalin injection. Sprague-Dawley rats were injected with 50 µL of 5% formalin in 0.9% NaCl into the dorsal surface of the right hind paw (Le Bars et al., 2001). Similarly, CD-1 mice were injected with 20 µL of 5% formalin solution. Hind paw licking time was recorded between 0 to 10 minutes (Phase 1) and between 15 and 40 minutes (Phase 2) after formalin injection in the rats. Licking time was recorded over 0-5 (Phase 1) and 20 to 30 (Phase 2) minutes in mice. Results are expressed as the mean ± SEM time spent licking paws during each phase of the study. These investigations were performed by MDS, Inc.

The complete Freund's adjuvant (CFA) model of persistent inflammatory pain

Rats were anesthetized with 2-3% isoflurane, and 50 uL of a *Mycobacterium tuberculosis* (1 mg/mL) suspended in CFA were injected subcutaneously into the plantar surface of the left paw (Stein et al., 1988). One week later, the baseline and post-drug treatment paw withdrawal thresholds to mechanical stimulus were measured as outlined below. These investigations were performed by Algos Therapeutics, Inc (MN, USA).

Models of Chronic Pain

The Spinal Nerve Ligation (SNL) Model.

The SNL model (Kim and Chung, 1992) was used to induce chronic neuropathic pain. Rats were anesthetized with isoflurane, the left L5 and L6

spinal nerves were exposed by removing the paravertebral muscle and a part of the left spinous process of the L5 vertebrae. The L5 and L6 spinal nerves were isolated and tightly ligated with 6-0 silk suture (NC-silk black, USP 5/0, metric 1, Braun). The muscle and adjacent fascia were sutured and the skin incision closed externally with wound clips. The clips were removed 10 days after surgery and the animals allowed to recover at least 2 weeks before testing. These investigations were performed by Porsolt & Partners and Algos Therapeutics.

Streptozotocin (STZ)-Induced Diabetic Neuropathy.

This model was utilized in studies performed by Cerep. Diabetes was induced by administration of a single dose of STZ (75 mg/kg, IP, Courteix et al., 1993), prepared as a solution in citrate buffer (pH 4.2). Twenty-three days after injection, the presence of diabetes was confirmed by measuring hyperglycemia using a glucometer with blood glucose strips (Glucotrend type 1895729 and Accu-Check strips, Roche Diagnostics, IN, USA). Animals with glucose levels lower than 250 mg/dL were not used for further studies.

Behavioral Testing Models of Chronic Pain

Mechanical hyperalgesia

Baseline and post-treatment values for mechanical nociceptive sensitivity in all models of chronic pain were evaluated using a Paw Pressure Analgesymeter

(7200, Ugo Basile, Comerio, Italy), which generates a linearly increasing mechanical force. For the assessment of mechanical hyperalgesia, the force was applied to the plantar surface of the injured rat hind paw via a cone-shaped stylus with a rounded plastic tip (2 mm^2) placed between the third and fourth metatarsus. The nociceptive threshold was defined as the force (g) at which the first pain behavior (paw withdrawal, struggle and/or vocalization) was expressed. The maximal force for testing was set at 390 g. The pain threshold was measured in both hind paws.

Thermal hyperalgesia.

Rats were placed into an acrylic box (17 x 11 x 13 cm, Ugo Basile 7371) on an elevated glass floor and left to habituate for 10 minutes. A mobile radiant heat source (96±10 mW/cm^2) was focused under the non-lesioned and lesioned hindpaws, and the paw-withdrawal latency automatically recorded. The cut-off latency was set for 45 seconds of exposure.

Mechanical allodynia

Rats were placed on a metal mesh and covered with a plastic dome. They were allowed to habituate until the exploratory behavior diminished (≈ 15 minutes). Mechanical sensitivity (paw withdrawal threshold) was evaluated at baseline, post-injury (day 14 post-surgery) and post-treatment (day 14 post-surgery) using 8 Semmes-Weinstein filaments (Stoelting, IL, USA) with varying stiffness (0.4, 0.7, 1.2, 2.0, 3.6, 5.5, 8.5, and 15 g) according to the up-down method (Chaplan

et al., 1994). Because this stimulus is normally not considered painful, significantly larger responses following surgery are interpreted as a measure of mechanical allodynia.

Side Effect Profile

Motor Function Assessments

The sedative, ataxic and myorelaxant effects of bicifadine and select tricyclic antidepressants were determined using the open field, rotarod, and grip strength meter (Popik et al., 2006). In the open field test, rats were placed in the corner of a dimly lit (40 lux) open field of black plywood (66 x 56 x 30 cm) beginning 60 minutes after drug or vehicle administration, and the distance traveled over a 21 minute period was measured using the Any-maze® tracking system. The presence of ataxia was assessed using a rotarod apparatus (ENV-577, MED Associates, VT, USA) rotating at 6 rpm. Those animals that did not fall off the apparatus within 2 minutes were considered to have normal balance and coordination. The degree of myorelaxation was determined next using a grip strength meter (Columbus Instruments, OH, USA). The forepaws of a rat were placed on the metal mesh attached to the meter, and the body was gently pulled until the rat released the grid. Three measures of grip strength were taken sequentially for each rat, averaged, and corrected for body weight.

Drug Administration

Bicifadine (5-100 mg/kg) was dissolved in distilled water (5 mL/kg, Randall-Selitto, tail-flick, colonic distension, CFA, SNL models) 0.9% saline (10 mL/kg, formalin) or 1% carboxymethylcellulose (10 mL/kg, Kaolin, STZ models) administered orally to mice or rats 60 minutes before measures of hyperalgesia or allodynia commenced. For the microdialysis studies, bicifadine (20 mg/kg IP) was administered in 0.9% saline solution (5 mL/kg) to freely moving animals during the procedure. In the local administration studies, all test substances were dissolved in distilled water and injected subcutaneously in a volume of 50 µL. All solutions were prepared immediately prior to administration.

Gabapentin (GBP, 300 mg/kg, PO), morphine sulphate (MOR, 128 mg/kg PO) or the κ-opioid receptor agonist U-50,488 (3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide, 10 mg/kg PO) were all dissolved in distilled and administered in a volume of 5 mL/kg either 60 or 30 minutes prior to testing as reference agents. The D_2 antagonist (-)-sulpiride (15 mg/kg IP, 5 mL/kg distilled water) was administered 60 minutes before testing. Indomethacin was administered orally in 1% methylcellulose vehicle (10 mL/kg). Animals were acclimated to the test room for at least one hour before testing. Drug solutions were coded by a separate experimenter uninvolved in conducting the behavioral testing. The blind was not broken until the end of the study. Test substances and vehicles were administered in random order by a blinded investigator.

Bicifadine was synthesized by ChemSyn Laboratories (MT, USA). Gabapentin (# 006569) was obtained from Hawkins Pharmaceutical Group (MN, USA). Indomethacin was purchased from either Sigma Aldrich or Fluka. Acetaminophen (A7085), codeine (C5901), complete Freund's adjuvant (F5881), desipramine (D3900), ibuprofen (I4883), morphine (M8777), streptozotocin (S0130), (-)-sulpiride (S7771), and U 50,488 (D8040) were obtained from Sigma-Aldrich.

Data Analysis.

IC_{50} and ED_{50} values were determined by fitting sigmoidal curves to the data using non-linear regression techniques (GraphPad Software, CA, USA). K_i values were determined using the Cheng-Prusoff correction. Parametric analysis of quantitative data was performed where indicated using a 2-tailed, unpaired t-test, a 1-way ANOVA followed by Dunnett's test, or 2-way ANOVA followed by the Bonferroni comparison matrix, where appropriate. Non-parametric analysis of the Gait Scores obtained using the Kaolin model was conducted using the Kruskal-Wallis test followed by Dunn's post-hoc comparison test, or the Mann-Whitney U test.

RESULTS

Biochemical Pharmacology

The ability of bicifadine to inhibit radioligand binding to a variety of receptors, including those implicated in nociception, was investigated in a radioligand receptor binding screen (Table 1). Bicifadine inhibited [^{125}I]RTI-55 binding to sites on recombinant human 5-HT, NE and DA transporters with moderate (μM) but equivalent affinities (1:2:2, respectively, Table 1). Subsequent functional tests (Table 2) indicated that bicifadine potently inhibited the uptake of [^{3}H]NE and [^{3}H]5-HT by cell lines expressing recombinant human monoamine transporters (IC_{50} = 55, 117 nM, respectively), while its potency in blocking [^{3}H]DA uptake was approximately one order of magnitude lower (IC_{50} = 910 nM) (1:2:17, respectively).

Microdialysis studies supported the action of bicifadine as a biogenic amine transport inhibitor. Bicifadine (20 mg/kg IP) increased extrasynaptic 5-HT and NE concentrations in the prefrontal cortex (Figure 1A). While NE levels increased more rapidly (t = 20 min) than did 5-HT, both levels of neurotransmitters reached a similar maxima (1017-1058% of baseline) by 40 minutes after bicifadine administration. The area under the curve (AUC_{0-180}, mean ± SEM) for the percentage change in neurotransmitter level above baseline values in the prefrontal cortex was 77000 ± 17000 for 5-HT and 75000 ± 5400 for NE. These values were not significantly different from each other (P = 0.94, t-test). NE levels in the locus coeruleus increased rapidly after bicifadine

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administration, with a maximal elevation of 970 ± 350% by 20 minutes, and remained elevated for 120 minutes (Figure 1B). The $AUC_{0\text{-}180}$ for the percentage increase in NE levels above baseline in the locus coeruleus after 20 mg/kg bicifadine was 83000 ± 20000. In comparison, bicifadine caused relatively small but significant elevations of 5-HT and DA in the striatum, while decreasing levels of their metabolites (Figure 1C-F). Extracellular 5-HT levels slowly increased, reaching a maximum of 320 ± 25 % of baseline at 60 minutes, and remained significantly elevated for 140 minutes after administration (Figure 1C). The $AUC_{0\text{-}180}$ for the percentage increase in striatal serotonin levels was 15000 ± 2400. 5-HIAA levels dropped below baseline by 80 minutes after bicifadine administration and remained depressed for the duration of the study (Figure 1D). Striatal DA levels were maximally elevated by 270 ± 26 % at 40 minutes after bicifadine administration, and remained significantly elevated above baseline until 140 minutes (Figure 1E). The $AUC_{0\text{-}180}$ for the percentage increase in striatal DA levels was 18000 ± 2500. DOPAC and HVA levels were significantly decreased 40 minutes after bicifadine administration and remained below baseline levels for the duration of the study (Figure 1F).

In addition to its interactions with biogenic amine transporters, bicifadine exhibits moderate affinity for α_1, α_2, and β_1 adrenergic, 5-HT_{1A}, 5-HT_{1B} serotonergic, and σ_1 receptors, as indicated by K_i values ≤ 10 μM for these sites (Table 1). Bicifadine inhibits radioligand binding to α_1 receptors (K_i = 773 nM) and acts as an antagonist of these receptors (IC_{50} = 18.6 μM), as indicated by the blockade of phenylephrine-stimulated contractions of rat vas deferens (Table

2). [^{3}H]Rauwolscine binding to α_2 receptors was inhibited by bicifadine with a K_i = 1.01 µM, but it exhibited lower affinities for recombinantly expressed human α_2 receptor subtypes (IC_{50}: α_{2A} = 6.0 µM, α_{2B} = 4.2 µM; α_{2C} = 23.9 µM, [^{3}H]RX 821002 [0.7 nM], [^{3}H]RX 821002 [2.5 nM], [^{3}H]MK 912 [0.2 nM], respectively). Bicifadine acted as an α_2 receptor agonist, reducing the neurogenic twitch response of the rat vas deferens with an EC_{50} = 6 µM. Radioligand binding to β_1 adrenoceptors was inhibited by bicifadine (Table 1), which acted as a relatively low potency antagonist at this site (Table 2).

In functional assays, bicifadine acted as a full agonist at the 5-HT_{1A} receptor, stimulating [^{35}S]GTPγS binding (EC_{50} = 10 µM), and as an antagonist at the 5-HT_{1B} receptor (using a rat tail artery ring contraction assay) with an IC_{50} = 22 µM (Table 2). Bicifadine inhibited radioligand binding to the σ_1 receptor (K_i = 1.2 µM) and decreased field-stimulated twitch contractions of the guinea pig vas deferens, causing complete inhibition of contractions at 10 µM, an effect consistent with an antagonist profile at the σ_1 receptor. However, the enhancement of field-stimulated contraction amplitude caused by the σ receptor agonist (+)SKF-10047 (10-100 µM) was suppressed by 100 µM bicifadine to levels 50% below control. These results suggest that the mechanism responsible for bicifadine suppression of vas deferens contractions did not involve σ receptors. Bicifadine exhibited low affinity for the NMDA receptor and electrophysiological studies demonstrated that bicifadine did not potently affect NMDA receptor function, blocking NMDA-gated currents in hippocampal pyramidal neurons *in vitro* (IC_{50} = 217 ± 18 µM). Bicifadine expressed a marginal

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affinity for the H_1 histamine receptor (44% inhibition of radioligand binding at 10 μM, Table 1), but subsequent assays (histamine stimulated [^{35}S]GTPγS binding to recombinant human receptors) showed no consistent (e.g., agonist or antagonist) effect on H_1 receptor function at concentrations up to 30 μM. Finally, bicifadine weakly inhibited veratridine-stimulated, tetrodotoxin-sensitive Na^+ fluxes in SK-N-SH cells *in vitro* (IC_{50} = 95 μM). However, it had no detectable effect on the *in vitro* activity of either recombinant human COX_1 or COX_2 at concentrations up to 10 μM.

Additional studies indicated that bicifadine did not significantly inhibit (<50% inhibition at the highest concentration tested) radioligand binding to: A_1, A_{2A} adenosine; AMPA; $\beta_{2,3}$ adrenergic; angiotensin$_1$; benzodiazepine; bradykinin$_{1,2}$; calcitonin-gene related peptide; cannabinoid$_{1,2}$; cholecystokinin$_A$; corticotropin releasing factor$_1$; $D_{1,2S,3,4}$ dopamine; endothelin$_A$; epidermal growth factor; $GABA_A$; glucocorticoid; glutamate binding site on NMDA receptor; melanocortin$_4$; neurokinin$_1$; neuropeptide Y_1; α_7 nicotinic cholinergic; $M_{1,2,3,4}$ cholinergic; μ, κ, δ opioid; orphanin$_1$; DP, $EP_{2,4}$ prostanoid; P_2X purine; 5-HT_3 serotonin; vanilloid$_1$; and vasopressin$_{1A}$ receptors (Table 2). Bicifadine had no measurable affinity for the glutamate binding site on NMDA receptors and exhibited low potency (IC_{50} = 23 ± 7.9 μM) in displacing [^{3}H]MK-801 from its binding site in the NMDA receptor ionophore. There was no evidence that bicifadine had significant affinity for the $\alpha_2\delta$ Ca^{++}, N-type Ca^{++}, K^+_{ATP}; saxitoxin-sensitive K^+_V; and hERG K^+_V channels.

The two principal metabolites of bicifadine, the lactam (5-*p*-tolyl-3-aza-bicyclo[3.1.0]hexan-2-one) and the lactam-acid (4-(4-oxo-3-aza-bicyclo[3.1.0]hexan-1-yl)benzoic acid), were also screened. Neither of these showed any affinity (< 50% inhibition) for any of the receptors or transporters investigated above at concentrations up to 30 µM (data not shown).

Pharmacokinetics

The plasma levels of bicifadine were determined 1, 2, 4, 8 and 24 hours after oral administration of single doses of 6, 20 or 60 mg/kg to male rats (Figure 2). The t_{max} was approximately 1 hour for all the doses tested, and the C_{max} values were 483, 1517 and 3361 ng/mL (approximately 2.3, 7.2 and 16 µM) following the administration of 6, 20 and 60 mg/kg, respectively.

Bicifadine Activity in Models of Acute Pain

The antinociceptive activity of bicifadine was examined in models of acute inflammatory, visceral and nociceptive pain. In the yeast-inflamed hind paw model of acute inflammatory pain (Randall-Selitto test, Table 3), bicifadine increased the threshold for paw withdrawal (Figure 3A). Compared to reference opiate and non-steroidal anti-inflammatory (NSAIDs) analgesics, bicifadine was approximately 15 times more potent (on a mg/kg basis, 25 fold on a µmol/kg basis) as an oral analgesic than acetaminophen and 4 times more potent than codeine (3.3 fold on a µmol/kg basis, Table 3). Based on its potency in the Randall-Selitto test (ED_{50} = 9.2 mg/kg PO, Table 3), and an LD_{50} = 370 mg/kg,

the therapeutic index (LD_{50}/ED_{50}) for orally administered bicifadine in the rat is approximately 40. Bicifadine was approximately equipotent in increasing the withdrawal threshold in the Randall-Selitto test following either subcutaneous (ED_{50} = 10.1 mg/kg) or oral administration, with a potency comparable to that of subcutaneously administered pentazocine, d-propoxyphene and codeine (data not shown). The analgesic action of a maximally effective dose of bicifadine (50 mg/kg PO) in the Randall-Selitto test was observed by 1 hour after administration, but was no longer apparent by 4 hours after administration (Figure 3B). Bicifadine was also an effective analgesic when administered locally in the Randall-Selitto test (Figure 3C). Bicifadine (4, 8, 12 µg/paw) significantly increased the paw withdrawal latency when administered into the plantar surface of the inflamed paw. Paw withdrawal thresholds for both the inflamed and uninflamed paws were not altered when bicifadine was administered into the uninflamed paw (8 µg/paw, Figure 3C). While locally administered bicifadine had no effect on inflamed paw volume (1.518 ± 0.045, 1.534 ± 0.077 ml, Veh and bicifadine, respectively), orally administered bicifadine (40 mg/kg) significantly reduced inflamed paw volume 21% (1.37 ± 0.04, 1.08 ± 0.06 mL, Veh and bicifadine, respectively, $P<0.05$, t-test). The antinociceptive effects of morphine and ibuprofen were comparable to bicifadine when locally administered at doses of 50 and 80 µg/paw, respectively.

Bicifadine demonstrated antinociceptive activity in the kaolin-induced model of acute arthritis (Figure 4), significantly improving the gait scores of rats with kaolin-induced acute arthritis at doses of 12.5 and 25 mg/kg PO, manifested

between 4 (Figure 4D) and 5 (Figure 4E) hours after the intra-articular administration of kaolin. While the antinociceptive potency of bicifadine at 4 and 5 hours post-kaolin administration was similar to that observed in the Randall-Selitto test, the antinociceptive efficacy of bicifadine declined at a dose of 50 mg/kg.

Bicifadine was also examined in models of acute nociception. Orally administered bicifadine did not show consistent efficacy in the tail flick test at high stimulus intensity (tail withdrawal latency of 6.14± 0.24 seconds in vehicle-treated mice) (Figure 5). However, bicifadine dose-dependently increased tail flick latencies (ED_{50} = 38.8, 30.7-49.0 mg/kg PO, mean, 95% CI, nonlinear regression analysis, 185 µmol/kg) when a lower intensity radiant heat stimulus was used (tail-withdrawal latency of 15.3 ± 0.35 seconds in vehicle treated mice), or following subcutaneous administration (data not shown). Similarly, ibuprofen (150 mg/kg IP) was more effective in increasing tail flick latency under low intensity stimuli (49.7 ± 8.0 MPE %) than under high intensity (9.0 ± 3.5 MPE %). Morphine was equieffective at both stimulus intensities (ED_{50} = 2.69, 2.51-2.88 mg/kg SC, 4.0 µmol/kg, low intensity, 2.84, 2.73-2.98 mg/kg SC, 4.2 µmol/kg , high intensity). Bicifadine (SC or PO) was ineffective in mice placed on a 59°C hot plate. However, when bicifadine (25 mg/kg SC, 30 minutes post-administration) was administered to mice placed on a hot plate maintained at 55°C, it significantly increased the response time by 43% above vehicle levels. By comparison, morphine (10 mg/kg SC, 15 µmol/kg 30 minutes post-administration) increased the response latency by 88%.

Bicifadine Activity in Models of Persistent Pain

Bicifadine was an effective analgesic in two models of tonic visceral pain, the PPQ-induced abdominal contraction test (Table 3) and the colonic distension test (Figure 6). Bicifadine suppressed the abdominal contractions induced by PPQ (ED_{50} = 13, 6-29 mg/kg PO, 4.8 mg/kg SC), with a potency equivalent to codeine (on a mg/kg basis, 0.5 fold less on a μmol/kg basis) and greater than acetaminophen (2.8 fold [mg/kg], 3.9 fold [μmol/kg], Table 3). In the colonic distension model, the number of abdominal contractions observed in response to acetic acid infusion followed by colonic distension was significantly reduced by bicifadine, with a minimum effective oral dose (MED) of 5 mg/kg, (23 μmol/kg) and a maximal effect comparable to the reference antinociceptive agent used in this study, the κ-opioid agonist U 50,488 (10 mg/kg, 24.6 μmol/kg, PO, Figure 6).

The antinociceptive efficacy of bicifadine was also examined in the formalin model of persistent pain processes. Bicifadine had a significant antinociceptive effect in rats (10, 20, 30 mg/kg PO, Figure 7A) and mice (5, 40, 60 mg/kg PO, Figure 7C) during Phase 1 of the formalin test when compared to vehicle-treated animals, dose-dependently decreasing the paw licking time by 54%, 77%, and 82% for rats, respectively, and by 33%, 52%, and 60% for mice. Bicifadine was also effective in suppressing responses in Phase 2 of the formalin test in rats, reducing the time spent paw licking by 47%, 79%, and 81% at 10, 20, and 30 mg/kg, respectively (Figure 7B). The antinociceptive actions of bicifadine in formalin-treated mice were more pronounced in Phase 2 than Phase 1, with all doses of bicifadine (5-60 mg/kg) significantly reducing the time spent licking by

as much as 89% at 60 mg/kg (Figure 7D). The antinociceptive effects of bicifadine in the rat formalin test were greatest at 20 mg/kg, with a maximal efficacy in both phases of the formalin test comparable to that of morphine (80 mg/kg PO). The antinociceptive actions of bicifadine (40, 60 mg/kg PO) in Phase 2 of the formalin test in mice were not significantly different from morphine (Figure 7D).

As in animal models of acute inflammatory pain, bicifadine was found to be active in a model of persistent inflammation. One week after the subplantar administration of CFA, bicifadine (40, 60 mg/kg, 190, 290 µmol/kg, PO) normalized the nociceptive thresholds at 1 and 3 hours after administration (Figure 8A, B), an effect comparable to that of indomethacin (30 mg/kg, 84 µmol/kg, PO). This antinociceptive effect was absent by 24 hours after administration of either drug (Figure 8C).

Bicifadine Activity in Models of Chronic Pain

Bicifadine was effective in two models of chronic, neuropathic pain. The effects of bicifadine on mechanical and thermal hyperalgesia were examined 14 days after separate groups of rats received unilateral spinal nerve ligations (SNL). The withdrawal thresholds and latencies for paws ipsilateral to the SNL were significantly reduced compared to the contralateral paws (Figure 9). One hour after administration, bicifadine dose-dependently increased the withdrawal threshold of lesioned paws (ED_{50} = 12.1, 7.4-19.6 mg/kg), reaching a maximal efficacy of 200% increase in withdrawal threshold at 40 mg/kg (191 µmol/kg,

Figure 9A), an effect comparable to that of gabapentin (300 mg/kg, 1.4 mmol/kg) on lesioned paw withdrawal thresholds. Bicifadine significantly increased paw withdrawal latencies in the thermal hyperalgesia assessment of SNL rats (Figure 9B), albeit in a non-dose-dependent fashion following the administration of 12.5, 25 and 100 mg/kg PO bicifadine. The average paw withdrawal latency following administration of 100 mg/kg (470 µmol/kg) bicifadine was not significantly different from that observed following morphine (128 mg/kg, 190 µmol/kg PO) administration, or for unlesioned paws. Bicifadine did not alter the responses of the unlesioned paw (data not shown).

In addition to its anti-nociceptive effects in the SNL model, bicifadine exhibited anti-allodynic properties (Figure 9C). A significant increase (250%) in the response threshold of the mechanical allodynia assessment was only observed 1 hour after administration of 40 mg/kg bicifadine, relative to the vehicle-treated, lesioned paw controls. The magnitude of this anti-allodynic effect was comparable to that of gabapentin (300 mg/kg, PO) and was not significantly different from the responses in the unlesioned vehicle control group. However, the highest dose of bicifadine tested (60 mg/kg PO) did not have a significant anti-allodynic effect. The effect of the D_2 dopamine receptor antagonist (-)-sulpiride on the anti-nociceptive actions of bicifadine was investigated in the mechanical hyperalgesia assessment using SNL rats (Figure 9D). Although bicifadine (40 mg/kg PO) effectively raised the lesioned paw withdrawal threshold to levels comparable to unlesioned paws, (-)-sulpiride (15 mg/kg SC, a non-sedating dose) alone had no significant effect. Combining (-)-

sulpiride with bicifadine significantly attenuated the bicifadine-induced elevation of the withdrawal threshold by 38%. The anti-allodynic actions of bicifadine (40 mg/kg) on the Von Frey thresholds (8.6 ± 2.1 g) were not significantly altered by (-)-sulpiride (15 mg/kg, 6.6 ± 1.5 g).

The temporal profile of the anti-nociceptive actions of bicifadine differed in the mechanical and thermal hyperalgesia assessments (Figure 10). Bicifadine (25 mg/kg PO) was maximally effective in increasing the force required to induce lesioned paw withdrawal at 60 minutes, with a significant antinociceptive effect observed up to 120 minutes after administration (Figure 10A). In contrast, bicifadine (25 mg/kg, 120 μmol/kg PO) was effective in the thermal hyperalgesia assessment as early as 30 minutes after administration, with significant activity at 90 and 240 minutes (Figure 10B). Comparable results in both assessments were observed following the administration of morphine (128 mg/kg PO).

There was no evidence of a change in the anti-nociceptive actions of bicifadine in the mechanical hyperalgesia assay following sub-chronic administration of 50 mg/kg/D of bicifadine for 5 days (Figure 11). The magnitude of the lesioned paw withdrawal threshold following subchronic bicifadine administration was significantly greater than that for the vehicle-treated, lesioned paw group, and was not different from the antinociceptive effects of either acutely administered bicifadine (50 mg/kg, 230 μmol/kg), morphine (128 mg/kg, 190 μmol/kg PO), or gabapentin (300 mg/kg, 1.4 mmol/kg PO). However, following 5 consecutive days of oral administration, the antinociceptive effects of morphine

were no longer significantly different from vehicle-treated control, consistent with the initial stages of the development of tolerance.

The anti-nociceptive actions of bicifadine were also manifested in a second model of chronic neuropathic pain, the STZ-treated rat (Figure 12). Twenty-three days after STZ administration, the force necessary to induce paw withdrawal in diabetic rats decreased 37%. Sixty minutes after administration of bicifadine (12.5, 25 mg/kg PO) to STZ-treated rats, the force inducing paw withdrawal was significantly higher than in the vehicle-treated diabetic rats and was not significantly different from non-diabetic rat response levels.

Side-Effect Profile

The effect of bicifadine on motor performance in the open field, rotarod, and weight-corrected grip strength was investigated and compared to that of desipramine. Bicifadine (12.5-100 mg/kg PO) had no significant effect on the distance traveled in the open field (Figure 13 A) at any time point monitored (3-21 minutes). The largest change observed was a non-significant decrement in distance traveled over the 21 minute monitoring period after the administration of 100 mg/kg (470 µmol/kg) bicifadine. In contrast, the distance traveled was significantly lower than vehicle travel distances between 3 and 18 minutes after administration of desipramine (25 and 50 mg/kg, 83 and 165 1.4 mmol/kg IP, respectively) (Figure 13B), with overall reductions of 73% and 90% in the overall distance traveled relative to vehicle control. The effects of bicifadine and desipramine on rotarod performance were not significant (Figure 13C, D),

although there was a trend towards reducing the amount of time spent on the rotarod following the administration of desipramine (25, 50 mg/kg). Desipramine (50 mg/kg) caused a small (18%) but significant ($P<0.05$) reduction in the weight-corrected grip strength (Figure 13F).

DISCUSSION

Bicifadine is a functional inhibitor of biogenic amine transporters, inhibiting radioligand binding to the DAT, NET and SERT with moderate affinity and suppressing [^{3}H]NE and [^{3}H]5-HT uptake with approximately an order of magnitude greater potency than [^{3}H]DA uptake. Such discrepancies between radioligand binding and neurotransmitter uptake assays are not unusual (Owens et al., 1997, Eshleman et al., 1999), and may reflect differences in the nature of the assays (an equilibrium binding assay versus a non-equilibrium functional test), substrate affinity (high affinity binding site probes versus lower affinity neurotransmitters), chemical structure of the radioligand, and the lack of contiguity between binding sites. Nonetheless, the ability of bicifadine to inhibit DA, NE and 5-HT uptake is supported by *in vivo* microdialysis studies, where bicifadine significantly increased extrasynaptic levels of these neurotransmitters in a number of brain regions. While the kinetics of the neurotransmitter changes differed slightly from region to region, the overall increases in neurotransmitter levels (as indicated by the $AUC_{0\text{-}180}$) were not significantly different in the prefrontal cortex (NE and 5-HT). This "balanced" inhibition of NET and SERT may yield an antinociceptive synergy resulting form the simultaneous activation of noradrenergic and serotonergic systems (Danzebrink and Gebhart, 1991, Iyengar et al., 2004, Leventhal et al., 2007), suggesting that dual uptake inhibitors may have an enhanced antinociceptive profile relative to uptake inhibitors more selective for the SERT or NET.

However, additional aspects of the molecular pharmacology of bicfadine may contribute to a novel antinociceptive profile. In addition to its actions as a functional DAT inhibitor, bicifadine is an α_2 adrenoceptor agonist at pharmacologically relevant concentrations (K_i = 1 μM; EC_{50}, neurogenic twitch = 6 μM). In addition, bicifadine is a full agonist at the 5-HT_{1A} receptor. These actions, combined with the blockade of biogenic amine neurotransmitter uptake may broaden the antinociceptive profile of bicifadine, or enhance its efficacy, particularly at doses where α_2 adrenoceptors and 5-HT_{1A} receptors are activated (Stone et al., 1997, Boyd, 2001).

Given a molecular pharmacology profile consistent with antinociceptive activity, bicifadine was tested *in vivo* in models of acute, persistent and chronic nociception. Bicifadine is readily absorbed following oral administration, producing maximal concentrations of 16 μM at 60 mg/kg, with an elimination half-life of approximately 3.5 hours. Bicifadine was found to be potent and fully efficacious in two models of acute (yeast-inflamed hindpaw and intra-articular kaolin) and persistent (CFA) inflammatory pain. While the efficacy of bicifadine was lost at higher doses (50 mg/kg) in the kaolin model, this is not likely to reflect a disruption of motor function that impacts gait (see below), but may reflect an inhibition of NE release by activation of presynaptic α_2 adrenoceptors. In addition to its systemic activity, bicifadine was active following local administration in the hindpaw inflammation model, consistent with its actions as an indirect β-adrenoceptor agonist inhibiting the secretion of pro-inflammatory agents by activated immunocytes (Elenkov et al., 2000). Nonetheless, bicifadine is not a

classic anti-inflammatory, as indicated by its lack of effect on COX-1 and COX-2 activity. Contrasting with its efficacy in models of acute inflammatory pain, bicifadine was less effective in the tail flick and hotplate models of acute nociception, and then only after reducing the stimulus intensity or administering bicifadine subcutaneously. These assays are particularly sensitive to opiates, and the relative lack of antinociceptive activity of bicifadine in these models is shared with a number of other analgesic classes, including other NET/SERT blockers, gabapentin, and NSAIDs (Le Bars et al., 2001).

The central actions of bicifadine are also important in its antinociceptive profile, as indicated by its high potency and efficacy in three models of persistent pain (PPQ, colonic distension and formalin tests). Activating central adrenergic pathways increase the threshold for the visceromotor response in visceral pain models (Danzebrink and Gebhart, 1990), an effect synergistically enhanced by stimulation of serotonergic systems (Danzebrink and Gebhart, 1991). Thus, the inhibition of NET and SERT and the direct activation of 5-HT_{1A} receptors may contribute to the efficacy and potency of bicifadine in these models (Rouzade et al., 1998). However, it is the antinociceptive profile of bicifadine in the formalin test that appears unique for a biogenic amine uptake inhibitor. Thus, bicifadine potently reduced both early and late phase paw licking after formalin injection. This effect on both phases of the response to formalin contrasts with other uptake blockers (e.g., duloxetine, amitriptyline) (Iyengar et al., 2004, Bomholt et al., 2005), NSAIDs (Hunskaar and Hole, 1987) and gabapentin (Field et al., 1997). The ability of bicifadine to completely suppress paw licking in Phase 1 is

consistent with its ability to inhibit acute pain by mechanisms independent of 5-HT or NE uptake blockade. Although opiates are effective analgesics in both phases of the formalin test, the mechanism of action of bicifadine does not involve direct activation of opioid pathways as indicated by its lack of affinity for opioid receptor subtypes and the inability of naloxone to inhibit its antinociceptive actions (data not shown). Alternatively, bicifadine may suppress Phase 1 licking by direct activation of serotonergic and adrenergic pathways (Buritova et al., 2005, Kanui et al, 1993).

Antinociceptive efficacy in the second phase of the formalin test is predictive of antihyperalgesic activity in neuropathic pain models (Le Bars et al., 2001, Fishbain et al., 2000). Bicifadine effectively suppressed mechanical hyperalgesia in both the SNL and STZ-induced models of neuropathic pain at doses comparable to those effective in treating acute and persistent pain syndromes. In these models, bicifadine exhibited greater oral potency and equivalent antinociceptive efficacy to morphine and gabapentin. Unlike morphine, the antinociceptive activity of bicifadine in the mechanical hyperalgesia assessment did not significantly decline following subchronic administration. While suppressing mechanical allodynia in the SNL model, further examination of the actions of bicifadine indicated that it was effective in suppressing thermal hyperalgesia, despite the insensitivity of this endpoint in nerve-ligation models of neuropathic pain (Wang and Wang, 2003, Dowdall et al., 2005). In the SNL model, the antinociceptive profile of bicifadine resembles that of dual uptake blockers. However, what appears to be unique is that a non-sedating dose of (-)-

sulpiride significantly inhibited the effects of bicifadine on mechanical hyperalgesia in SNL rats without altering its actions on mechanical allodynia. Although consistent with the involvement of bicifadine-induced elevations in striatal DA in its antinociceptive actions, these observations contrast with previous reports of the involvement of dopaminergic mechanisms in mechanical allodynia in this model (Pedersen et al., 2005). Investigating the effect of D_2 antagonists on the anti-allodynic effects of bicifadine using a full dose-response curve may be a more sensitive way to establish the role of dopaminergic pathways in bicifadine-induced antinociception.

Bicifadine shares many antinociceptive attributes with other uptake inhibitors, but it does not share their side-effect profile. Desipramine significantly reduced motor activity in the open field, reduced grip strength and showed a trend towards inducing ataxia, all at antinociceptive doses (Sawynok and Reid, 2001, Bomholt et al., 2005). In contrast, bicifadine had no significant effects on motor activity. Although striatal DA levels are increased by antinociceptive doses of bicifadine, there is no evidence of bicifadine-induced hyperlocomotion or stereotypy, even at doses 10 fold greater than its antinociceptive ED_{50} in the Randall-Selitto test. The α_1 antagonist properties of bicifadine may serve to suppress any motor hyperactivity or stereotypy resulting from dopaminergic activation given the role of α_1 adrenoceptors in mediating these activities (Drouin et al., 2002). Conversely, the lack of sedation, myorelaxation, or ataxia associated with bicifadine administration may result from its low affinity for muscarinic cholinergic and H_1 histamine receptors. In addition to these

preclinical observations, clinical studies administering analgesic doses of bicifadine (600 mg PO) report no significant changes in basic cardiovascular function relative to placebo (4 mm Hg increase in systolic blood pressure, 3 mm Hg increase in diastolic blood pressure), and observed no effect on pulse, respiration rate, or body temperature (Stern et al., 2006). Finally, while many psychostimulants with abuse liability (e.g., amphetamine, cocaine) inhibit dopamine uptake, bicifadine does not induce dependency in rodents or primates after as much as 48 days of administration, nor does it substitute for cocaine in discrimination studies with cocaine-trained rats at doses that do not impair bar-pressing performance (R. Balster, personal communication).

Dual NET/SERT inhibitors have demonstrated clinical utility analgesics, but recent data suggests that the inhibition of DAT (and NET) may have a further salutary effect on the treatment of peripheral neuropathic pain, as evidenced by the NNT score (Number of patients needed to treat to obtain a positive response [50% reduction in pain intensity]). Analgesic NNT values for an SSRI are as low as 6.8, rising with a SERT/NET inhibitor (venlafaxine) to 5.5, TCAs = 2.2, reaching 1.6 for the DAT inhibitor bupropion (Sindrup et al., 2005). The results of this trend suggest that the transporter inhibition profile of bicifadine, a functional inhibitor of DAT, NET and SERT with antinociceptive activity, may provide greater clinical efficacy in treating neuropathic pain states than currently available SNRIs and TCAs, but without the side effect profile associated with TCAs, a hypothesis that awaits further study in the clinic.

REFERENCES

Bomholt SF, Mikkelsen JD and Blackburn-Munro G (2005) Antinociceptive effects of the antidepressants amitriptyline, duloxetine, mirtazapine and citalopram in animal models of acute, persistent and neuropathic pain. *Neuropharmacology* **48**:252-263

Boyd RE (2001) Alpha$_2$-adrenergic receptor agonists as analgesics. *Curr Top Med Chem* **1**:193-197.

Buritova J, Larrue S, Aliaga M, Besson J-M and Colpaert F (2004) Effects of the high-efficacy 5-HT$_{1A}$ receptor agonist, F-13540 in the formalin pain model: A c-Fos study. *Eur J Pharmacol* **514**:121-130.

Chaplan SR, Bach FW, Pogrel JW, Chung JM and Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* **53:**55-63.

Chudler EH and Dong WK (1995) The role of the basal ganglia in nociception and pain. *Pain* **60:**3-38.

Courteix C, Eschalier A, and Lavarenne J (1993) Streptozotocin-induced diabetic rats: behavioral evidence for a model of chronic pain. *Pain* **53:**81-88.

Danzebrink RM and Gebhart GF (1991) Intrathecal coadministration of clonidine with serotonin receptor agonists produces supra-additive visceral antinociception in the rat*. Brain Res* **555:**35-42.

Drake DF, Harkins S and Qutubuddin A (2005) Pain in Parkinson's disease: pathology to treatment, medication to deep brain stimulation. *NeuroRehabilitation* **20**:335-341.

Drouin C, Darracq L, Trovero F, Blanc G, Glowinski J, Cotecchia S and Tassin J-P (2002) α1b-Adrenergic receptors control locomotor and rewarding effects of psychostimulants and opiates. *J Neurosci* **22:**2873-2884.

Elenkov IJ, Wilder RL, Chrousos GP and Vizi ES (2000) The sympathetic nerve-an integrative interface between two supersystems: the brain and the immune system. *Pharmacol Rev* **52**: 595-638.

Ertas M, Sagduyu A, Arac N, Uludag B and Ertekin C (1998) Use of levodopa to relieve pain from painful symmetrical diabetic polyneuropathy. *Pain* **75**:257-259

Eshleman AJ, Carmolli M, Cumbay M, Martens CR, Neve KA and Janowsky A (1999) Characteristics of drug interactions with recombinant biogenic amine transporters expressed in the same cell type. *J Pharmacol Exp Ther* **289**:877-885.

Field MJ, Oles RJ, Lewis AS, McCleary S, Hughes J and Singh L (1997) Gabapentin (neurontin) and S-(+)-3-isobutylgaba represent a novel class of selective antihyperalgesic agents. *Br J Pharmacol* **121**:1513-1522.

Fishbain DA, Cutler R, Rosomoff HL and Rosomoff RS (2000) Evidence-based data from animal and human experimental studies on pain relief with antidepressants: a structured review. *Pain Medicine* **4:**310-316.

Hagelberg N, Jaaskelainen SK, Martikainen IK, Mansikka H, Forssell H, Scheinin H, Hietala J, Pertovaara A (2004) Striatal dopamine D2 receptors in modulation of pain in humans: A review. *Eur J Pharmacol* **500**:187-192.

Hertz F, Ranson M and Lwoff JM (1980) Pharmacological properties of a new anti-inflammatory agent: 2-(2-isopropyl-5-indanyl)propionic acid (UP 517-03). *Arzneimittelforsch* **30**:1549-1557.

Hunskaar S and Hole K (1987) The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* **30**:103-114.

Iyengar S, Webster AA, Hemrick-Luecke SK, Xu JY and Simmons RMA (2004) Efficacy of duloxetine, a potent and balanced serotonin-norepinephrine reuptake inhibitor in persistent pain models in rats. *J Pharmacol Exp Ther* **311**:576-584.

Kanui TI, Tjolsen A, Lund A, Mjellem-Joly N and Hole K (1993) Antinociceptive effects of intrathecal administration of alpha-adrenoceptor antagonists and clonidine in the formalin test in the mouse. *Neuropharmacology* **32**:367-371.

Kim SH and Chung JM (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* **50:**355–363.

Lang E, Hord AH, and Denson D. (1996) Venlafaxine hydrochloride (Effexor) relieves thermal hyperalgesia in rats with an experimental mononeuropathy. *Pain* **68:**151-155.

Le Bars D, Gozariu M and Cadden SW (2001) Animal models of nociception. *Pharmacol Rev* **53**:597-652.

Leventhal L, Smith V, Hornby G, Andree TH, Brandt MR and Rogers KE (2007) Differential and synergistic effects of selective norepinephrine and serotonin reuptake inhibitors in rodent models of pain. *J Pharmacol Exp Ther*. **320**:1178-1185.

Lin MT, Wu JJ, Chandra A and Tsay BL (1981) Activation of striatal dopamine receptors induces pain inhibition in rats. *J Neural Trans* **51:** 213-222.

Magnusson JE and Fisher K (2000) The involvement of dopamine in nociception: The role of D1 and D2 receptors in the dorsolateral striatum. *Brain Res* **855:** 260-266.

Millan MJ (2002) Descending control of pain. *Prog Neurobiol* **66**:355-474.

Nahum-Levy R, Tam E, Shavit S and Benveniste M (2002) Glutamate but not glycine agonist affinity for NMDA receptors is influenced by small cations. *J Neurosci* **22**: 2550-2560.

Owens MJ, Morgan WN, Plott SJ and Nemeroff CB (1997) Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. *J Pharmacol Exp Ther* **283**: 1305-1322.

Pedersen LH, Nielsen AN and Blackburn-Munro G (2005) Anti-nociception is selectively enhanced by parallel inhibition of multiple subtypes of monoamine transporters in rat models of persistent and neuropathic pain. *Psychopharmacol (Berlin)* **182:** 551-561.

Popik P, Kostakis E, Krawczyk M, Nowak G, Szewczyk B, Krieter P, Chen Z, Russek SJ, Gibbs TT, Farb DH, Skolnick P, Lippa AS and Basile AS (2006) The anxioselective agent DOV 51892 is more efficacious than diazepam at enhancing GABA-gated currents at α_1 subunit containing $GABA_A$ receptors. *J Pharmacol Exp Ther* **319**:1244-1252.

Rouzade, M-L, Fioramonti J and Bueno L (1998) Decrease in gastric sensitivity to distension by 5-HT_{1A} receptor agonists in rats. *Dig Dis Sci* **43**:2048-2054.

Sawynok J and Reid A (2001) Antinociception by tricyclic antidepressants in the rat formalin test: differential effects on different behaviors following systemic and spinal administration. *Pain* **93**: 51-59.

Semenchuk MR and Davis B (2000) Efficacy of sustained-release bupropion in neuropathic pain: an open-label study. *Clin J Pain* **16:**6-11.

Sindrup SH, Otto M, Finnerup NB and Jensen TS (2005) Antidepressants in the treatment of neuropathic pain. *Basic Clin Pharmacol Toxicol* **96:**399-409.

Skagerberg G, Bjorklund A, Lindvall O and Schmidt RH (1982) Origin and termination of the diencephalo-spinal dopamine system in the rat. *Brain Res Bull* **9:** 237-244.

Stein C, Millan MJ and Herz A (1988) Unilateral inflammation of the hindpaw in rats as a model of prolonged noxious stimulation: alterations in behavior and nociceptive thresholds. *Pharmacol Biochem Behav.* **31**:445-451.

Stern W, Pontecorvo M, Czobor P, Wang Q, Waldron D and Apfel S (2006) A multi-center, randomized, active comparator-controlled study to evaluate the long-term safety and efficacy of bicifadine for the treatment of chronic low back pain. *J Pain* **7**(S2):752

Stone LS, MacMillan LB, Kitto KF, Limbird LE and Wilcox GL (1997) The α_{2a} adrenergic receptor subtype mediates spinal analgesia evoked by α_2 agonists and is necessary for spinal adrenergic-opioid synergy. *J Neurosci* **17**:7157-7165.

Tamae A, Nakatsuka T, Koga K, Kato G, Furue H, Katafuchi T and Yoshimura M (2005) Direct inhibition of substantia gelatinosa neurons in the rat spinal cord by activation of dopamine D2-like receptors. *J Physiol* **568:** 243-253.

Westlund KN (1992) Anatomy of noradrenergic pathways modulating pain, in: *Towards the use of noradrenergic agonists for the treatment of pain* (Besson JM and Guilbaud G eds) pp 91-118, Elsevier, Amsterdam.

LEGENDS FOR FIGURES

Figure 1. Bicifadine-induced changes in the extracellular levels of biogenic amine neurotransmitters and their metabolites as determined using microdialysis in normal rats. Serotonin (5-HT, $F_{(11,1,11)}$ = 83 treatment; 7.7 time; 7.5 interaction, all $P<0.01$) and norepinephrine (NE, $F_{(11,1,11)}$ = 299 treatment; 25.4 time; 24.8 interaction, all $P<0.01$) levels were measured in the prefrontal cortex (PFCtx, [A]) 40 minutes before and up to 180 minutes after administration of bicifadine to freely moving rats. NE levels were measured in the locus coeruleus (LC, [B], $F_{(8,1,8)}$ = 28.8 treatment; 2.6 time; 2.5 interaction, all $P<0.01$) following bicifadine administration. In the striatum (Str), levels of 5-HT ([C], $F_{(11,1,11)}$ = 95.9 treatment; 8.5 time; 9.1 interaction, all $P<0.01$), dopamine (DA, [E], $F_{(11,1,11)}$ = 127 treatment; 7.8 time; 8.3 interaction, all $P<0.01$) and their metabolites 5-HIAA ([D], $F_{(11,1,11)}$ = 54.1 treatment; 4.3 time; 4.5 interaction, all $P<0.01$), DOPAC ([F], $F_{(11,1,11)}$ = 285 treatment; 6.2 time; 12.6 interaction, all $P<0.01$) and HVA ([F], $F_{(11,1,11)}$ = 87.5 treatment; 5.7 time; 5.4 interaction, all $P<0.01$) were measured following bicifadine administration. The dose of bicifadine administered was 20 mg/kg IP in all cases. Each sample of microdialysate was accumulated over a 20 minute period, with each point representing the mean percentage change (± SEM) from baseline levels for bicifadine (solid symbols, n = 4 [B] or 8 [A, C-F]) and vehicle-treated (open symbols, n = 4, all panels) rats. Absolute baseline levels (in pg/10 µl) were: 2.8 ± 0.2 (5-HT, PFCtx, [A]), and 3.4 ± 0.2 (NE, PFCtx [A]); 0.6± 0.15 (NE, LC [B]); 2.9 ± 0.1 (5-HT, Str, [C]); 615± 29 (5-HIAA, Str [D]); 10.2 ± 0.8 (DA, Str, [E]); 2418 ± 159 (DOPAC, Str, [F]); and 1829 ± 120 (HVA, Str, [F]). *:

Individual points, or point ranges falling within the brackets were significantly different from the mean baseline level, $P<0.05$, 2-way ANOVA followed by Bonferroni's post-hoc comparison matrix.

Figure 2. Plasma concentrations of bicifadine in male rats. Bicifadine was administered as a single oral dose of 6 (■), 20 (●), or 60 (▲) mg/kg, and blood drawn at the indicated times. Plasma levels of bicifadine were determined using a validated LC-MS/MS method. Data represent the mean ± SD of observations from 3 animals.

Figure 3. The antinociceptive effects of bicifadine in a model of acute inflammatory pain (Randall-Selitto test). (A): Dose-response curve for bicifadine in the Randall-Selitto test. Rats received a subplantar injection of brewer's yeast suspension, followed one hour later by orally administered test substances or saline vehicle. Testing began one hour after administration of the test substances. Bicifadine (■) dose-dependently increased the threshold pressure for paw withdrawal with an ED_{50} = 11.3, (3.38, 37.7) mg/kg (mean, 95% CI), with 20 mg/kg bicifadine increasing the withdrawal threshold to 206 ± 22 g. In comparison, indomethacin (□, 20 mg/kg PO) increased the nociceptive threshold to 142 ± 20.8 g. Response to vehicle (dashed line across bottom of graph) was 91 ± 13.5 g. Each point represents the mean ± SEM of data from n = 10 animals. The sigmoidal curve was fitted to bicifadine data, and the ED_{50} determined using non-linear regression. (B): Duration of action of bicifadine. Bicifadine was

administered orally at t = 0, at a dose of 50 mg/kg. Data represents the mean (n = 8) pressure threshold for withdrawal of inflamed paw 2 hours after subplantar injection of yeast suspension. (C): Bicifadine exhibits antinociceptive activity following local administration. Sixty minutes after injecting the yeast suspension into the plantar surface of the hind paw, the test substances (bicifadine, ibuprofen [Ib], morphine [Mor], 50 µL) were injected into the dorsal surface of the inflamed paw, and withdrawal thresholds measured in the inflamed paws 60 minutes later. In one cohort (dotted bars), bicifadine (8 µg) was injected into the normal paw, and withdrawal thresholds were measured in the bicifadine-treated inflamed (BI) and normal (BN) paws 60 minutes later. Bicifadine (1, 4, 8, 12 µg/paw, solid bars) reduced the paw withdrawal threshold when injected into the inflamed paw, as did ibuprofen (80 µg/paw, cross-hatched bar) and morphine (50 µg/paw, hatched bar). Dashed line: mean antinociceptive activity achieved following administration of 20 mg/kg bicifadine, PO. Data represent mean ± SEM of 10-20 observations. **: Significantly different from vehicle-treated, inflamed paw, $P<0.01$, respectively, 1-way ANOVA followed by Dunnett's post-hoc comparison test, ($F_{(4,55)} = 7.50$, $P<0.01$; $F_{(2,37)} = 17.9$, $P<0.01$). ##: Significantly different from vehicle-treated, inflamed paw, (Ibuprofen $t_{28} = 5.29$; morphine $t_{28} = 5.39$; all $P<0.01$). aa: Significantly different from vehicle-treated, uninflamed paw, ($t_{38} = 7.58$, $P<0.01$). Veh: Vehicle injected normal paw. Veh Infl: vehicle injected inflamed paw.

Figure 4. Bicifadine is an effective antinociceptive agent in the kaolin-induced arthritis model. Arthritis was induced by the intra-articular injection of kaolin suspension. One hour after kaolin administration, the test substances or saline vehicle were orally administered. Gait scores were recorded hourly, starting one hour after kaolin administration (A) and hourly for up to five hours (E). Bicifadine (12.5, 25 mg/kg, closed bars) and indomethacin (3 mg/kg PO, hatched bars) significantly improved the gait scores of kaolin-treated rats only at 4 (D) and 5 (E) hours after administration. **: Significantly different from vehicle-treated control animals, $P<0.01$, respectively, Kruskal-Wallis test followed by Dunn's post-hoc comparison matrix. ##: Significantly different from vehicle-treated control animals, $P<0.01$, Mann-Whitney U test.

Figure 5. Bicifadine activity in the tail-flick test. The antinociceptive actions of orally administered bicifadine (25, 50, 75, 100 mg/kg) were investigated in the tail flick test at high and low stimulus intensities in albino Swiss mice. The heat source was adjusted for testing subjects under high intensity (12 second cut-off latency, open symbols) or low intensity (25 second cut-off latency, closed symbols) stimuli. Vehicle-treated mice responded to the high and low intensity stimuli with an average latency of 6.3 ± 0.18 and 16.8 ± 0.41 seconds, respectively. The tailflick latencies at low intensity of all doses of bicfadine-treated animals (squares) are significantly different from vehicle-treated control, $P< 0.01$, 1-way ANOVA and Dunnett's post-hoc comparison test (Bicifadine, $F_{(4,44)} = 13.48$, $P<0.01$). Responses of the ibuprofen (150 mg/kg IP)-treated

animals (circles) were significantly different from vehicle-treated controls under low intensity stimuli, $P<0.01$, ($t_{17} = 5.286$).

Figure 6. Bicifadine reduces the number of abdominal contractions in the rat colonic distension model. Anesthetized rats received colonic irrigation with an irritant (acetic acid) solution, followed by oral administration of bicifadine. Sixty minutes later, a balloon catheter was inserted into the colon, inflated, and the number of abdominal contractions counted. Bicifadine (solid bars) significantly reduced the number of abdominal contractions at all doses tested, with a maximal efficacy comparable to that of the reference agent, U 50,488 (10 mg/kg PO, hatched bar). **: Significantly different from vehicle-treated control animals, $P< 0.01$, 1-way ANOVA and Dunnett's post-hoc comparison test ($F_{(4,45)} = 15.6$, $P<0.01$). ##: Significantly different from vehicle-treated control animals, unpaired 2-tailed t-test, $t_{18} = 8.17$, $P< 0.01$,

Figure 7. Bicifadine is an effective antinociceptive agent in both phases of the formalin test in rats and mice. One hour after administration of bicifadine, rats and mice received a mid-plantar injection of 5% formalin solution. For rats, hind paw licking time was recorded between 0 to 10 minutes (Phase 1, panel A) and between 15 and 40 minutes (Phase 2, panel B) after formalin injection, while for mice, the licking time was recorded over 0-5 (Phase 1, panel C) and 20 to 30 (Phase 2, panel D) minutes. Bicifadine (solid bars, 10, 20, 30 mg/kg PO, rats, or 5, 10, 20, 40, 60 mg/kg PO, mice) dose-dependently decreased the amount of

time spent licking in both phases of the test, and in both species of rodent, with maximal efficacies comparable to or greater than that of the reference agent, morphine (Mor, hatched bars, 80 mg/kg, PO, rats, 60 mg/kg, PO, mice). *, **: Significantly different from vehicle-treated control, P<0.05, 0.01, respectively, 1-way ANOVA and Dunnett's post-hoc comparison test (A: $F_{(6,63)}$ = 17.0, P<0.01; B: $F_{(6,63)}$ = 19.1, P<0.01; C: $F_{(5,54)}$ = 6.0, P<0.01; D: $F_{(5,54)}$ = 10.7, P<0.01). ##: Significantly different from vehicle-treated control, P<0.01, 2-tailed, unpaired t-test (A: $t_{(18)}$ = 3.0; B: $t_{(18)}$ = 3.8; C: $t_{(18)}$ = 13.1; D: $t_{(18)}$ = 6.2).

Figure 8. Duration of the antinociceptive actions of bicifadine in the CFA model of persistent inflammatory pain. Animals received an intraplantar injection of CFA (1 mg/ml) under anesthesia 1 week prior to administration of test substances (bicifadine: 5, 10, 20, 40, 60 mg/kg PO, solid bars; indomethacin: 30 mg/kg PO, hatched bars). Mechanical hyperalgesia (paw withdrawal latency) was assessed at 1 (A), 3 (B) and 24 (C) hours after administration of the test substances. Both bicifadine (20, 40 mg/kg) and indomethacin (Indo) significantly elevated the nociceptive threshold of the lesioned paw at 1 and 3 hours after administration. *,**: Significantly different from vehicle-treated control, P<0.05, 0.01, 1-way ANOVA and Dunnett's post-hoc comparison test (A: $F_{(5,54)}$ = 5.9, P<0.01; B: $F_{(5,54)}$ = 4.48, P<0.01). #, ##: Significantly different from vehicle-treated control, P<0.05, 0.01, 2-tailed, unpaired t-test (A: $t_{(18)}$ = 2.8; B: $t_{(18)}$ = 2.1).

Figure 9. The antinociceptive effects of bicifadine in the SNL rat model of neuropathic pain: mechanical (A) and thermal (B) hyperalgesia and mechanical allodynia (C). Bicifadine (20, 40, and 60 mg/kg PO, closed bars) and gabapentin (Gbp, 300 mg/kg PO, hatched bar) significantly increased the withdrawal threshold of lesioned paws above those observed in vehicle-treated animals (Veh, open bar) in the mechanical hyperalgesia assessment (A). In a separate group of animals, bicifadine (12.5, 25, 100 mg/kg PO) and morphine (Mor, 128 mg/kg PO, hatched bar) significantly increased the withdrawal latency for lesioned paws above those for vehicle-treated paws (Veh, open bar) (B). Bicifadine (40 mg/kg PO) and gabapentin (300 mg/kg PO) also increased the response threshold to Von Frey hairs in a test of mechanical allodynia (C). Evidence for the involvement of dopamine in the suppression of mechanical hyperalgesia by bicifadine is indicated in panel D. The antihyperalgesic action of bicifadine (40 mg/kg, closed bar) was inhibited by pretreatment with the D_2 antagonist (-)-sulpiride (15 mg/kg, cross-hatched bar), a dose that did not alter responsiveness in this assay (grey bar). Values represent the mean ± SEM of results from 9-10 rats per group. *,**: Responses are significantly different from those for lesioned paws from vehicle-treated rats, $P<0.05$, 0.01, respectively, 1-way ANOVA followed by Dunnet's post-hoc comparison test. (Panel A: $F_{(5,51)} = 6.9$, $P<0.01$; Panel B: $F_{(4,45)} = 4.7$, $P<0.01$; Panel C: $F_{(5,53)} = 6.3$, $P<0.01$). **: Responses are significantly different from those for lesioned paws from vehicle-treated rats, $P< 0.01$, and from (-)-sulpiride and Bic/Sulpiride groups, $P<0.01$, respectively, 1-way ANOVA followed by Tukey's post-hoc comparison matrix;

(Panel D: $F_{(3,32)}$ = 18.0, P<0.01). #, ##: Significantly different from vehicle-treated control, P<0.05, 0.01, respectively, 2-tailed, unpaired t-test, (Panel A: $t_{(18)}$ = 2.9; Panel B: $t_{(18)}$ = 4.1; Panel C: $t_{(18)}$ = 3.1; Panel D: $t_{(15)}$ = 2.4).

Figure 10. The duration of action of bicifadine in assessments of mechanical (A) and thermal (B) hyperalgesia in the SNL rat model of neuropathic pain. The force inducing lesioned paw-withdrawal was significantly increased above vehicle-treated control levels at 60 and 120 minutes following administration of bicifadine (25 mg/kg PO, A). The latency to withdraw the lesioned paw was significantly increased in rats 30, 90 and 240 minutes after administration of bicifadine (25 mg/kg PO, B). In contrast, morphine (128 mg/kg, PO) significantly increased the force inducing paw withdrawal at 60 minutes, and the latency to paw withdrawal at 15, 60, 120 and 240 minutes after administration. Values represent the mean ± SEM of results from 8 rats per group. *, **: Significantly different from the contemporaneous, vehicle-treated lesioned paw response, P<0.05, 0.01, 2-way ANOVA followed by Bonferroni's post-hoc comparison matrix. A: Bicifadine, $F_{(6,1,6)}$ = 18.7 treatment, P<0.01; Morphine, $F_{(3,1,3)}$ = 36 treatment, 11.7 time, 11.1 interaction, all P<0.01; B: Bicifadine, $F_{(1,6,6)}$ = 47.2 treatment, 2.1 time, 2.7 interaction, all P<0.01; Morphine, $F_{(1,3,3)}$ = 68.9 treatment, P<0.01).

Figure 11. The antinociceptive properties of bicifadine are maintained following subchronic administration. The force inducing lesioned paw withdrawal in SNL

rats (mechanical hyperalgesia assessment) was significantly increased above vehicle control levels following either a single dose of bicifadine (Bic, 50 mg/kg PO, dotted bar), or after subchronic administration (50 mg/kg/D, 5 D, PO, black bar). Acute, but not subchronic morphine (Mor, 128 mg/kg, PO, hatched bar) and acute gabapentin (Gbp, 300 mg/kg PO, vertical stripes) also significantly increased the paw withdrawal force above vehicle control. Values represent the mean ± SEM of results from 10 rats per group. *, **: Significantly different from vehicle-treated lesioned paw response, P<0.05, 0.01,1-way ANOVA followed by Dunnet's test, $F_{(4,44)} = 6.9$, P<0.01. ##: Significantly different from vehicle-treated control, P< 0.01, 2-tailed, unpaired t-test, $t_{(18)} = 5.0$.

Figure 12. The antinociceptive actions of bicifadine in the STZ-treated rat model of diabetic neuropathy. Bicifadine (6.25, 12.5, 25 mg/kg, PO) significantly increased the paw withdrawal force in rats with STZ-induced mechanical hyperalgesia above vehicle-treated, diabetic controls. Values represent the mean ± SEM of results from 10 rats per group. **: Response thresholds are significantly different from those from vehicle-treated rats, P< 0.01, 1-way ANOVA followed by Dunnet's post-hoc comparison test, $F_{(4,45)} = 8.3$, P<0.01.

Figure 13. Effects of bicifadine and desipramine on motor activity, rotarod performance and grip strength. Bicifadine (12.5-100 mg/kg, PO) had no significant effect on the distance traveled by rats in the open field (A), while desipramine (25, 50 mg/kg IP, B) significantly reduced the distance traveled in

the open field relative to vehicle-treated rats. Bicifadine (C) did not cause a decrement in rotarod performance, while rats treated with desipramine (D) showed a trend towards decreased rotarod performance which did not reach significance (E). Bicifadine (F) did not alter grip strength, while rats treated with desipramine (50 mg/kg) showed a significant decrement in grip strength (G). *, brackets: Significantly different from vehicle control, $P<0.05$, 2-way ANOVA and Bonferroni-corrected post-hoc comparison test, $F_{(2,6,12)} = 654$, Treatment, 328, Time, 150 Interaction, all $P<0.01$. *: Significantly different from vehicle control, $P<0.05$, 1-way ANOVA and Dunnett's post-hoc comparison test, $F_{(2,19)} = 6.2$, $P<0.01$.

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Table 1. Bicifadine interactions with neurotransmitter transporters, receptors, and ion channels: Radioligand-receptor binding screen

Transporter/ Receptor/ Ion Channel	**Source**	**Assay Conditions (Radioligand, [nM]/Non-specific binding ligand, [μM])**	**Affinity**
Norepinephrine transporter	Human recombinant (HEK 293 cells)	[^{125}I]RTI-55, 0.04/mazindol, 5[1]	K_i = 5.0 ± 1.1 μM
Serotonin transporter	Human recombinant (HEK 293 cells)	[^{125}I]RTI-55, 0.04/imipramine, 5[1]	K_i = 2.4 ± 0.7 μM
Dopamine transporter	Human recombinant (HEK 293 cells)	[^{125}I]RTI-55, 0.04/mazindol, 5[1]	K_i = 5.2 ± 1.2 μM
A_1 Adenosine	Human recombinant (CHO cells)	[^{3}H]DPCPX,1/DPCPX,1[2]	<25% inhibition at 10 μM
A_{2A} Adenosine	Human recombinant (HEK 293 cells)	[^{3}H]CGS 21680, 6/NECA,10[2]	<25% inhibition at 10 μM
α_1 Adrenergic	Rat brain	[^{3}H]Prazosin, 0.25/prazosin, 0.1[3]	K_i = 773 nM
α_2 Adrenergic	Rat cerebral cortex	[^{3}H]Rauwolscine, 0.7/yohimbine, 1[3]	K_i = 1.01 μM
β_1 Adrenergic	Human recombinant (Rex 16 cells)	[^{125}I]Cyanopindolol, 0.03/S(-)-propranolol, 100[3]	K_i = 1.55 μM

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β_2 Adrenergic	Human recombinant (CHO cells)	[^{3}H]CGP-12177, 0.2/ICI-118551, 10^3	41% inhibition at 10 μM
β_3 Adrenergic	Human recombinant (HEK 293 cells)	[^{125}I]Cyanopindolol, 0.05/alprenolol, 1000^3	<25% inhibition at 10 μM
Angiotensin$_1$	Human recombinant (CHO cells)	[^{125}I][Sar^1,Ile^8]-Angiotensin II, 0.05/angiotensin II, 10^3	<25% inhibition at 10 μM
Benzodiazepine	Rat brain (- cerebellum)	[^{3}H]Flunitrazepam, 1/diazepam, 10^3	<25% inhibition at 10 μM
Bradykinin$_1$	Human IMR-90 cells	[^{3}H](des-Arg^{10})-Kallidin, 0.5/(Des-Arg^9,Leu^8)-bradykinin, 0.00017^3	<25% inhibition at 10 μM
Bradykinin$_2$	Human recombinant (CHO cells)	[^{3}H]Bradykinin, 0.2/bradykinin, 5^3	<25% inhibition at 10 μM
Calcitonin-gene related peptide;	Human SK-N-MC cells	[^{125}I]CGRP, 0.01/α-CGRP (8-37), 1^3	<25% inhibition at 10 μM
Cannabinoid$_1$	Human recombinant (HEK 293 cells)	[^{3}H]WIN 55212-2, 2/WIN 55212, 10^2	<25% inhibition at 10 μM
Cannabinoid$_2$	Human recombinant (CHO-K1 cells)	[^{3}H]WIN 55212-2, 2.4/R(+)-WIN 55212-2, 0.0049^3	<25% inhibition at 10 μM
Cholecystokinin$_A$	Human recombinant (NIH-3T3 cells)	[^{3}H]L-364718, 0.8/L-364718, 1^3	<25% inhibition at 10 μM

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Cholinergic receptor, M_1 Muscarinic	Human recombinant (CHO cells)	[^{3}H]N-Methylscopolamine 0.8/atropine, 1[3]	<25% inhibition at 10 μM
Cholinergic receptor, M_2 Muscarinic	Human recombinant (CHO cells)	[^{3}H]N-Methylscopolamine 0.8/atropine, 1[3]	<25% inhibition at 10 μM
Cholinergic receptor, M_3 Muscarinic	Human recombinant (CHO cells)	[^{3}H]N-Methylscopolamine 0.8/atropine, 1[3]	38% inhibition at 10 μM
Cholinergic receptor, M_4 Muscarinic	Human recombinant (CHO cells)	[^{3}H]N-Methylscopolamine 0.8/atropine, 1[3]	<25% inhibition at 10 μM
Cholinergic receptor, α_7 Nicotinic	Rat brain (-cerebellum)	[^{125}I]α-Bungarotoxin, 0.6/ α-bungarotoxin,1[3]	<25% inhibition at 10 μM
Corticotropin releasing factor$_1$	Human recombinant (CHO-K1 cells)	[^{125}I](Tyr^0)-CRF, 0.05/urocortin, 0.1[3]	<25% inhibition at 10 μM
D_1 Dopamine	Human recombinant (CHO cells)	[^{3}H]SCH 23390, 1.4/(+)-butaclamol, 10[3]	<25% inhibition at 10 μM
D_{2S} Dopamine	Human recombinant (CHO cells)	[^{3}H]Spiperone, 0,3/(+)butaclamol, 10[3]	<25% inhibition at 10 μM
D_3 Dopamine	Human recombinant (CHO cells)	[^{3}H]Spiperone, 0.7/S(-)-sulpiride, 25[3]	<25% inhibition at 10 μM
$D_{4.4}$ Dopamine	Human recombinant (CHO cells)	[^{3}H]Spiperone, 0.3/(+)butaclamol, 10[2]	IC_{50} >30 μM

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$Endothelin_A$	Human recombinant (CHO cells)	[^{125}I]Endothelin-1, 0.03/endothelin-1, 0.1[3]	<25% inhibition at 10 μM
Epidermal growth factor	Human A431 cells	[^{125}I]EGF, 0.08/EGF, 0.1[3]	<25% inhibition at 10 μM
$GABA_A$	Rat brain (- cerebellum)	[^{3}H]Muscimol, 1/muscimol, 0.1[3]	<25% inhibition at 10 μM
Glucocorticoid	Human HeLa S3 cells	[^{3}H]Dexamethasone, 6/dexamethasone, 20[3]	<25% inhibition at 10 μM
Glutamate receptor, AMPA type	Rat cerebral cortex	[^{3}H]AMPA, 5/L-glutamic acid, 1000[3]	<25% inhibition at 10 μM
Glutamate receptor, NMDA type, agonist binding site	Rat cerebral cortex	[^{3}H]CGP-39653, 2/L-glutamic acid, 1000[3]	<25% inhibition at 10 μM
Glutamate receptor, NMDA type, ionophore binding site	Rat cerebral cortex	[^{3}H]MK-801, 5/PCP, 100[4]	K_i = 23.7±7.9 μM
H_1 Histamine	Human recombinant (CHO cells)	[^{3}H]Pyrilamine, 1.2/pyrilamine, 1[3]	44% inhibition at 10 μM
$Melanocortin_4$	Human recombinant (HEK-293 cells)	[^{125}I]NDP-α-MSH, 0.05/ NDP-α-MSH, 1[2]	<25% inhibition at 10 μM

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Neurokinin$_1$	U-373MG cells	[^{3}H]Sar^9, Met(O$_2$)^{11}]-Substance P, 1.2/ Sar^9, Met(O$_2$)^{11}]-SP, 1[2]	<25% inhibition at 10 μM
Neuropeptide Y$_1$	Human SK-N-MC cells	[^{125}I]Peptide YY, 0.015/ Neuropeptide Y, 1[3]	<25% inhibition at 10 μM
μ Opioid	Human recombinant (CHO cells)	[^{3}H]DAMGO, 0.5/naloxone, 10[2]	IC$_{50}$ >30 μM
κ Opioid	Guinea-pig cerebellum	[^{3}H]U 69593, 0.7/naloxone, 10[2]	IC$_{50}$ >30 μM
δ$_2$ Opioid	Human recombinant (CHO cells)	[^{3}H]DADLE, 0.5/naltrexone, 10[2]	<25% inhibition at 30 μM
Orphanin$_1$	Human recombinant (HEK 293 cells)	[^{3}H]Nociceptin, 0.6/orphanin-FQ, 1[3]	<25% inhibition at 10 μM
DP Prostanoid	Human recombinant (CHO K1 cells)	[^{3}H]PGD$_2$, 1.7/PGD$_2$, 1[3]	<25% inhibition at 10 μM
EP$_2$ Prostanoid	Human recombinant (HKE-293 cells)	[^{3}H]PGE$_2$, 4/PGE$_2$, 10[3]	<25% inhibition at 10 μM
EP$_4$ Prostanoid	Human recombinant (HKE-293 cells)	[^{3}H]PGE$_2$, 0.3/PGE$_2$, 10[3]	<25% inhibition at 10 μM

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P_2X Purine	NZW Rabbit urinary bladder	[^{3}H]α,β-Methylene-ATP, 8/β,γ-Methylene-ATP, 100^3	<25% inhibition at 10 μM
5-HT_{1A} Serotonin	Human recombinant (HKE-293 cells)	[^{3}H]8-OH-DPAT, 0.5/8-OH-DPAT, 10^2	K_i = 1.7 μM
5-HT_{1B} Serotonin	Rat cerebral cortex	[^{125}I]Cyanopindolol, 0.1/serotonin, 10^2	K_i = 2.9 μM
5-HT_3 Serotonin	Human recombinant (HEK-293 cells)	[^{3}H]BRL 43694, 0.5/MDL 72222, 10^2	37% inhibition at 10 μM
σ_1	Jurkat T-cells	[^{3}H](+)Pentazocine, 8/haloperidol, 10^2	K_i = 1.2 μM
σ_2	Rat cerebral cortex	[^{3}H]DTG, 5, (+)pentazocine, 300 nM/haloperidol, 10^2	K_i = 11 μM
$Vanilloid_1$	Rat spinal cord	[^{3}H]Resiniferatoxin, 0.2/resiniferatoxin, 0.1^3	<25% inhibition at 10 μM
$Vasopressin_{1A}$	Human recombinant (CHO cells)	[^{3}H]AVP, 0.3/AVP, 1^2	<25% inhibition at 10 μM
$\alpha_2\delta$ Ca^{++} Channel	Rat cerebral cortex	[^{3}H]Gabapentin, 20/gabapentin, 100^3	<25% inhibition at 10 μM
N-type Ca^{++} Channel	Rat frontal cortex	[^{125}I]ω-Conotoxin GVIA, 0.010/ ω-conotoxin GVIA, 0.10^3	<25% inhibition at 10 μM
K^+_{ATP} Channel	Rat cerebral cortex	[^{3}H]Glibenclamide, 0.1/glibenclamide, 1^2	<25% inhibition at 10 μM

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Saxitoxin-sensitive K^+_V Channels	Rat cerebral cortex	[^{125}I]α-Dendrotoxin, 0.01/ α-dendrotoxin, 0.050[2]	<25% inhibition at 10 μM
K^+_V hERG	Human recombinant (HEK-293 cells)	[^{3}H]Astemizole, 1.5/astemizole, 10[3]	<25% inhibition at 10 μM

The receptors tested, their sources, the radioligands used to define the binding sites and the relative affinity of bicifadine for these receptors are indicated. Additional information on the assay conditions can be obtained from the contractor's website. Bicifadine showed significant affinity (K_i < 10 μM) for the α_1, α_2, and β_1 adrenergic, 5-HT_{1A} and 5-HT_{1B} serotonergic and σ_1 receptors. Studies performed at: [1]Oregon Health Sciences University/VA Medical Center; [2]CEREP; [3]MDS; [4]Polish Academy of Sciences.

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Table 2. Functional activity of bicifadine at neurotransmitter receptors and transporters.

Receptor /Transporter System	**Preparation**	**Stimulus**	**Actions**
Norepinephrine transporter	Recombinant human transporters[1]	[^{3}H]NE uptake by intact cells	Antagonist, inhibits uptake, IC_{50} = 55 ± 3.4 nM
Serotonin transporter	Recombinant human transporters[1]	[^{3}H]5-HT uptake by intact cells	Antagonist, inhibits uptake, IC_{50} = 117 ± 26 nM
Dopamine transporter	Recombinant human transporters[1]	[^{3}H]DA uptake by intact cells	Antagonist, inhibits uptake, IC_{50} = 910 ± 140 nM
α_1 Adrenergic	Rat vas deferens[2]	Phenylephrine (2 µM)-induced contractions	Antagonist, inhibits phenylephrine induced contractions, IC_{50} = 18.6 µM
α_2 Adrenergic	Rat vas deferens[2]	Neurogenic twitch	Agonist, reduces twitch response, EC_{50} = 6 µM
β_1 Adrenergic	Guinea pig left atrium[2]	Isoproterenol (50 nM)-induced positive inotropy	Antagonist, blocks inotropy, IC_{50} = 22.4 µM
5-HT_{1A} Serotonergic	Recombinant human receptors[3]	5-HT (30 nM) stimulated [^{35}S]GTPγS binding	Agonist, enhances binding, EC_{50} =10 µM
5-HT_{1B} Serotonergic	Rat caudal artery rings[2]	Serotonin (1 µM)-stimulated contractions	Antagonist, blocks contractions, IC_{50} = 22 µM

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NMDA Receptor	Primary cultures of hippocampal pyramidal neurons[4]	Glycine dependent, NMDA (5-1000 μM)-stimulated ion currents	IC_{50} = 217 ± 18 μM.

The functional activity of bicifadine was tested in physiological or biochemical systems where its binding affinity (K_i) was determined to be less than 10 μM (Table 2). Studies performed at: [1]Oregon Health Sciences University/VA Medical Center; [2]MDS; [3] CEREP; [4]Rehovot University.

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Table 3. Potency of bicifadine and reference antinociceptive agents in models of acute inflammatory and visceral pain.

Drug	**Route, Time**	**Randall-Selitto**		**PPQ Contractions**	
		ED_{50} (mg/kg)	n/dose	ED_{50} (mg/kg)	n/dose
Bicifadine	PO, 1 hr	9.2 (5.0-16.8)	8	13.2 (6-29)	5-10
Codeine	PO, 2 hr	43 (29-62)	12	9.0 (7.4-11)	5-10
Acetaminophen	PO, 2 hr	150 (98-231)	12	37 (11-123)	5-10

ED_{50} values presented as the mean, 95% confidence interval for the increase in withdrawal threshold (Randall-Selitto test) and decreasing the number of contractions/3 minutes (PPQ test).

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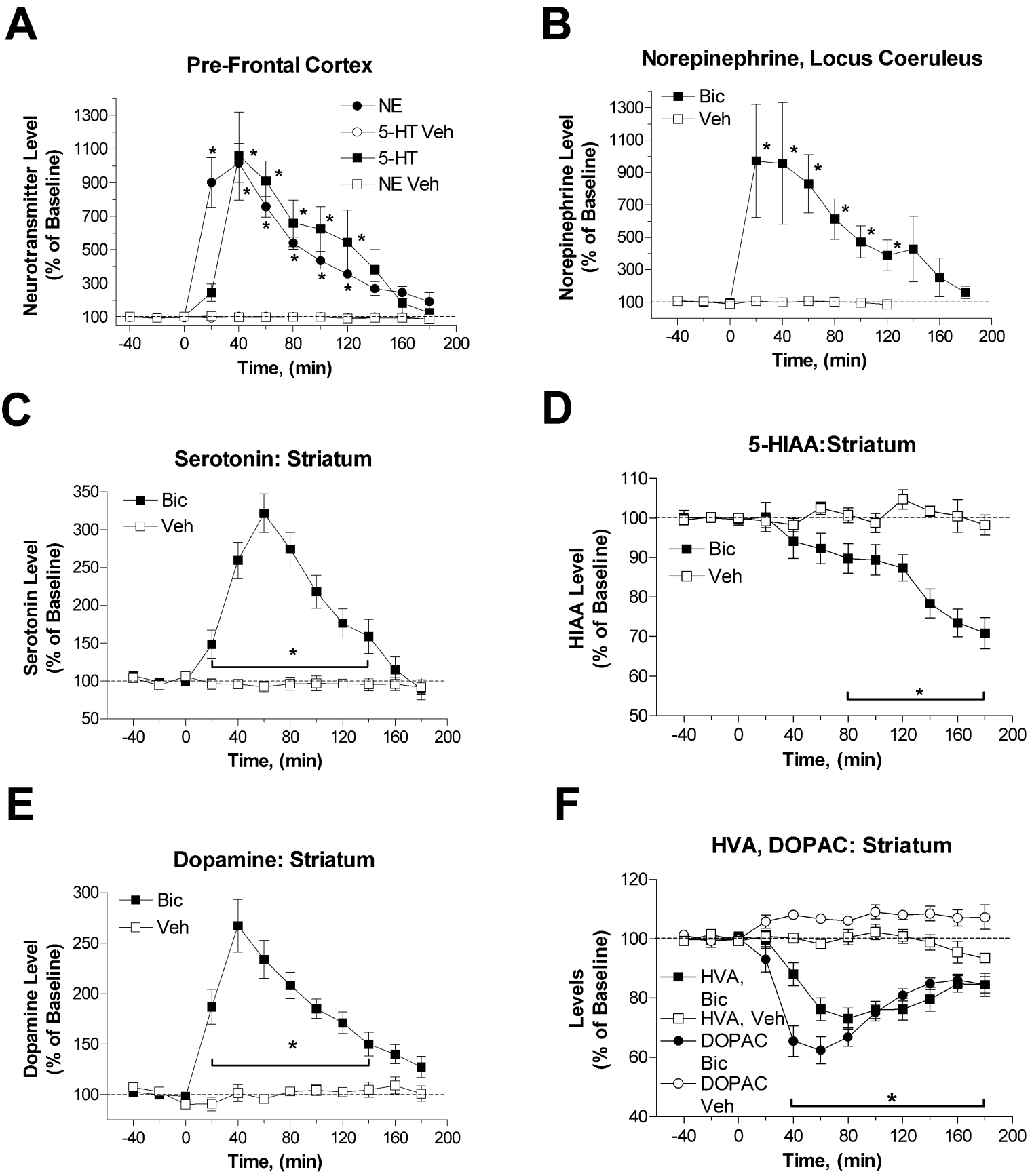

Figure 1

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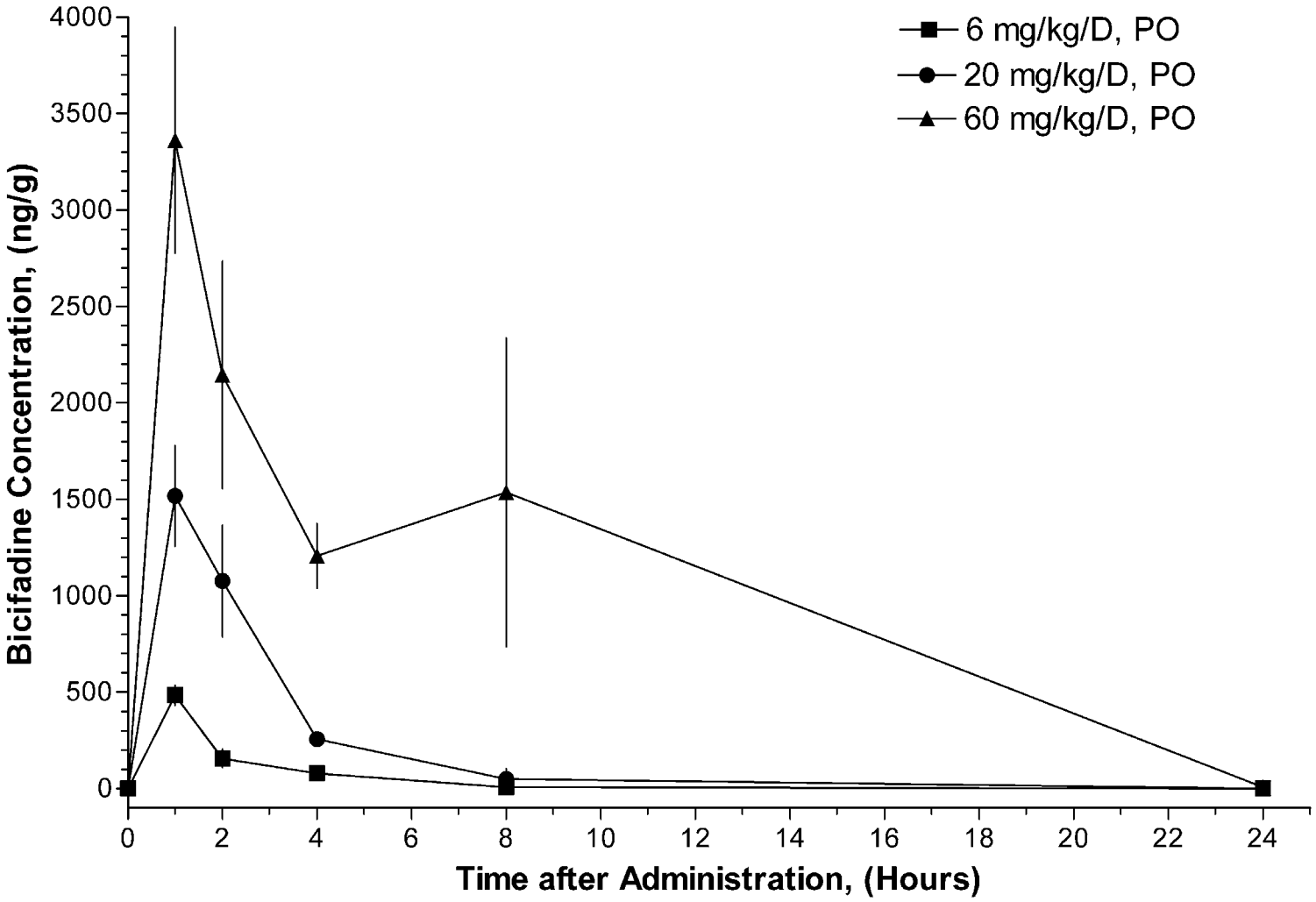

Figure 2

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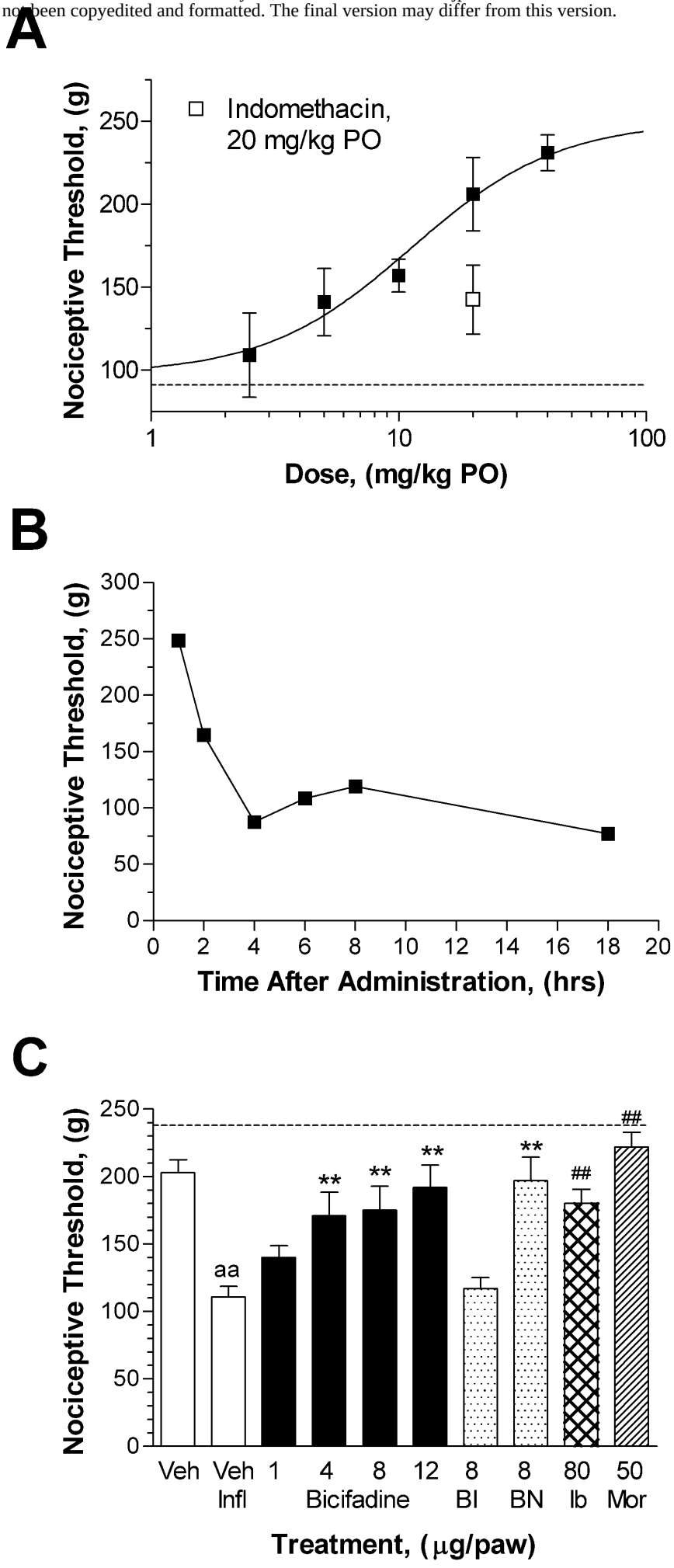

Figure 3

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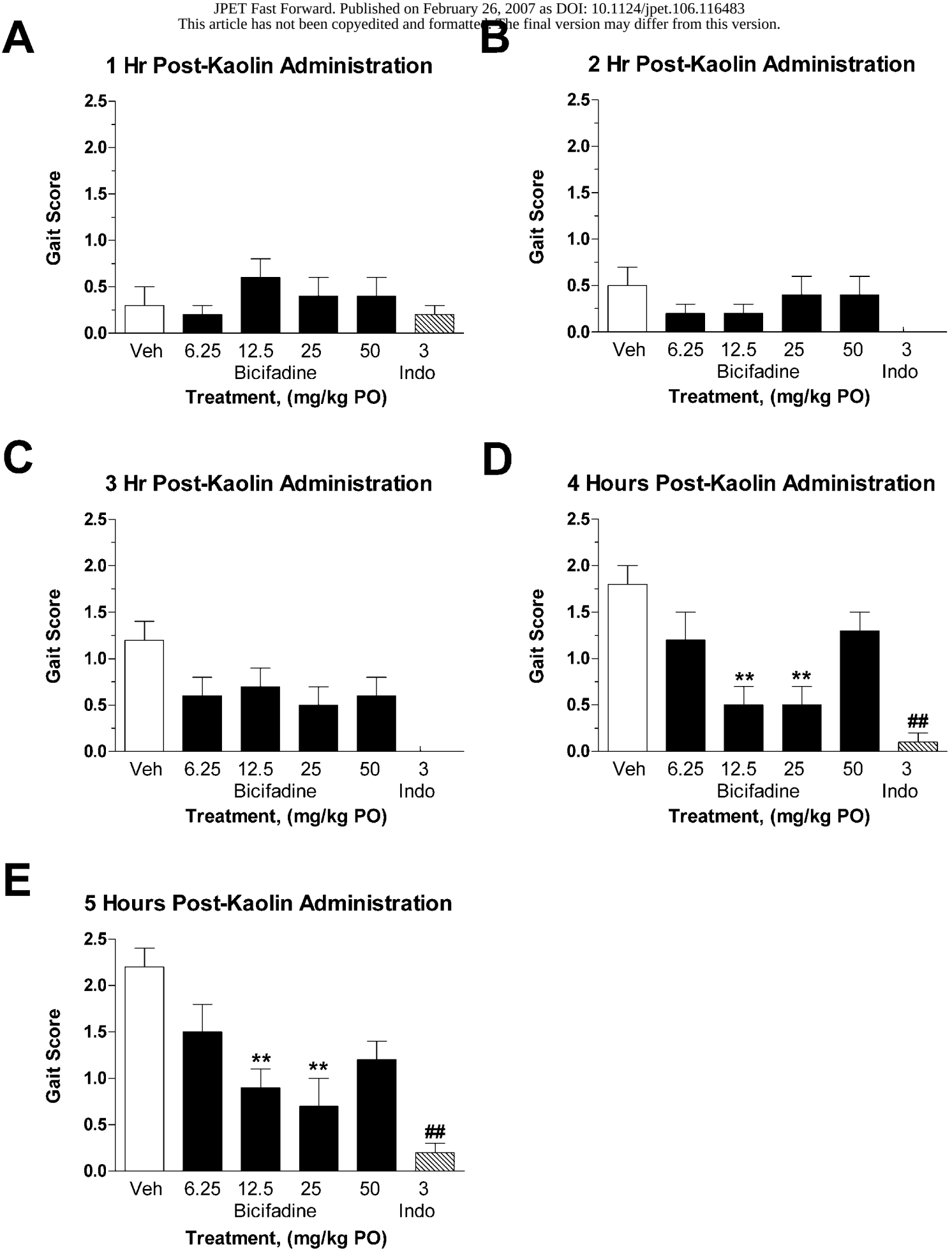

Figure 4

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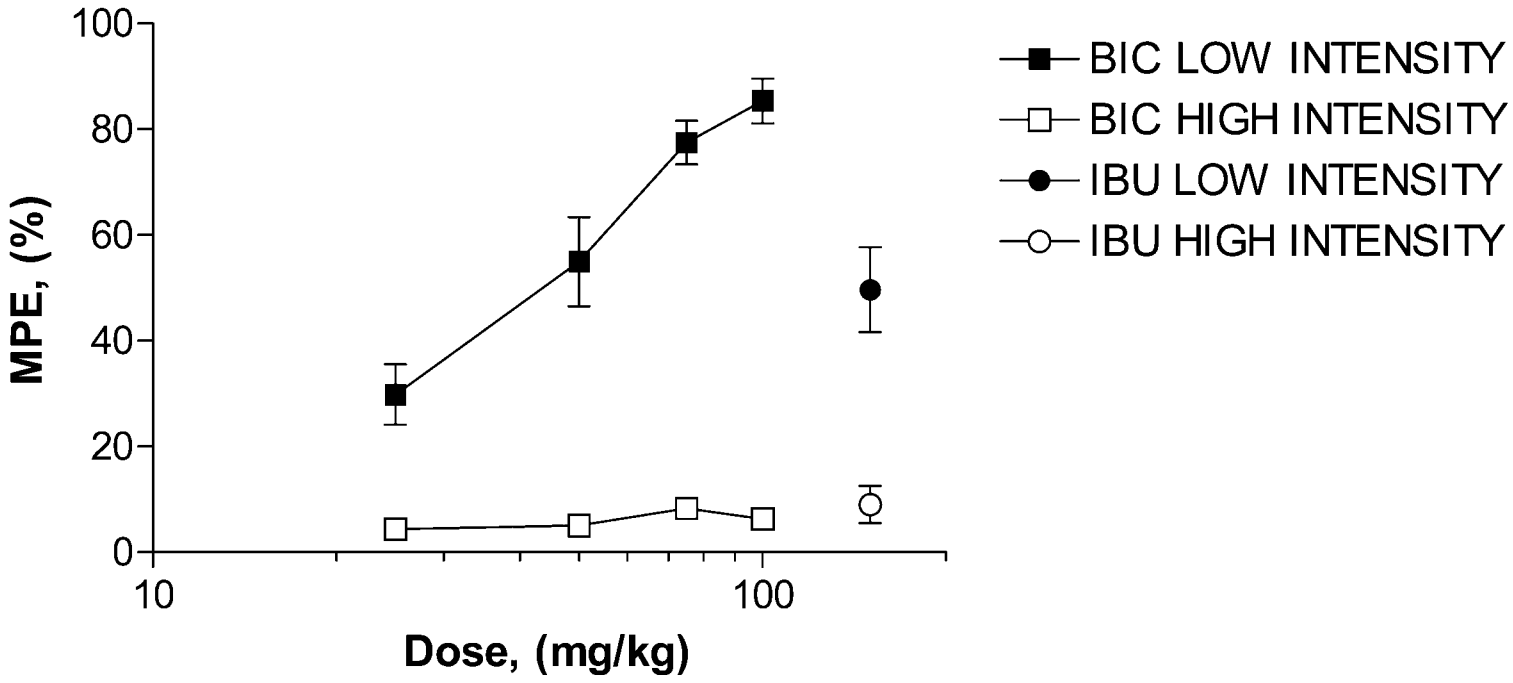

Figure 5

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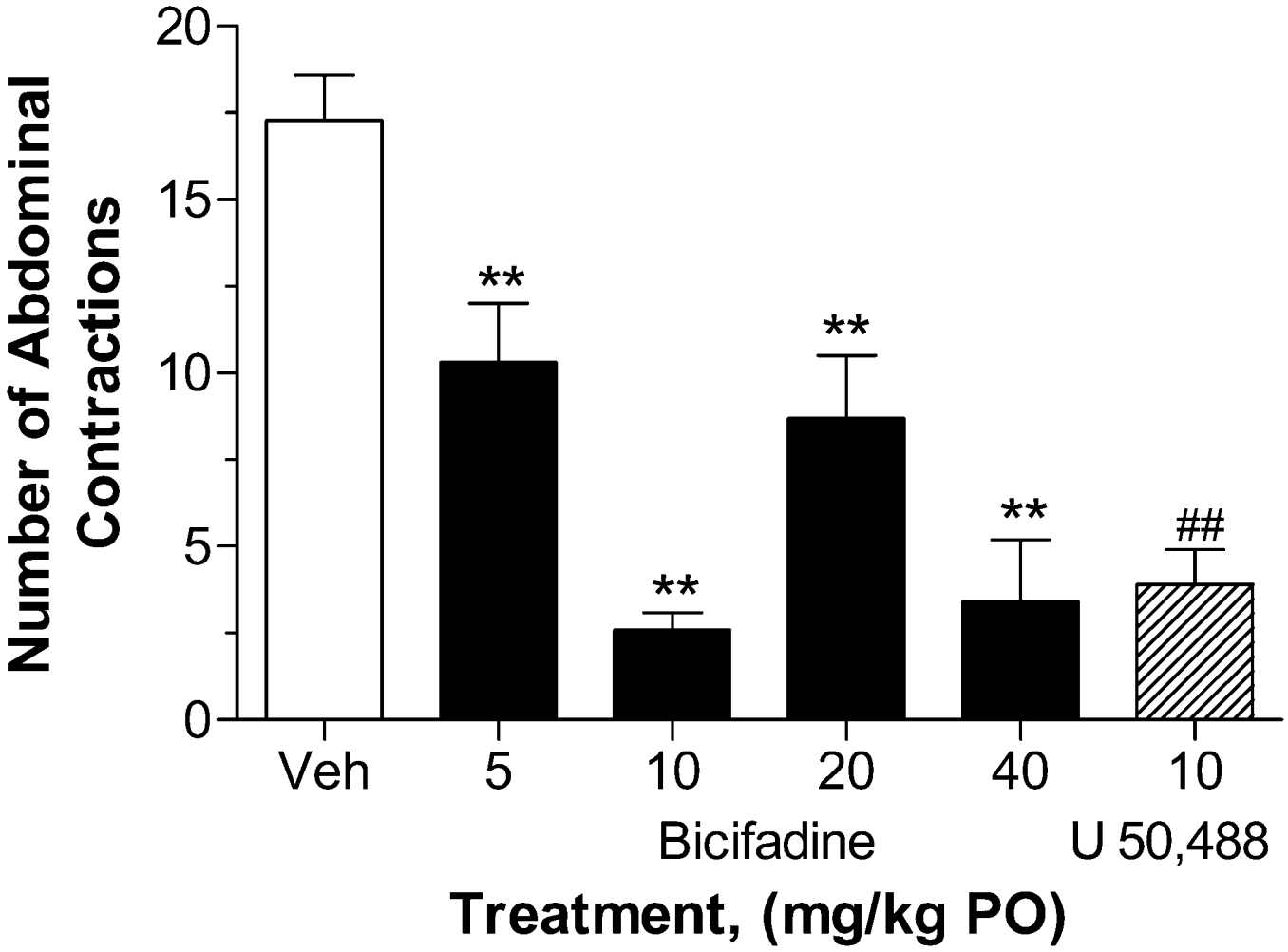

Figure 6

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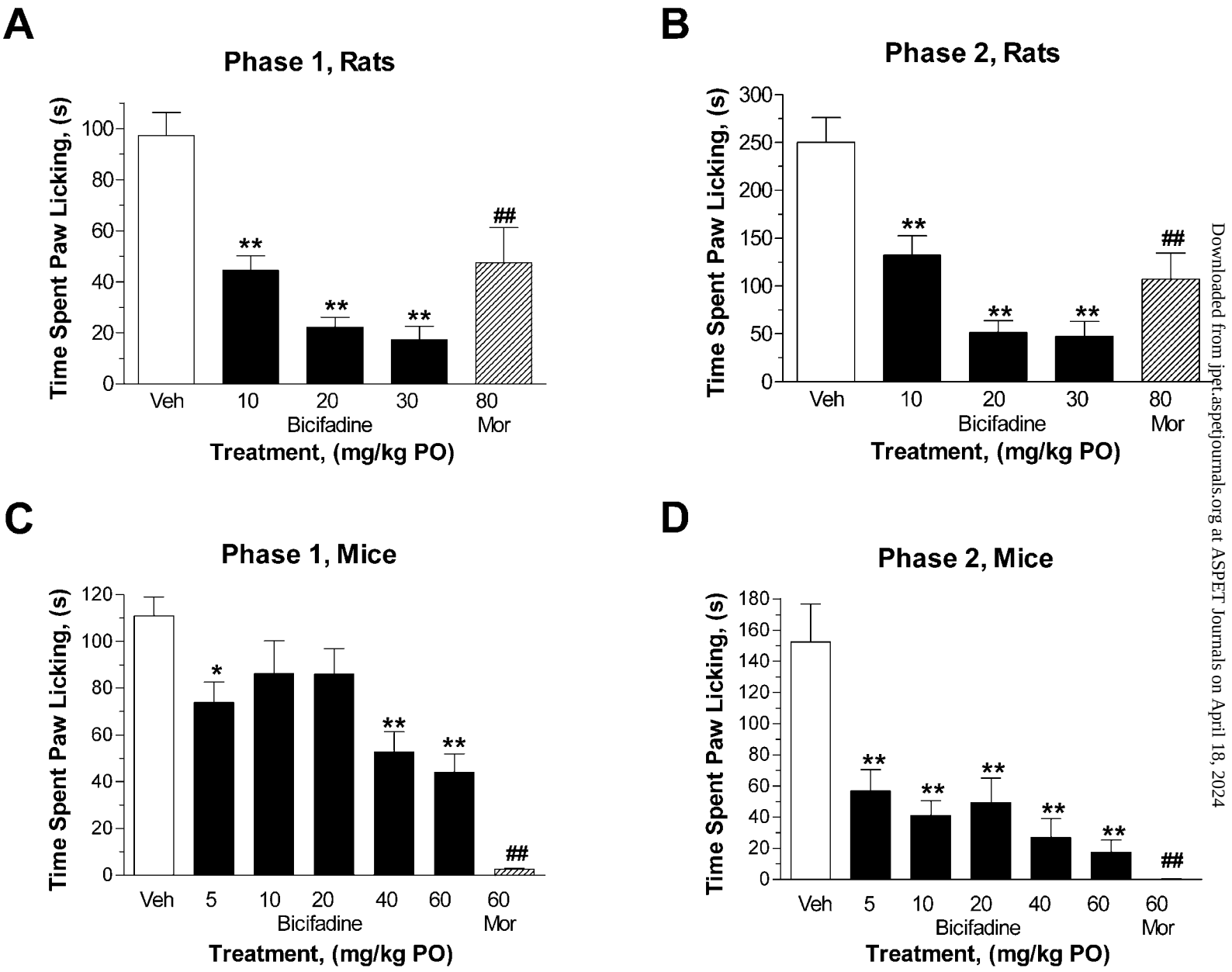

Figure 7

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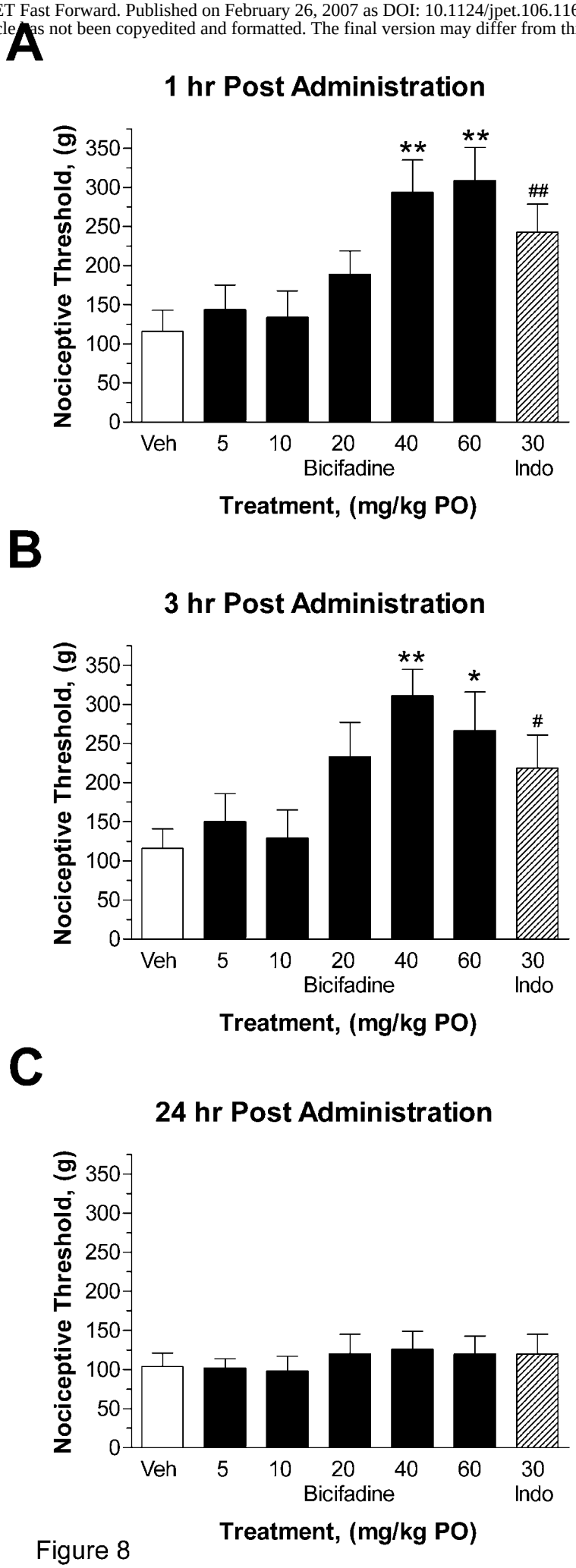

Figure 8

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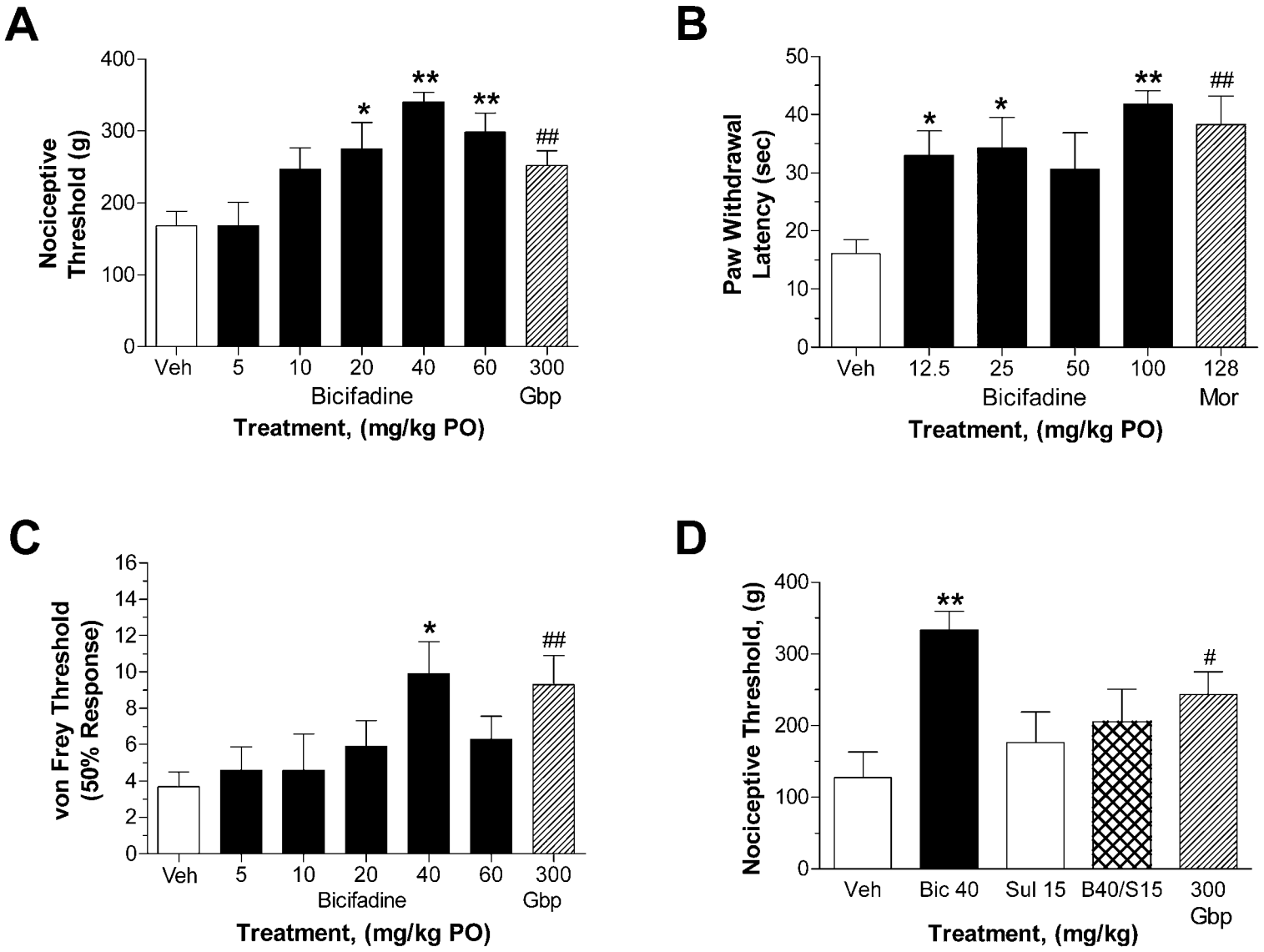

Figure 9

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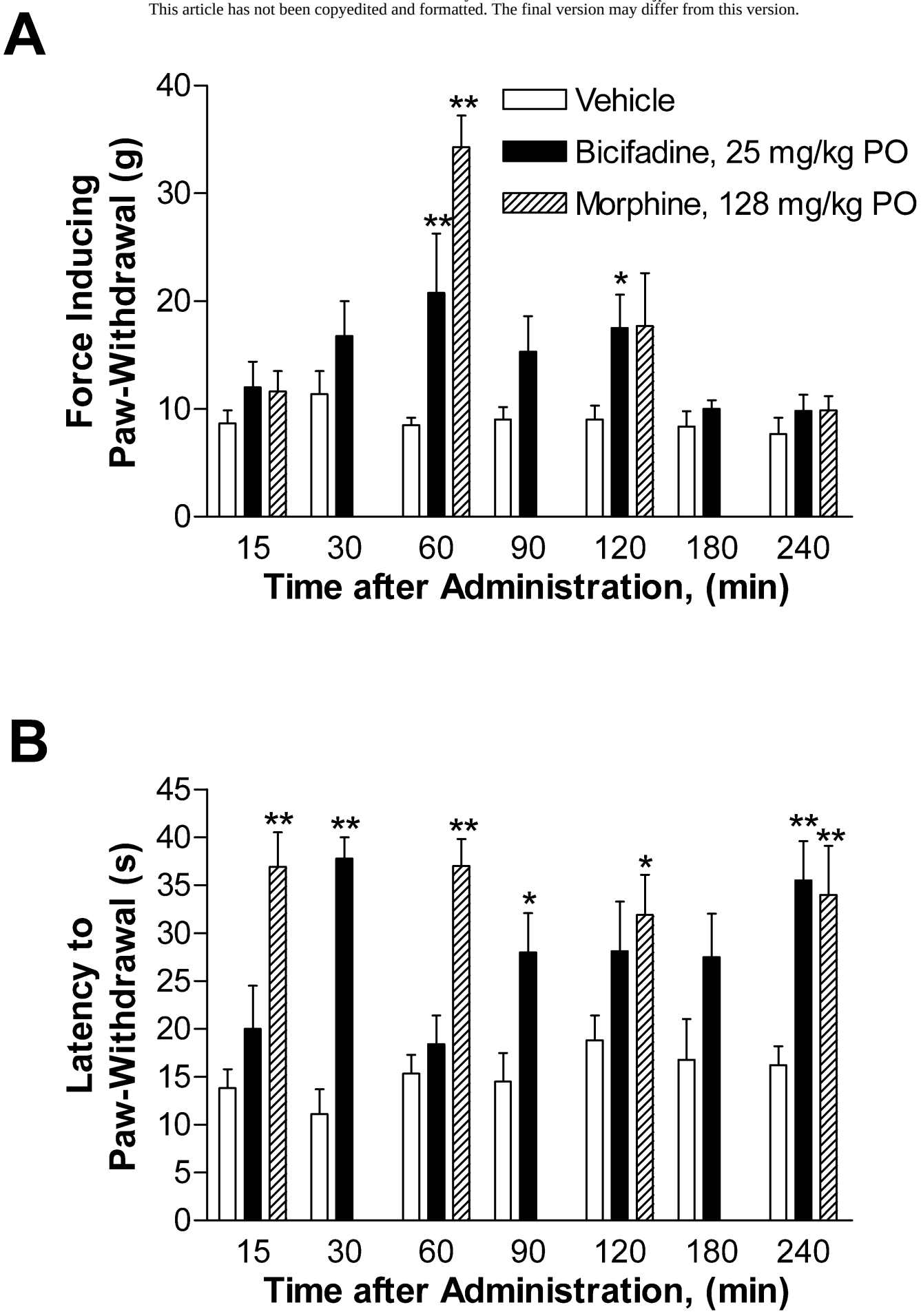

Figure 10

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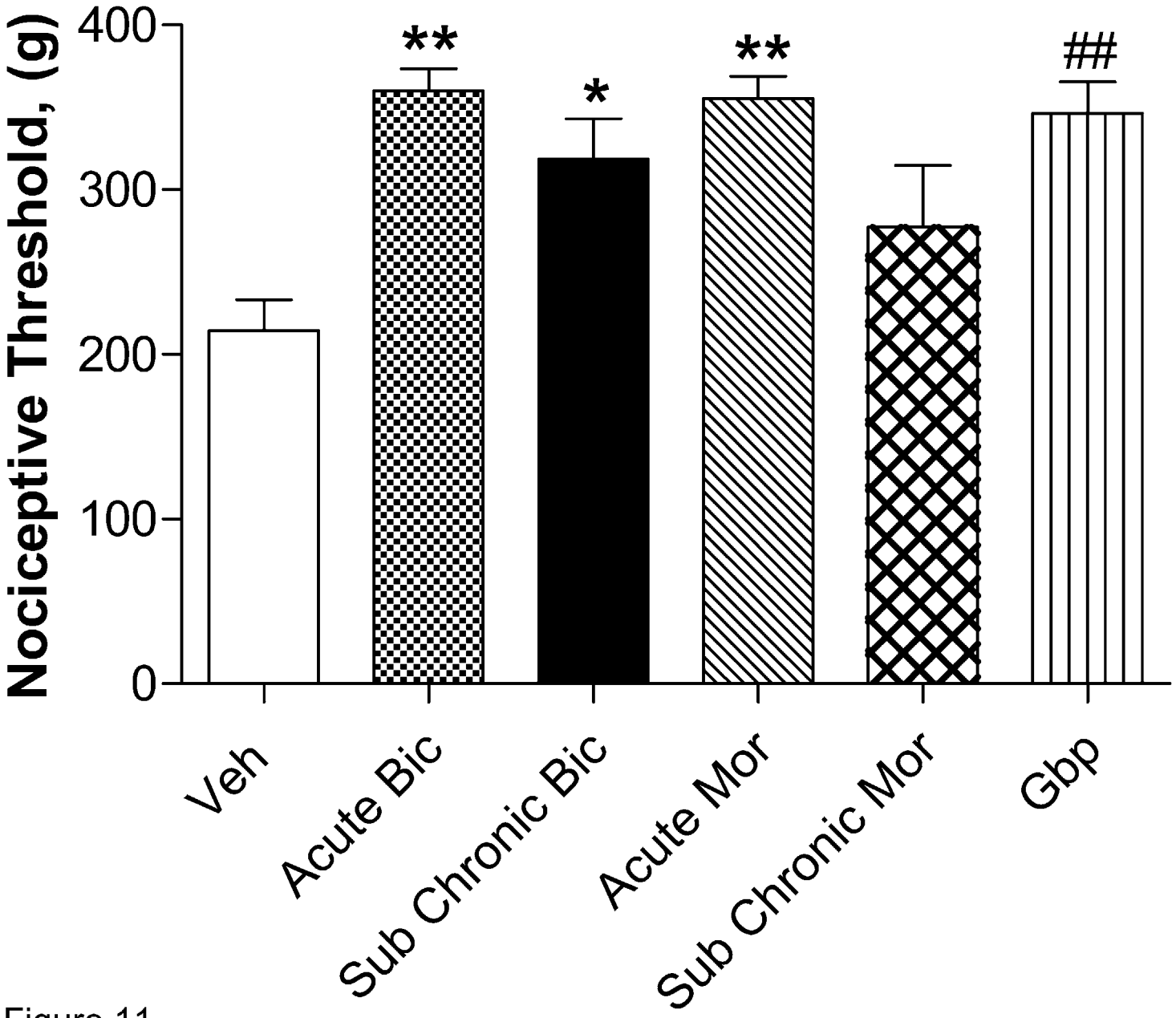

Figure 11

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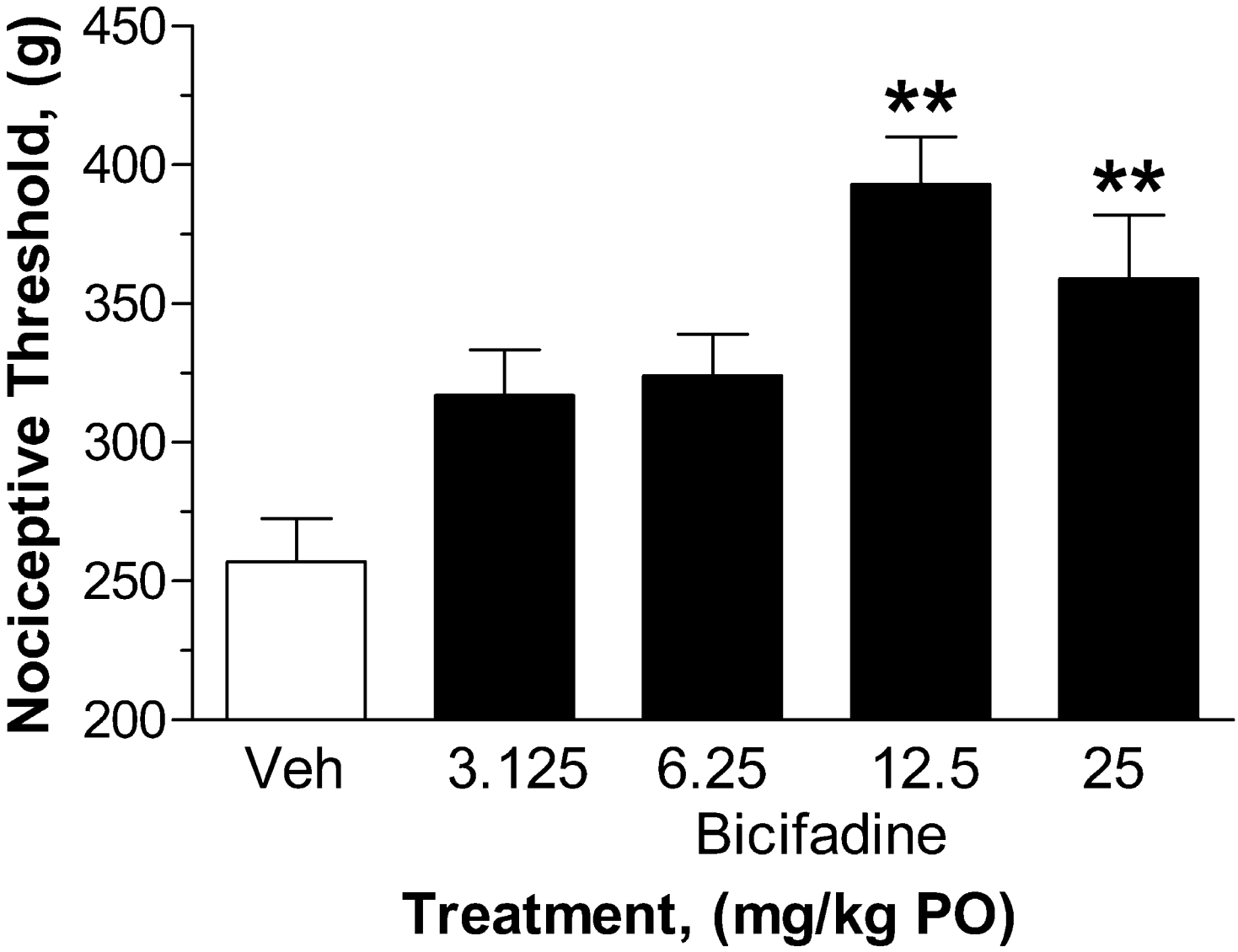

Figure 12

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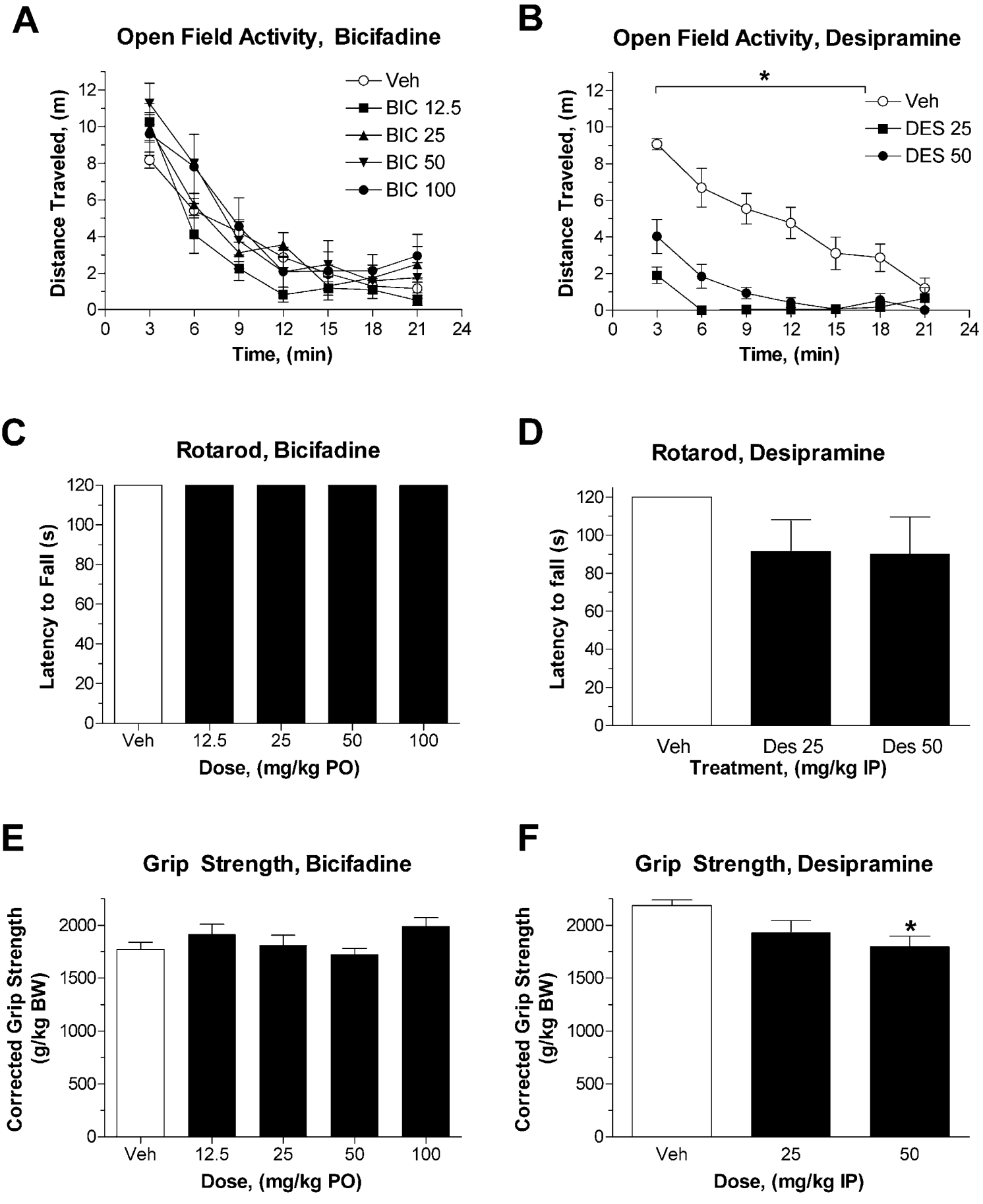

Figure 13