The α2,3-selective potentiator of $GABA_A$ receptors, KRM-II-81, reduces nociceptive-associated behaviors induced by formalin and spinal nerve ligation in rats

J. M. Witkin[1,2,7], R. Cerne[1], P. G. Davis[3], K. B. Freeman[4], J. M. do Carmo[5], J. K. Rowlett[4], K. R. Methuku[2], A. Okun[1], S. D. Gleason[1], X. Li[1], M. J. Krambis[1], M. Poe[2], G. Li[2], J. M. Schkeryantz[1], R. Jahan[2], L. Yang[1], W. Guo[1], L. K. Golani[2], W. H. Anderson[1], J. T. Catlow[1], T. M. Jones[1], F. Porreca[6], J. L. Smith[7], K. L. Knopp[1], and J. M. Cook[2]

[1]The Lilly Research Labs, Eli Lilly and Company,
Indianapolis, IN, USA

[2]Department of Chemistry & Biochemistry
University of Wisconsin-Milwaukee, Milwaukee, WI, USA

[3] Millsaps College; Jackson, MS, USA

[4]Department of Psychiatry and Human Behavior,
University of Mississippi Medical Center, Jackson, MS, USA

[5]Department of Physiology and Biophysics
University of Mississippi Medical Center; Jackson, MS, USA

[6]Department of Pharmacology, University of Arizona, Tucson, AZ, USA

[7]Laboratory of Antiepileptic Drug Discovery
Department of Neurological Surgery
Indiana University School of Medicine, Indianapolis, IN, USA

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Correspondence:
J. M. Witkin
Laboratory of Antiepileptic Drug Discovery
Department of Neurological Surgery
Indiana University School of Medicine, Indianapolis, IN, USA
witkinconsult@gmail.com

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Non-Standard Abbreviations

HZ-166: HZ-166 ethyl 8-ethynyl-6-(pyridin-2-yl)-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepine-3-carboxylate

KRM-II-81: 5-(8-ethynyl-6-(pyridin-2-yl)-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepin-3-yl)oxazole

Abstract

Clinical evidence indicates that positive allosteric modulators (PAMs) of $GABA_A$ receptors have analgesic benefit in addition to efficacy in anxiety disorders. However, the utility of $GABA_A$ receptor PAMs as analgesics is compromised by the central nervous system side effects of non-selective potentiators. A selective potentiator of $GABA_A$ receptors associated with $\alpha 2/3$ subunits, KRM-II-81(5-(8-ethynyl-6-(pyridin-2-yl)-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepin-3-yl)oxazole), has demonstrated anxiolytic, anticonvulsant, and antinociceptive effects in rodents with reduced motoric side effects. The present study evaluated the potential of KRM-II-81 as a novel analgesic. Oral administration of KRM-II-81 attenuated formalin-induced flinching; in contrast, diazepam was not active. KRM-II-81 attenuated nociceptive-associated behaviors engendered by chronic spinal nerve ligation (L5/L6). Diazepam decreased locomotion of rats at the dose tested in the formalin assay (10 mg/kg) whereas KRM-II-81 produced small decreases that were not dose-dependent (10-100 mg/kg). Plasma and brain levels of KRM-II-81 were used to demonstrate selectivity for $\alpha 2/3$- over $\alpha 1$-associated $GABA_A$ receptors and to define the degree of engagement of these receptors. Plasma and brain concentrations of KRM-II-81 were positively-associated with analgesic efficacy. GABA currents from isolated rat dorsal-root ganglion cultures were potentiated by KRM-II-81 with an ED_{50} of 32nM. Measures of respiratory depression were reduced by alprazolam whereas KRM-II-81 was either inactive or produced effects with lower potency and efficacy. These findings add to the growing body of data supporting the idea that $\alpha 2/3$-selective $GABA_A$ receptor PAMs will have efficacy and tolerability as pain medications including those for neuropathic pain. Given their predicted anxiolytic effects, $\alpha 2/3$-selective $GABA_A$ receptor PAMs offer an additional inroad into the management of pain.

Management and control of pain continues to be a serious gap in medical practice with multiple alternative medicines that either lack critical efficacy and/or produce unacceptable side-effects (Papanas and Ziegler, 2016; Yan et al., 2017). One approach that has been underexploited is the enhancement of GABAergic neurotransmission, an area overlooked in part because of the general lack of efficacy of the 1,4-benzodiazepine GABA modulators (see Ralvenius et al., 2015). However, a strong rationale for the potential value of GABAergic modulators in pain states exists: 1) GABA is the primary inhibitory neurotransmitter in the mammalian nervous system and is critically involved in pain (Hammond and Drower, 1984; Dirig and Yaksh, 1995; Enna and McCarson, 2006, Zeilhoffer et al., 2015; Etlin et al., 2016); 2) GABA and some positive modulators of GABA have demonstrated efficacy in pain states (Besson et al., 2015; Jonsson et al., 2015; Ralvenius et al., 2016); 3) $GABA_A$ receptor-regulated pathways are integral to pain processing (Zeilhoffer et al., 2015); 4) data from transgenic mouse lines and selective pharmacological tools confirm an absolute requirement for α2/3-containing $GABA_A$ receptors in pain therapeutics (Ralvenius et al., 2015); 5) α2/3-containing $GABA_A$ receptors are present in central pathways modulating pain sensation (doral horn) and pain perception (Paul et al., 2012; Lian et al., 2012; Lorenzo et al., 2014); and 6) compounds that selectively amplify α2/3-containing $GABA_A$ receptor signaling are effective in multiple pain models in rodents at exposures lower than those producing motor side effects (Ralvenius et al., 2015).

The present set of experiments was undertaken to evaluate whether a newly-dicovered potentiator of $GABA_A$ receptors associated with α2/3 protein subunits would attenuate nociceptive-associated behaviors induced by formalin or spinal nerve ligation. Although some drugs that selectively modulate α2/3-containing $GABA_A$ receptors have undergone clinical investigation (anxiety) (see Atack, 2011), no drug of this mechanism has been evaluated in human pain patients. However, the side-effect profile of this mechanism has been assessed in humans and validates the tolerability and side-effect the value proposition (Atack, 2011; Atack et al., 2011; Nickolls et al., 2018).

Poe et al. (2016) proposed a novel structural class of compounds with functional selectivity for $GABA_A$ receptors associated with α2/3 subunits (see Li et al., 2018 for a more efficient synthetic strategy); KRM-II-81 is more potent in potentiating GABA-induced currents in HEK293 cells with human $\alpha2\beta3\gamma2$ or $\alpha3\beta3\gamma2$ receptors compared to cells with $\alpha_1\gamma_3\beta_2$ receptors (Poe et al., 2016) (see also Lewter et al., 2017 for functional selectivity using Xenopus oocytes) Moreover, they solved a long-standing issue with benzo[*f*]imidazo[1,5-*a*][1,4]diazepines; that is, the lack of reliable systemic bioavailability. For example, the ester, HZ-166, produced relatively little plasma exposure (Poe et al., 2016) and was not active as an anticonvulsant in several rodent models; efficacy was achieved with KRM-II-81 (Witkin et al., 2018). KRM-II-81 (5-(8-ethynyl-6-(pyridin-2-yl)-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepin-3-yl)oxazole) produced exposure of plasma and brain after oral dosing in rats to levels equal to or above the EC_{50} for activity at associated $GABA_A$ receptors containing α2/3 subunits and retained selectivity over $\alpha1-$associated $GABA_A$ receptors (Poe et al. 2016) that are known to confer motor-impairing and ataxic effects (McKernan et al., 2005). KRM-II-81 produced anxiolytic (Poe et al., 2016; Witkin et al., 2017), antidepressant (Methuku et al., 2017), and anticonvulsant (Witkin et al., 2018) effects in mice and rats. KRM-II-81 was also recently reported to have efficacy against acid-induced pain provocation (Lewter et al., 2017; Moerke et al., 2019) and structural analogs protected against inflammatory pain (Fischer et al., 2017; Kannampalli et al., 2017). To date, there are no data on the effects of this mechanism against chronic neuropathic pain.

The present report provides data on effects of KRM-II-81 on nociceptive behaviors controlled by the inflammatory impact of formalin injection and on pain-related behaviors of rats undergoing chronic ligation of spinal nerves L5/6. Both models have been used to evaluate a host of other drugs that act as analgesics or are candidate molecules for pain therapeutics (Carter and Shieh, 2010; Johnson et al., 2017; Tjølsen et al., 1992; Wang and Wang, 2003).

Methods

Rodent behavioral assays. All studies were performed in accordance with guidelines of the National Institutes of Health and by local animal care and use committees. The local animal care and use committee and veterinary staff provided direct oversight of the animals by inspections, protocol reviews, laboratory site visits, and animal health monitoring. Animals were housed in a quiet, ventilated-, temperature- and humidity-controlled vivarium that met AAALAC accreditation. Lighting was controlled with a 12 h light-dark cycle (lights on at 6 am). Food and water were available to the animals at all times when the animals were in their home cages. Male, Sprague Dawley rats weighing 225-350 g (Harlan Sprague Dawley, Indianapolis, IN) were used in these experiments. However, in the dorsal root ganglion studies, 2 month old male rats were used. Animals were maintained in the colony room for at least 3 days before testing. Animals were moved to a quiet room 1 h prior to the start of the test. All efforts were made to decrease bias and to increase reproducibility (Andrews et al., 2016) as described in the method details below.

Formalin model. Intraplanar injection of formalin initiates dramatic increases in spontaneous activity of c-fibers and discharge of wide dynamic range neurons, observed initially in spinal cord neurons (Chapman and Dickenson, 1995). Behaviorally, this neuronal excitability manifests as biphasic bouts of nocifensive responding. In the present experiments, male, Rats were used to assess the ability of KRM-II-81 to reduce tactile hypersensitivity induced by formalin injection into the rat paw by methods previously described (Johnson et al., 2017). Briefly, rats were acclimated to the test apparatus (SR-Lab Startle Response System, San Diego Instruments, San Diego, CA) and then injected with 50 µL of 5% formalin solution (in 0.9% saline) into the plantar surface of the right hind paw.

Behaviors of the rat were assessed by a force-transducer monitoring the chamber floor over 50 min. The number of events was defined as the number of 1 s bins with a change in force exceeding values observed when the rats were just resting or sniffing. Data were separately analyzed for early- (0-5 min) and late-phase (10-50 min) behaviors by ANOVA followed by post-hoc Dunnett's test with $p<0.05$ being considered statistically different than vehicle control values.

L5/6 ligation model. Male, Sprague Dawley rats weighing between 225-350 g were studied (Harlan Sprague Dawley, Indianapolis, IN). Animals were anesthetized using 1-5% isoflurane anesthesia. Eye lubricant was placed in the eyes to prevent drying of the corneas during anesthesia. No analgesics are given due to the need for pain response measurement. The dorsal side of the animals was shaved and cleaned from the lower thoracic region to the base of the tail. Rats were warmed with a heating pad and maintained on isoflurane.

Surgery. A small cranial/caudal skin incision was made just to the left of the spinal column above the L6 / S transverse processes and a small cranial/caudal incision (approx. ¼ in) was made along the epaxial muscles. The muscles were then separated from the spinous process via a small retractor exposing the transverse processes of the L6 / S1 vertebrae. The L6 transverse process was then removed via a small orthopedic rongeur, being careful not to nick the L4 and L5 nerves. Using a small glass hooked rod, the L5 nerve was isolated and firmly ligated with 4-0 6-0 silk strand. The L6 nerve was subsequently isolated from beneath the S1vertebrae by positioning the glass hook caudal and ventral to the S1 transverse process and gently moving the hook cranially against the ventral side of the vertebrae, being careful not to

elongate or break the nerve. Once the L6 nerve was isolated and ligated (as per the L5 nerve), it was gently repositioned beneath the S1vertebrae. The L4 nerve was then located and lightly stroked with the glass hooked rod approximately 20 times to maximize induction of long-term nociceptive behavioral hyper-responsivity. The L5 and L4 nerves were then gently replaced underneath the surrounding muscle. The epaxial muscles are then sutured closed and the skin closed.

Sham procedure. A sham operation was conducted as per the procedure describe above with the exception that lumbar nerves were neither ligated nor stroked.

Post-surgical care. After the surgical procedure the animals were transferred to a warm recovery cage and monitored until fully recovered. Animals were then returned to their home cages and monitored at least once daily for signs of distress that would be more than expected after surgical intervention such as respiratory distress, wound abnormalities, etc. In the event of such observations, veterinarian help would be summoned. In the present set of experiments, there were no observations that required reporting.

Von Frey testing. Two separate studies were conducted with separate surgical cohorts of rats. In the first study, rats were tested for nociceptive-associated behaviors from 90 to 104 days post surgery. No a priori critieria were utilized to select them for this study. These rats were not sensitized to the Von Frey testing procedure (see Okun et al., 2016) as described previously (Simmons et al., 2014). In the first experiment, we utilized i.p. dosing but then used p.o. dosing in the second experiment. Testing was conducted with Von Frey hairs of 1, 2, 4, 6, 8, 15 g force in ascending order The second experiment used the sensitization training method of Simmons et al. (2014) to reduce the baseline nociceptive reactivity scores. Rats were studied from 28-35 days post surgery. Von Frey testing was conducted with rats in suspended chambers with wire mesh bottoms. After habituation to the chamber for 30 min, a series of calibrated von Frey filaments were applied perpendicular to the plantar surface of the ipsilateral hindpaw until the filament buckled. Filaments ranging from 0.2 to 15 g were utilized as per Chaplan et al. (1994). For both experiments, the upper limit was set at 15 g such that a value of 15g in the results represents $\geq$ 15g force. The up-down method was used to determine the 50% withdrawal threshold with the Dixon nonparametric test as described previously (Chaplan et al., 1994). Testing was performed blinded to treatment.

Data were analyzed by repeated measures (time) two-way ANOVA followed by post-hoc multiple comparison tests. In the first experiment, data were also analyzed using only one time period using one-way ANOVA followed by Dunnett's test comparing vehicle to effects of gabapentin or KRM-II-81. In all analyses, alpha was set at 0.05.

Plasma and brain exposures. Plasma exposure of KRM-II-81 was studied in rats in order to define the exposures associated with antinocicptive drug effects. For this, three rats each were dosed with 10 mg/kg KRM-II-81 by i.p. or p.o. routes and blood was taken by decapitation at 0.25, 0.5, 1, 2, 4, 8, and 12 h post dosing. This one dose was selected in order to conserve test material and experimental animals. Three Study samples of KRM-II-81 in plasma and brain were analyzed by LC-MS/MS using a Sciex API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS; Foster City, CA) equipped with a Turbo-Ion-Spray interface, and operated in positive ion mode. The analyte was chromatographically separated using a Betasil C18 5µm 20x2.1mm Javelin (Thermo Electron Corp. Cat# 70105-022106 (pk of 4)). The pumps were Shimadzu LC-10AD units with an SCL-10A controller (Kyoto, Japan), and a Leap CTC liquid handler (LEAP Technologies, Carrboro, NC) was used as the autosampler. Water/1M NH_4HCO_3 (2000:10, v/v) (Mobile Phase A), and MeOH/1M NH_4HCO_3 (2000:10, v/v) (Mobile Phase B) were

used. For KRM-II-81 the gradient profile changed from 10% B at 0 min, 47% B at 0.01 min to 0.20 min, 77% B at 0.30 to 0.40 min, and 98% at 0.41 to 0.72 min. The flow rate was 1.5 mL/min from 0 to 0.49 min, and increased to 2.5 mL/min from 0.51 to 0.72 min. Chromatography was performed at ambient temperature, with flow directed to the mass spectrometer between 0.25 and 0.50 min. The selected reaction monitoring (SRM) (M+H) transition m/z was 352.2 > 297.1 for KRM-II-81. The Turbo Ion Spray temperature was maintained at 750 °C, with collision, curtain, nebulizing, and desolvation gas (nitrogen) settings of 40, 10, 70, and 50, respectively. The ion spray voltage was set to 4500 V, while the respective declustering, entrance, and exit potentials were 80, 10, and 8.

Calibration standards were prepared by serial dilution of a 100 ug/mL analyte stock solution (95:5:1 formamide:water:5N HCl) with methanol/water (1:1, v/v) w/0.1% Acetic Acid, which were then used to fortify control plasma and brain homogenates to yield analyte concentrations of 1, 5, 10, 20, 50, 100, 500, 1000, 2000, 4000, and 5000 ng/mL. A 25 uL aliquot of each study sample, appropriate calibration standard and control matrix were transferred to a 96-well plate. The study samples, calibration standards, and control matrix sample were then mixed with 25 uL of acetonitrile/ methanol (1:1, v/v) w/0.1% acetic acid containing internal standard and 150 uL of acetonitrile. After mixing, the samples were centrifuged to pellet the precipitated proteins, and 150 uL of the resulting supernatants were transferred to a clean 96-well plate and evaporated under nitrogen at 40C. Samples were then reconstituted with 50 uL of 0.5% HFBA in water. Ten microliter aliquots were analyzed by LC-MS/MS.

Although data for plasma were collected only at 10 mg/kg, estimated exposures at 30 mg/kg were made from the current data combined with those of Poe et al. (2016) and higher dose exposure data from Witkin et al. (2018). Likewise brain levels were estimated from brain/plasma relationship data in Poe et al. (2016). The brain concentration at 60 min estimated in this way was exactly as found by experiment.

Dorsal root ganglion electrophysiology. Primary culture of DRG neurons were prepared as described (Bleakman et al., 1996). Briefly, DRG were isolated from 2-month old rats (235 – 310 g) and digested enzymatically with collagenase (type II, 3 mg/ml) for 50 min at 37 °C. After 50 min digestion the tissue was mechanically dissociated with a series of sterile fire-polished glass pipettes of decreasing diameter. The dissociated neurons were plated directly on Nunc 35x10 tissue culture dishes and incubated in Nb Active1 (Brainbits LLC, Springfield, IL) supplemented with 5% dialyzed fetal bovine serum, 0.25% Glutamax (Gibco). Cell cultures were maintained in a tissue incubator (37 °C, 5% CO_2) and used within seven days.

DRG neurons were recorded at room temperature using conventional whole-cell voltage clamp methods as described (Cerne et al., 2016). We attempted to record from relatively small diameter neurons like those involved in nociception. Other markers of nociceptive neurons were also not evaluated. Therefore, the present recordings should be considered as deriving from primary afferent neurons involved in sensory transmission. Glass capillary tubing was pulled in three stages to yield micropipettes with tip diameters of approximately 1-2 microns. Pipettes were filled with a solution containing (in mM): 70 CsCl, 60 CsF, 5 NaCl, 10 EGTA, 10 mM Hepes at pH 7.4. After forming a high resistance (>10 MΩ) seal between the plasma membrane and the pipette, the patch of membrane inside the pipette was ruptured by applying negative pressure. Throughout the experiment, cells were bathed in a solution containing (in mM): 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 0.2 $MgCl_2$, 10 glucose, 10 Hepes at pH 7.4. Using these solutions, liquid junction potentials were less than 4 mV. No correction for this voltage was made. The voltage across the cell membrane was controlled and the sodium currents were measured using an

Axopatch 200B amplifier controlled by Clampex 10.3 software and used in combination with a Digidata 1440A A/D converter (Molecular Devices, Sunnyvale, CA). Data were analyzed off-line using Clampfit 10.4 (Molecular Devices, Sunnyvale, CA) and GraphPad Prism 6.02 (GraphPad Software, San Diego, CA). Sample frequency was 30 kHz. Voltage errors were minimized by series resistance compensation (75-85%), and the capacitance artifact was canceled using the amplifier's built-in circuitry. Cells were voltage clamped at -80 mV for the duration of experiment. GABA currents were activated by 4s perfusion with GABA at an EC_{10} concentration using a ValveBank II eight channel perfusion system (AutoMate Scientific, Berkley, CA). Stock solutions of test compounds were prepared in DMSO and diluted into the external solution or the GABA EC_{10} solution such that DMSO concentrations did not exceed 0.1% (v/v). Compound was perfused for two minutes followed by 4s co-administration with GABA. During the recording the Nunc 35x10 tissue culture dishes were continuously perfused with external solution using a gravity flow perfusion system.

The data were normalized to the baseline GABA EC_{10}–evoked current amplitude and reported as mean ± standard error of the mean (SEM). The concentration–response data were fit using the four parameter Hill equation.

Respiratory Effects. These experiments were conducted as an independent assessment of comparative potency and efficacy of KRM-II-81 to decrease respiratory parameters compared to alprazolam, a drug found most often in benzodiazepine over-dose victims from emergency room samples (Jann et al., 2014). Thus, the present study was conducted to estimate relative safety of KRM-II-81 to alprazolam. Doses were selected as described below and were counterbalanced within-subject where each rat received each dose. Doses were not selected to fully overlap those used in the pain assessment assays. Eight rats were tested. Using a method adapted from Bassi et al. (2015), rats were allowed 60 min to acclimate to the recording chamber before each dose was tested. Next, the chamber was opened, the animal was administered drug or vehicle, and it completed either a 30-min pretreatment after KRM-II-81 administration (1.0 mg/kg, 3.2 mg/kg, and 10 mg/kg, i.p.) or a 15 min pretreatment after alprazolam administration (0.32 mg/kg, 1.0 mg/kg, and 3.2 mg/kg, i.p.). Pretreatment times were selected based on the peak-plasma concentrations (Poe et al., 2016). Subjects spent their respective pretreatment times in the plethysmograph chamber. The selection of doses for each drug was made to capture anxiolytic doses of KRM-II-81 (3.2 mg/kg - unpublished elevated zero maze data to 10 mg/kg for Vogel conflict data in rats in Poe et al., 2016) and alprazolam (1.0 mg/kg; Griebel et al., 1996). Subsequently, the chamber was filled with 100% oxygen, sealed, and pressure fluctuations were measured for 1 min. One mL of air was then pumped into the chamber for pressure calibration. All rats received both drugs and all doses, and the order of treatments was counterbalanced across subjects.

Pulmonary ventilation measures were compared across treatment conditions using a repeated measure one-way ANOVA, with Dunnett's multiple comparison tests assessing for differences from vehicle. Pulmonary ventilation was calculated using a plethysmography method adapted from Bassi et al. (2015). Respiratory frequency (f) and tidal volume (VT) were determined for each baseline and drug condition by selecting data within the 1-min sampling period that contained rhythmic breathing (i.e., signals that included oscillations caused by gross body movement were excluded from analysis) using PowerLab data acquisition software. Minute Volume was determined by formulas adapted from Bassi et al. (2015). All statistical tests were performed with Prism 6 (GraphPad Software, San Diego,CA).

Locomotor Activity. Locomotor activity was measured with a photobeam activity system (San Diego Instruments, San Diego, CA, USA) with seven photocells per activity areana. Locomotor activity was recorded as the number of ambulations, where ambulation was defined as the breaking of adjacent photobeams. Rats were dosed with KRM-II-81 (10, 30, or 100 mg/kg, p.o., comparable to the spinal nerve ligation study #2) and placed individually into polypropylene cages (40.6 x 20.3 x 15.2 cm, no bedding) after 60 min. Locomotion was then automatically assessed for the next 60 min period. Diazepam was run as a comparator in the locomotor activity assay in rats with i.p. dosing, 30 min prior to testing as per dosing paramaters for the formalin experiment.

Compounds. KRM-II-81 was synthesized by K.R. Methuku and G. Li, University of Wisconsin-Milwaukee, as previously described (Poe et al., 2016, and Li et al., 2018). Gabapentin (Neurontin) was obtained from Pfizer, Groton, CT, USA). In the respiration studies, alprazolam (Toronto Research Chemicals, Inc - Ontario, Canada) and KRM-II-81 were dissolved in 60% propylene glycol and 40% sterile water. Tramadol HCl (Sigma Aldrich) was dissolved in distilled water and dosed i.p. Diazepam (Sigma Aldrich Corporation, St. Louis, MO, USA) was suspended in in 1% hydroxyethylcellulose carboxymethylcellulose/0.05% Tween 80/0.25% Dow antifoam and dosed i.p. KRM-II-81was also suspended in the media used for diazepam and dosed in a volume of 1 ml/kg in rats below doses of 30 mg/kg; 30 mg/kg (dosed at 3 ml/kg), 60 mg/kg (dosed at 6 ml/kg). Gabapentin was dosed orally in a volume of 5 ml/kg. All other drugs were given in a dose volume of 1 ml/kg.

Results

Formalin pain model. Diazepam (1-10 mg/kg, i.p.) decreased early phase nociceptive events at doses of 3 and 10 mg/kg ($F_{4,39}$ = 7.19, p<0.001). Significant reversal of late phase pain behaviors was not observed up to 10 mg/kg with diazepam when compared to vehicle control values ($F_{3,31}$ = 1.96, p=0.14). In contrast, tramadol (80 mg/kg, i.p.) produced a 69.7 $\pm$ 11.7% reversal of pain-associated behaviors during the late phase recordings. (**Fig. 1**).

KRM-II-81 (i.p.) dose-dependently reduced nociceptive behaviors in the late phase of testing with a minimal effective dose of 30 mg/kg ($F_{4,38}$=13.6, p<0.001). The 30 mg/kg dose produced effects equally efficacious to the positive control tramadol (80 mg/kg, i.p.). Early phase behaviors were significantly affected only by a dose of 100 mg/kg KRM-II-81 and by tramadol ($F_{4,38}$ = 4.84, p<0.01) (**Fig. 1**).

L5/6 ligation pain model. Spinal nerve-ligated rats were tested for nociceptive behaviors either in the presence of vehicle, KRM-II-81 (30 mg/kg, i.p.) or gabapentin (50 mg/kg, i.p.), used as a positive control. When analyzed over the 4 hr period including the baseline period, there were no significant differences across treatments ($F_{2,12}$=1.62, p=0.24). However, there was a significant effect of time ($F_{4,48}$=10.9, p<0.0001), matched subjects ($F_{12,48}$=3.24, p<0.01) and a significant interaction ($F_{8,48}$=3.15, p<0.01). Based upon these findings, a post-hoc analysis was performed on the treatment effects at the 1 hr time period. ANOVA demonstrated a significant effect of treatments ($F_{2,12}$=6.12, p<0.05) and multiple comparison analysis by Dunnett's test revealed that both KRM-II-81 (p<0.01) and gabapentin (p<0.05) significantly separated from vehicle control (**Fig. 2**).

A second study was performed using oral dosing and with a method known to reduce baseline behavioral response scores (Simmons et al., 2014). In this experiment, baseline levels of paw withdrawal thresholds were reduced by the sensitization methods of training prior to testing (**Fig. 3**). With the sensitization method, control levels for paw withdrawal thresholds were 0.65 ± 0.21 g compared to 5.1 ± 2.8 g in the previous experiment (**Fig. 2**). Dose-effect data for KRM-II-81 were analyzed by two-way ANOVA without the baseline data were there was found to be a significant effect of treatment (KRM-II-81 and positive control) ($F_{4,205}$=32.3, p<0.001) and time ($F_{4,205}$=18.5, p<0.001). There was also a significant treatment x time interaction ($F_{16,205}$=3.04, p<0.001). Both 30 and 100 mg/kg KRM-II-8, p.o, significantly increased paw withdrawal thresholds in these rats as did the positive control drug gabapentin (75 mg/kg, p.o.). The time-course for gabapentin was generally as previously reported with a trend for activity at 1 hr (Johnson et al., 2017).

Exposure of KRM-II-81. Plasma exposure of KRM-II-81 was obtained at different time points to compare drug exposures to effects in the pain models. The unbound plasma exposure of KRM-II-81 (10 mg/kg) after either oral or intraperitoneal dosing in rats is shown in **figure** 4 (left panel). For both dosing routes, there were time-dependent changes in plasma exposure ($F_{6,28}$=15.4, p<0.0001). There were also differences in exposures that were dependent upon the route of administration ($F_{1,28}$=7.68, p<0.01) but not time x route interaction ($F_{6,28}$=0.83, p=0.56).

PK-PD Relationships. Based upon the exposure data (**Fig. 4, left panel**), relationships between exposure and antinociceptive effects were evaluated. In the spinal nerve ligation study, where a time-course of behavioral effects was obtained, the antinociceptive effects of 10

mg/kg KRM-II-81 increased as a function of plasma drug concentration, reaching a maximum of 3-fold enhancement at 2 hr post dosing (**Fig. 4, right panel**).

Table 1 shows the unbound plasma and brain concentrations of KRM-II-81 at 1, 2, and 4 hr post oral administration (Fig. 4) (time points for which behavioral data were collected in the spinal nerve ligation study) (**Fig. 3**) in relationship to in vitro potencies to enhance GABA currents either in HEK293 cells expressing $\alpha x\beta 3\gamma 2$ $GABA_A$ receptor subtypes or in primary DRG cultures. Measures of target engagement at 10 and 30 mg/kg KRM-II-81 were calculated as the ratio of plasma concentration/in vitro potency (**Table 1**). Thus, target-engagement ratios of < 1 are exposure levels that are less than the specified biological metric (e.g., EC_{50} or EC_{90}). Values greater than 1 are for exposure concentrations that exceed these biological endpoints.
For the recombinant functional assays target engagement ratios were greater for $\alpha 2\beta 3\gamma 2$, and $\alpha 3\beta 3\gamma 2$ than for $\alpha 1\beta 3\gamma 2$. Using EC_{50} levels of biological response, (**Table 1, left columns**), the target engagement ratios are ~1 for $\alpha_1\gamma_3\beta_2$ receptors and > 1 for the other biological measures. When the EC_{90} was used, the target engagement ratios were markedly less than those at the EC_{50} level (**Table 1, right columns**). For $\alpha 1\beta 3\gamma 2$ receptors, this ratio was between 0.1 and 0.2 for all conditions. For the $\alpha 2/3$-associated $GABA_A$ receptors, the target engagement ratio for plasma levels was substantially higher but still <1 for 10 mg/kg KRM-II-81. At 30 mg/kg, the target engagement ratio increased but reached only 70% at 60 min post dosing when antinocicpive measurements began. In contrast, brain concentrations of KRM-II-81 (30 mg/kg) were equal to the EC_{90} value for $\alpha 2\beta 3\gamma 2$ receptors. The EC_{90} values for GABA enhancement in dorsal root ganglia were >1. **Dorsal Root Ganglion Electrophysiology.** The effect of KRM-II-081 on native GABA currents was examined in DRG neurons isolated from rats. The negative current evoked by saturating GABA (1mM) application showed a rapid activation followed by a slow desensitization (**Fig. 5A**). When GABA was applied at the concentration which only partially activates GABA channels (EC_{10}), it evoked a slowly activating current with no pronounced desensitization. Co-application of 10 µM KRM-II-081 potentiated the partially activated GABA current (EC_{10}) to 355%. The potentiation of partially activated GABA currents by KRM-II-081 was concentration dependent with an EC_{50} of 32nM and maximum efficacy of 421% (**Fig. 5B**).

Respiratory effects of KRM-II-81. The non-alpha-selective, $GABA_A$ receptor potentiator, alprazolam, significantly decreased respiration rate at 0.32 mg/kg ($p<0.01$) and 3.2 mg/kg ($p<0.01$). KRM-II-81 significantly decreased respiration rate at only the highest dose tested, 10.0 mg/kg ($p<0.05$). All doses of both drugs tested produced no significant change in volume. Alprazolam decreased overall minute volume / kg at the highest dose tested ($p<0.05$). However, KRM-II-81 did not produce any decrease in minute volume / kg (**Fig. 6**).

Locomotor Activity. KRM-II-81 was studied in doses of 10 to 100 mg/kg after oral dosing in rats after a 60 min pretreatment as used in the spinal nerve ligation study. KRM-II-81 decreased locomotor activity ($F_{3,168} = 7.2$, $p<0.001$) as a function of time post dosing ($F_{5,168} = 209$, $p<0.001$). Post-hoc analysis demonstrated effects of 10, 30, and 100 mg/kg only at the 10 min time period ($p<0.05$ by Bonferonni test). There was not a significant dose x time interaction ($F_{15,168} = 1.38$, $p>0.05$). Although there was an overall effect of dose on locomotion, this effect was small and not dose-dependent. Two-way ANOVA comparing 10, 30, and 100 mg/kg showed a lack of effect of dose ($F_{2,126} = 2.02$, $p>0.05$) with a significant effect of time ($F_{5,126} = 118$, $p<0.001$) and no dose x time interaction ($F_{10,126} = 0.434$, $p>0.05$) (**Fig. 7**).

Diazepam was run as a comparator in the locomotor activity assay in rats. After i.p. dosing, 30 min prior, as used in the formalin assay, diazepam significantly decreased locomotion

($F_{1,60}$ =89, p<0.001) with time as a significant factor ($F_{5,60}$= 263, p<0.001) and a significant dose x time interaction ($F_{5,60}$ =21.5, p < 0.001) (**Fig. 7**).

Discussion

The potential utility of positive allosteric modulators (PAMs) of $GABA_A$ receptors containing α2/3 subunits has recently gained serious scientific attention. The pioneering work on single amino acid substitutions to create mouse lines with precise α-subtype composition has been a major factor driving interest in this mechanism (Knabl et al., 2009; Ralvenius et al., 2015, 2016). Subsequently, the α2/3-selective $GABA_A$ receptor PAM, KRM-II-81 (Poe et al., 2016), was shown to be active in several assays utilizing acid as a pain provocation (Lewter et al., 2017; Moerke et al., 2019) and structural analogs were active against inflammatory pain (Fischer et al., 2017; Kannampalli et al., 2017). Work on inflammatory bladder pain has, in addition, shown that there are significant reductions in the levels of $GABA_A(\alpha2)$ in the lumbosacral spinal cord in the pain-induced rats (zymosan-treatment) (Kannampalli et al., 2017). The present series of experiments extends this work documenting the efficacy of KRM-II-81 against formalin-induced, and spinal nerve L5/6 ligation induced pain states in rodents. The spinal nerve ligation model is notable as it is a model of chronic pain (Okun et al., 2016). The systematic replication of efficacy in the spinal nerve ligation model studied under different conditions, highlights the robustness of the activity of KRM-II-81. However, we did not evaluate chronic dosing with KRM-II-81 in the spinal nerve ligation model, a set of experiments that would help inform the reality of therapeutic need for patients with chronic neuropathic pain.

KRM-II-81 also potentiated GABA currents in dorsal root ganglia isolated from rat spinal cord in the present study. These ex-vivo data are consistent with the $GABA_A$ potentiator profile of KRM-II-81 gleaned from other methods (Poe et al., 2016). The activity of KRM-II-81 in dorsal root ganglia is significant in the present context for showing activity in sensory neurons that might be involved in pain signaling. However, we have not done experiments here to define either the specific neuronal populations from which the recordings were made or the presence of $GABA_A$ receptors containing α2/3 subunits on these neurons or on innovating neurons. If some of the sensory neurons recorded were nociceptors, there is evidence for the presence of α2- and α3-containing $GABA_A$ receptors on those neurons and for their involvement in pain perception (Lian et al., 2012; Lorenzo et al., 2014; Paul et al., 2014; Ralvenius et al., 2018; Witschi et al., 2011). The potency of KRM-II-81 was about an order of magnitude greater in DRG neurons than in human recombinant receptors in our study. We do not have a definitive explanation of this potency difference at this time. Differences in species and tissue preparations (recombinant vs native) are likely contributors. For example, in native neurons GABA receptors make complexes with other membrane proteins which in turn modify pharmacology (cf., Chen and Olsen, 2006).

The data on KRM-II-81 presented here complement the findings of KRM-II-81 and analogs as a modulator of acid-induced pain (Lewter et al., 2017; Moerke et al., 2019) and inflammatory pain with a structural analog (Fischer et et., 2017). However, it is important to note some discrepancies that require further investigation. Doses of KRM-II-81 active in the previous reports were lower than the 30 mg/kg reported here. Whether this is due to the use of mice in the prior reports and/or the nociceptive behaviors studied needs to be determined. Most striking, however, was the report that the structural analog, KRM-II-18B, was also active in the acid-induced pain models (Lewter et al., 2017). Since KRM-II-18B was not selective for α2/3-containing $GABA_A$ receptors (Lewter et al., 2017), the positive findings raise concern that this structural class might be working through mechanism disparate from or overlapping with the positive allosteric modulation of α2/3-associated $GABA_A$ receptors. Although other mechanisms might play a role, this possibility requires further experimental scrutiny, especially for KRM-II-18B. In the meantime, the data for KRM-II-81 to be acting as an α2/3-selective $GABA_A$ receptor

PAM appear on solid ground that is substantiated from the functional selectivity data (Poe et al., 2016; Lewter et al., 2017), the lack of activity at a host of ancillary protein targets (Poe et al., 2016), the separation of $GABA_A$-driven efficacy from motor side effects (Poet et al., 2016; Witkin et al., 2017, 2018).

KRM-II-81 exposes plasma (Poe et al., 2016; Witkin et al., 2018; present study) and brain (Poe et al., 2016; Witkin et al., 2018) after systemic dosing. Comparison of time-course data for plasma exposure of KRM-II-81 and the time-course for effects against pain-evoked tactile hypersensitive demonstrated a positive association between these measures (Fig. 3). Calculation of target engagement ratios (Table 1), enabled assessments of the selectivity of KRM-II-81 for $\alpha2\beta3\gamma2$ and $\alpha3\beta3\gamma2$ over $\alpha1\beta3\gamma2$ receptors from a pharmacokinetic perspective. Plasma and brain exposures of KRM-II-81 produced less engagement of $\alpha_1\gamma_3\beta_2$ confirming electrophysiological functional measures of target selectivity based upon drug concentration. Although in vitro data, summarized above, demonstrate the selectivity of KRM-II-81 for α2/3-associated $GABA_A$ receptors, the mechanism of action of KRM-II-81 in vivo requires further investigation. In the present study, we have begun to generate the necessary PK/PD relationships for this purpose. Target engagement ratios generated in the present experiments enabled assessments of the degree of engagement of a mechanism needed for analgesic efficacy. These data showed that greater than EC_{50} levels need to be reached to generate significant analgesic effect with KRM-II-81. Brain levels reaching EC_{90} concentration levels were needed to produce efficacy and corresponded to a dose of 30 mg/kg. In contrast, the degree of target engagement to activate dorsal root ganglia was shown to be somewhere between concentrations that achieve EC_{50} and EC_{90} levels of activation. The relatively high levels of target engagement are in line with predictions of the high levels of receptor occupancy for $\alpha2\beta3\gamma2$ needed for analgesia (Ralvenius et al., 2015). Overall, these data relating drug exposure to biological effect begin, for the first time, to define the in vivo mechanism associated with the analgesic effects of KRM-II-81. However, additional PK/PD data are needed to provide definitive linkage of behavioral effects to central mechanisms. For example, brain microdialysis studies (e.g., Hu et al., 2018) would enable determination of exact values of free brain concentrations, which could then be directly correlated with in vitro and in vivo findings.

$GABA_A$ receptor PAMs that are not selective for specific alpha protein compositions have also been demonstrated to affect nociceptive responses under some conditions but results have potentially been confounded by motor impairment (Rosland et al., 1987; Grégoire et al., 2012). The models utilized in the present experiment to evaluate pain sensitivity are also dependent upon motor performance. As such, dramatic impairments of motor performance present a potential confounding factor in the interpretation of either positive or negative findings. For example, diazepam is not typically active in rodent models of increased nociceptive sensitivity, as in the present study with formalin (no data were collected in the spinal nerve ligation assay), due to its motor incapacitating effects. If the motor-impairing effects of diazepam are mitigated (e.g., by inactivation of the $GABA_A$-associated $\alpha1$ protein) (Knabl et at., 2009; Ralvenius et al., 2015), efficacy of diazepam against pain can be rescued. In the present study, doses up to 10 mg/kg were not active in the formalin model as assessed in the late-phase behavioral responses. Diazepam (3 and 10 mg/kg) significantly decreased early-phase behaviors which can be used to estimate potential motoric impairment. Explicit evaluation of effects of diazepam on motor performance showed significant impact upon locomotor activity at 10 mg/kg. A comparable situation arose when KRM-II-81 and diazepam were compared in antidepressant-detecting mouse models. In these experiments, KRM-II-81,

but not diazepam, was active. However, if the motor-impacting effects of diazepam were attenuated by the α1-selective $GABA_A$ receptor antagonist β-CCT, diazepam also produced an antidepressant-like behavioral signature (Methuku et al., 2017). Similarly, ataxic effects of diazepam were significantly attenuated by β-CCT without altering its anticonvulsant effects (Witkin et al., 2018).

Previous assessments of motor-impairing effects of KRM-II-81 in rats have shown an improvement in this side-effect profile over that of diazepam. In that regard, this mechanism has demonstrated reduced motor effects in humans already (Atack, 2011; Atack et al., 2011; Zuiker et al., 2016). Using an inverted-screen test of motor impairment, Witkin et al. (2018) reported a minimal impairing dose of diazepam of 10 mg/kg, i.p. and of 170 mg/kg for KRM-II-81, a value 5.7 x greater than required to produce efficacy in the formalin and spinal nerve ligation models reported here. The locomotor activity findings of the present paper are also supportive of a reduction of motoric effects with KRM-II-81 relative to diazepam. In addition, although diazepam decreased early phase formalin responses at 3 and 10 mg/kg, KRM-II-81 did so only at 100 mg/kg. As these early phase responses could be interpreted as a read out of motor-impacting effects of drugs, these data too are consistent with the greater liability induced by diazepam. Structural analogs of KRM-II-81 have been made that need to be more fully profiled for potential improvement in motor-impacting effects (Poe et al., 2016). A recent report has attempted to address the sedative liabilities of GABA ligands by an alternative route. A novel compound with reduced sedative liability, acting upon β2/3-subunit-containing extrasynaptic $GABA_A$ receptors, reversed both thermal- and tactile- induced hypersensitivity in spinal nerve ligated rats (Johnstone et al., 2018).

Another side-effect of $GABA_A$ augmentation is respiratory depression, withbenzodiazepines and alprazolam in particular (Isbister et al., 2004; Jann et al., 2014), as culprits in overdose and emergency room visits (Warner et al., 2016). In the present study, we studied the respiratory-depressant effects of KRM-II-81 relative to the non-alpha-subtype-selective $GABA_A$ receptor potentiator, alprazolam. Dose ranges encompassed anxiolytic doses for each drug. Thus, these doses are lower by about ½ log unit from those active in the pain assessments reported here but encompass the antinociceptive doses previously published (Lewter et al., 2017). In multiple measures of respiratory function, KRM-II-81 was either inactive or less potent than alprazolam. Notably, in the measure of Minute Volume, which accounts for both respiration rate and depth, only alprazolam produced significant respiratory-depressant effects. Other side-effects of $GABA_A$ receptor potentiation also need exploration to help define margins between efficacy and side effect. These should include memory, development of tolerance and dependence and propensity for drug abuse. In this regard, it has recently been reported that KRM-II-81 did not develop tolerance after subacute dosing at doses efficacious in rodent pain models (JM Cook, personal communication).

In conclusion, KRM-II-81 was efficacious in mitigating the increased nociceptive sensitivity induced by formalin and by chronic spinal nerve ligation. These findings add to evidence for analgesic efficacy of KRM-II-81 demonstrated previously against acid-induced (Lewter et al., 2017; Moerke et al., 2019) and against inflammatory pain produced by of analogs of KRM-II-81 (Fischer et al., 2017; Kannampalli et al., 2017). These are the first data showing efficacy of KRM-II-81 and the a2/3-linked $GABA_A$ receptor mechanism in chronic mechanical hypersensitivity induced by spinal nerve ligation. Along with the non-intrusive motor side-effects of this mechanism (Atack, 2011; Atack et al., 2011; Fisher et al., 2017; Poe et al., 2016; Witkin et al., 2017), the present findings add to the growing data base supporting the potential value of positive allosteric modulators of α2/3-containing $GABA_A$ receptors in the treatment of chronic

pain and other pain states (Ralvenius et al., 2015). Given their predicted anxiolytic effects, α2/3-associated $GABA_A$ receptor PAMs offer an additional inroad (c.f., Bandelow, 2015; Atta et al., 2018) into the management of pain.

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New TABLE for revision

Table 1. Unbound plasma and brain concentrations of KRM-II-81 after oral administration and the target-engagement ratio (TER) for each assay GABA potentiation assay[1].

Tissue – Time (min)[2]	[KPM–II–81]	α1 EC_{50} (489.1)	α2 EC_{50} (118)	α3 EC_{50} (205.3)	DRG EC_{50} (32)		α1 EC_{90} (4401)	α2 EC_{90} (1062)	α3 EC_{90} (1800)	DRG EC_{90} (288)
Plasma – 60										
10 mg/kg	610	1.2	5.2	3.0	19		0.1	0.6	0.3	2.1
30 mg/kg	793	1.6	6.7	3.9	25		0.2	0.7	0.4	2.8
Plasma – 120										
10 mg/kg	512	1.0	4.3	2.5	16		0.1	0.5	0.3	1.8
30 mg/kg	666	1.4	5.6	3.2	21		0.2	0.6	0.4	2.3
Plasma – 240										
10 mg/kg	336	0.7	2.8	1.6	10		0.1	0.3	0.2	1.2
30 mg/kg	437	0.9	3.7	2.1	14		0.1	0.4	0.2	2.5
Brain – 60										
10 mg/kg	644	1.3	5.5	3.1	20		0.1	0.6	0.3	2.2
30 mg/kg	1030	2.1	8.7	5.0	32		0.2	1.0	0.6	3.6

[1]The alpha-1, 2, and 3 data are from Poe et al. (2016, Supplemental S1) and represent potencies to potentiate GABA-induced electrophysiological repsonses in human recominant constructs of αxβ3γ2 preparations in HEK293 cells. DRG electrophysiological data are from the present manuscript. Values are in nM.

[2]The values for 10 mg/kg are from experiment (Figure 4). The values for 30 mg/kg are estimated from PK data from Figure 4 and Poe et al. (2016) as described in Results.

DRG : dorsal root ganglia; data from present manuscript
TER: target-engagement ratio = [plasma] or [brain]/EC_X

Legends for Figures

Figure 1. Diazepam (i.p., 30 min prior) did not reduce paw-withdrawal thresholds in the formalin assay during the late phase but decreased early phase behavioral events. KRM-II-81 (i.p., 30 min prior) significantly decreased nociceptive responses in the late phase of the formalin assay but only significantly affected early phase behaviors in the early phase at a dose ½ unit higher. Tramadol (80 mg/kg, i.p., 30 min prior) was active as a positive control in both studies. Each point represents the mean ± SEM of the same 8 rats *p<0.05 compared to vehicle control data.

Figure 2. KRM-II-81 produces analgesic effects in rats undergoing chronic nociceptive hypersensitivity induced by spinal nerve ligation. KRM-II-81 (50 mg/kg) was dosed i.p. 30 min prior to testing. Gabapentin (75 mg/kg) was dosed i.p., 30 min prior to testing. Each point represents the mean ± SEM of the same 5 rats tested at each time point. *p<0.05 compared to vehicle control values. BL: baseline paw withdrawal latencies prior to drug administration.

Figure 3. KRM-II-81 (p.o., 60 min prior) produces analgesic effects in rats after chronic spinal nerve ligation under conditions of sensitization training. KRM-II-81 or gabapentin (75 mg/kg) were dosed orally. BL: baseline paw withdrawal latencies prior to spinal nerve ligation (SNL). Post-SNL: paw withdrawal latencies after SNL.

Figure 4. Left Panel: Unbound plasma exposure of KRM-II-81 after either oral (p.o.) or intraperitoneal (i.p.) dosing at 10 mg/kg. Each bar represents the mean ± S.D. of 3 rats. Right Panel: Increase in paw-withdrawal thresholds as a function of unbound plasma concentration of KRM-II-81 (10 mg/kg, p.o.) (figure 4) in rats exposed to chronic spinal nerve ligation (left panel). Each point represents the mean ± S.E.M. of the same 9 or 10 (30 mg/kg) rats tested at each time point. *p<0.05.

Figure 5. Effects of KRM-II-81 as a potentiator of GABA currents in dorsal root ganglia preparations from rats. **A:** Manual patch clamp recording of currents evoked by saturating GABA concentration (1mM) and by EC_{10} concentration of GABA (5 µM). GABA current evoked with 5µM GABA was potentiated by KRM-II-081(10 µM). **B:** Concentration response curve of KRM-II-081 effect on currents evoked with EC_{10} concentration of GABA. The potentiation is expressed as percent of increase in the amplitude of partially activated GABA current (GABA EC_{10}). All values shown are mean ± SEM for a minimum of 4 replicates.

Figure 6. Comparative effects of KRM-II-81 (i.p., 30 min prior) or to alprazolam (i.p., 15 min prior) on measures of respiration in rats. **a)** Respiration rate, **b)** Tidal Volume **c)** Minute Volume / Kg. Data are means ± S.E.M. for 8 rats. *p<0.05. Veh: vehicle.

Figure 7. Effects of KRM-II-81 (p.o., 60 min prior) or diazepam (i.p., 30 min prior) on locomotor activity of rats. Each point represents the mean ± S.E.M. of 8 (KRM-II-81) or 6 rats (diazepam).

Highlights

- KRM-II-81 is an orally-bioavailable selective potentiator of α2/3 $GABA_A$ receptors.
- GABA currents in rat dorsal root ganglia were potentiated by KRM-II-81.
- KRM-II-81 suppressed pain-associated behaviors in formalin and spinal nerve ligation models in rats
- PK data predicted that high occupancy of α2/3 receptors is required for efficacy.

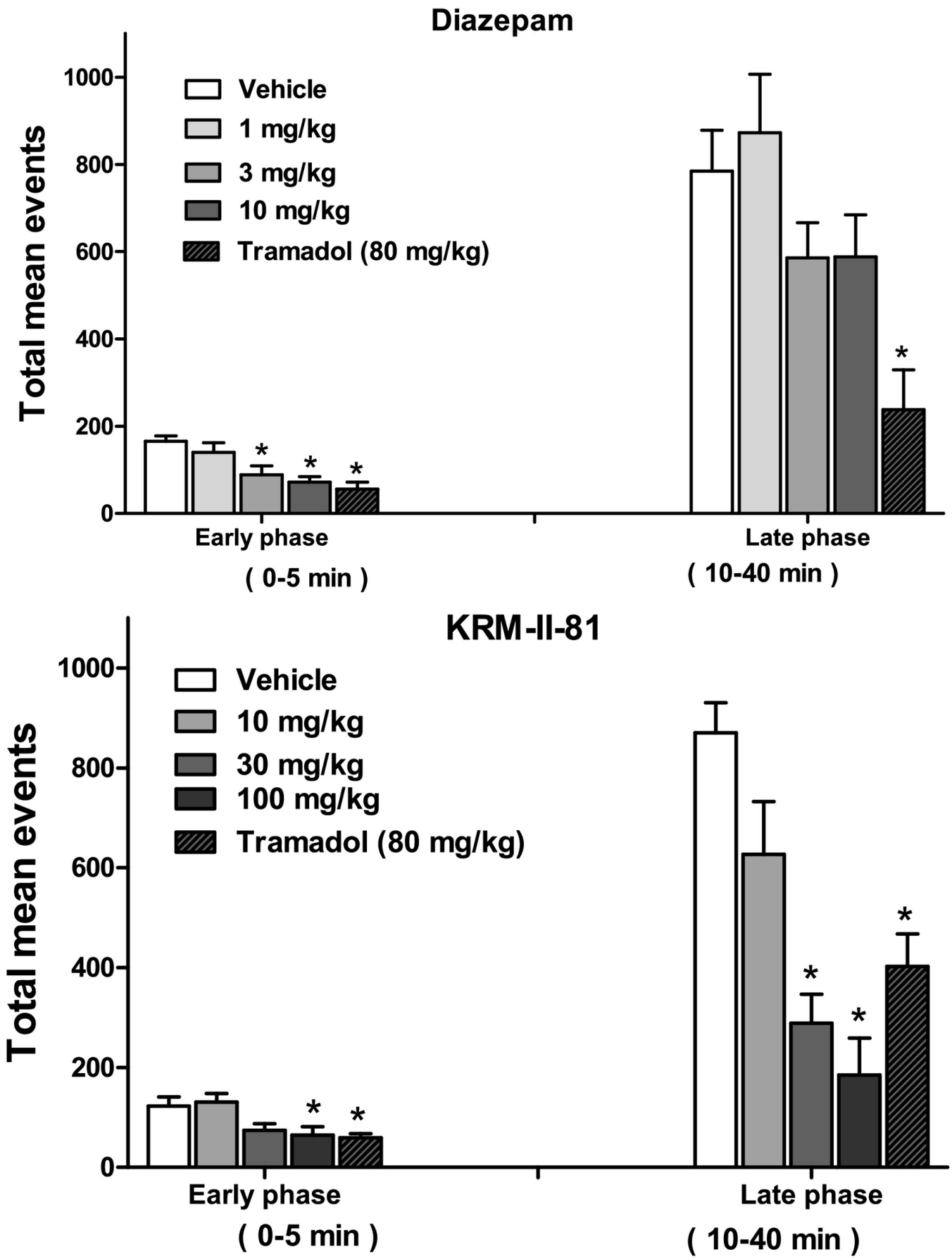

Diazepam
Total mean events
1000
800
600
400
200
0
Vehicle
1 mg/kg
3 mg/kg
10 mg/kg
Tramadol (80 mg/kg)
*
*
*
*
Early phase
(0-5 min)
Late phase
(10-40 min)
KRM-II-81
Total mean events
1000
800
600
400
200
0
Vehicle
10 mg/kg
30 mg/kg
100 mg/kg
Tramadol (80 mg/kg)
*
*
*
*
Early phase
(0-5 min)
Late phase
(10-40 min)

Figure 1

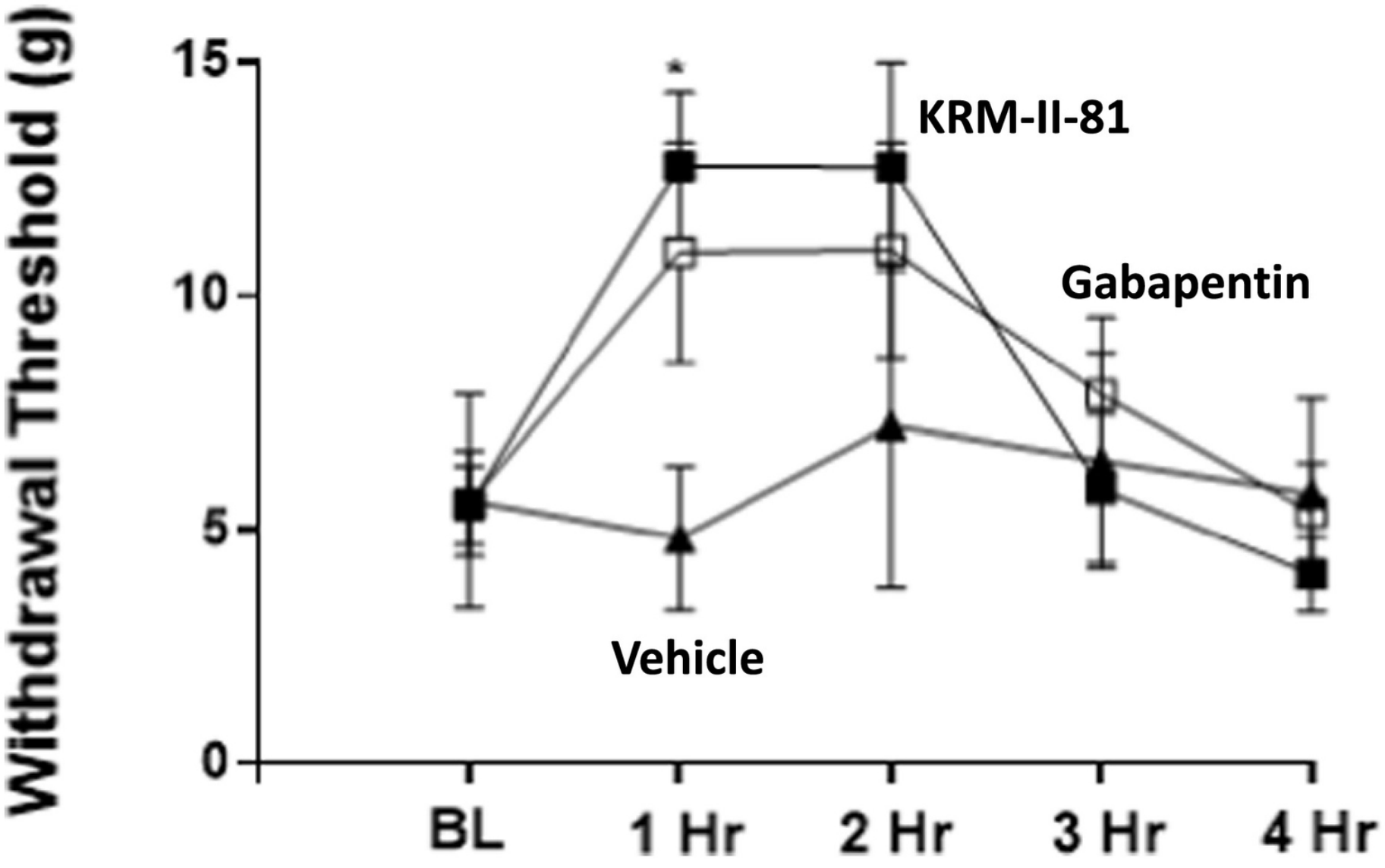

Withdrawal Threshold (g)
15
10
5
0
*
KRM-II-81
Gabapentin
Vehicle
BL
1 Hr
2 Hr
3 Hr
4 Hr

Figure 2

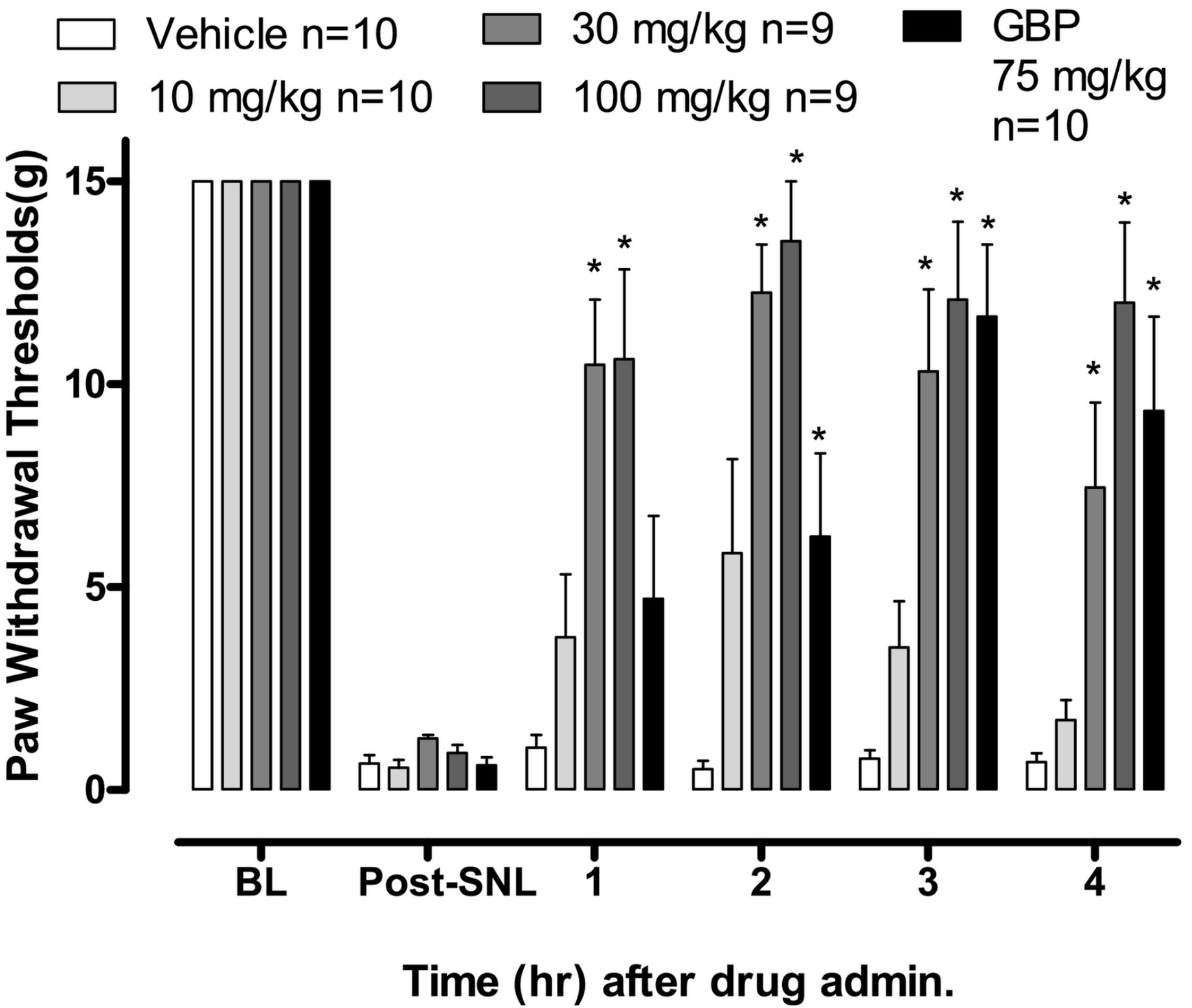
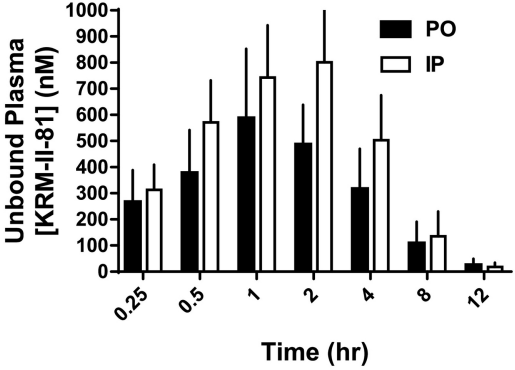

Vehicle n=10
10 mg/kg n=10
30 mg/kg n=9
100 mg/kg n=9
GBP
75 mg/kg
n=10
Paw Withdrawal Thresholds(g)
15
10
5
0
BL
Post-SNL
1
2
3
4
Time (hr) after drug admin.

Figure 3


Unbound Plasma [KRM-II-81] (nM)
1000
900
800
700
600
500
400
300
200
100
0
PO
IP
0.25
0.5
1
2
4
8
12
Time (hr)

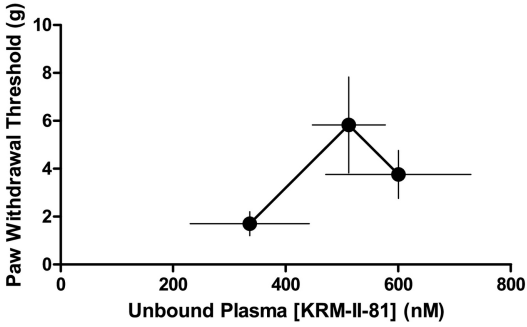

Paw Withdrawal Threshold (g)
10
8
6
4
2
0
0
200
400
600
800
Unbound Plasma [KRM-II-81] (nM)

Figure 4

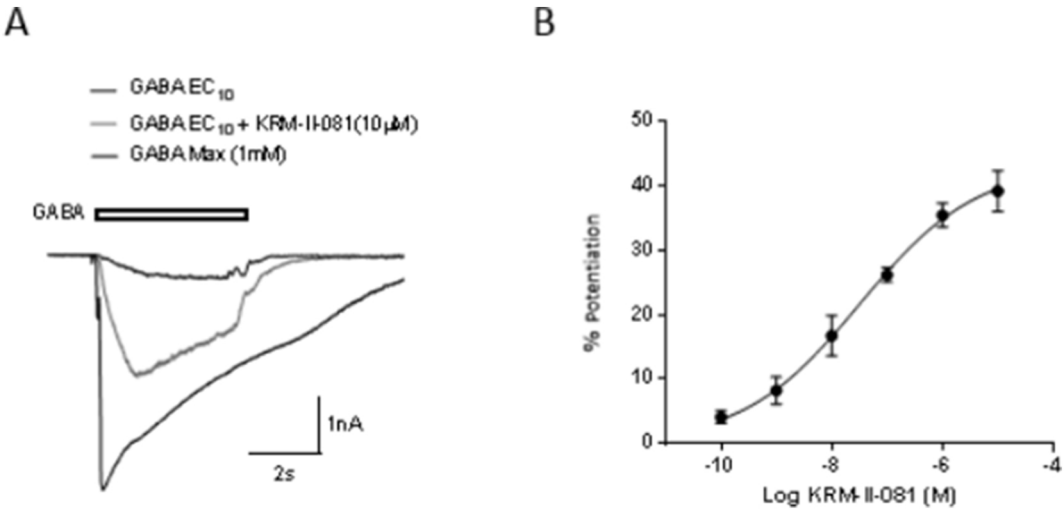

A
GABA EC10
GABA EC10 + KRM-II-081(10μM)
GABA Max (1mM)
GABA
1nA
2s
B
% Potentiation
50
40
30
20
10
0
-10
-8
-6
-4
Log KRM-II-081 (M)

Figure 5

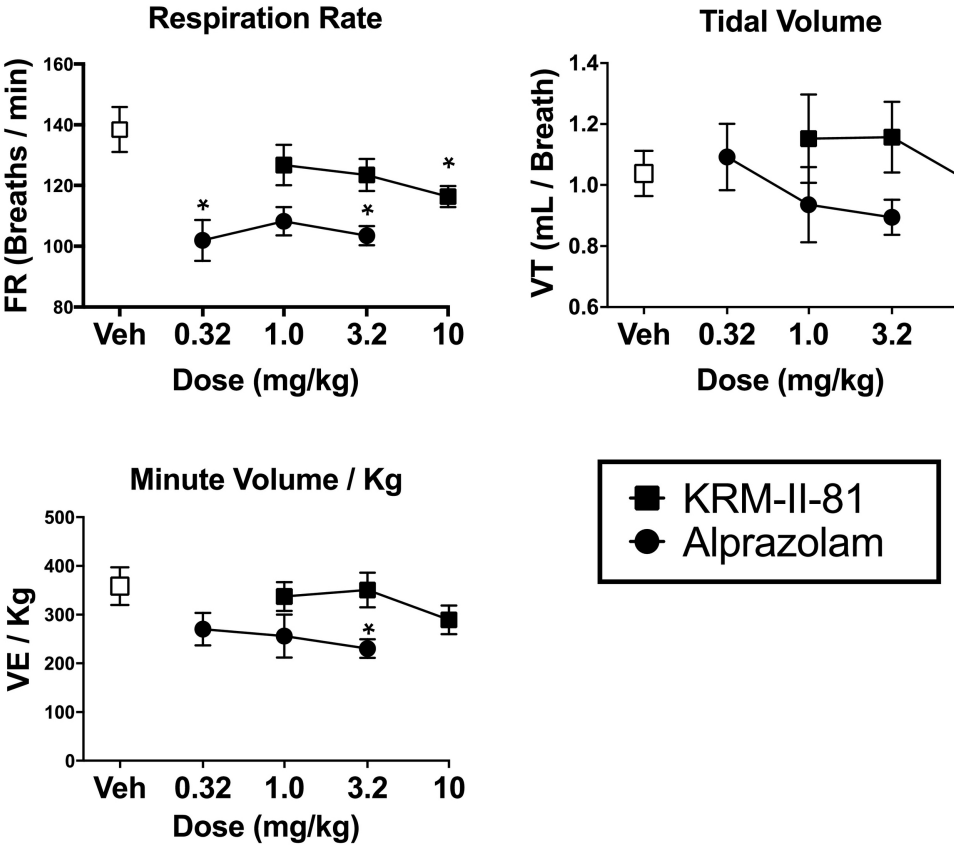
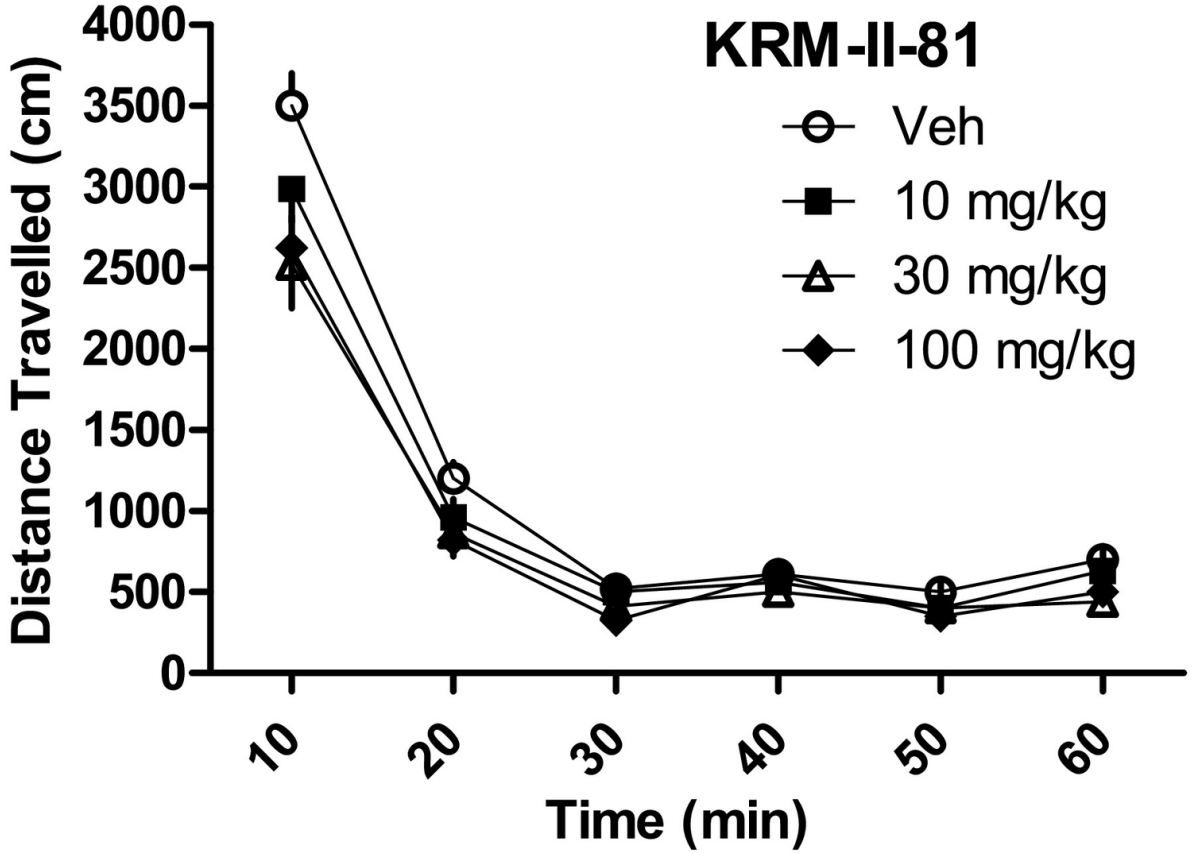

Respiration Rate
FR (Breaths / min)
160
140
120
100
80
Veh
0.32
1.0
3.2
10
Dose (mg/kg)
Tidal Volume
VT (mL / Breath)
1.4
1.2
1.0
0.8
0.6
Veh
0.32
1.0
3.2
10
Dose (mg/kg)
Minute Volume / Kg
VE / Kg
500
400
300
200
100
0
Veh
0.32
1.0
3.2
10
Dose (mg/kg)
KRM-II-81
Alprazolam

Figure 6


KRM-II-81
Veh
10 mg/kg
30 mg/kg
100 mg/kg
Distance Travelled (cm)
4000
3500
3000
2500
2000
1500
1000
500
0
10
20
30
40
50
60
Time (min)

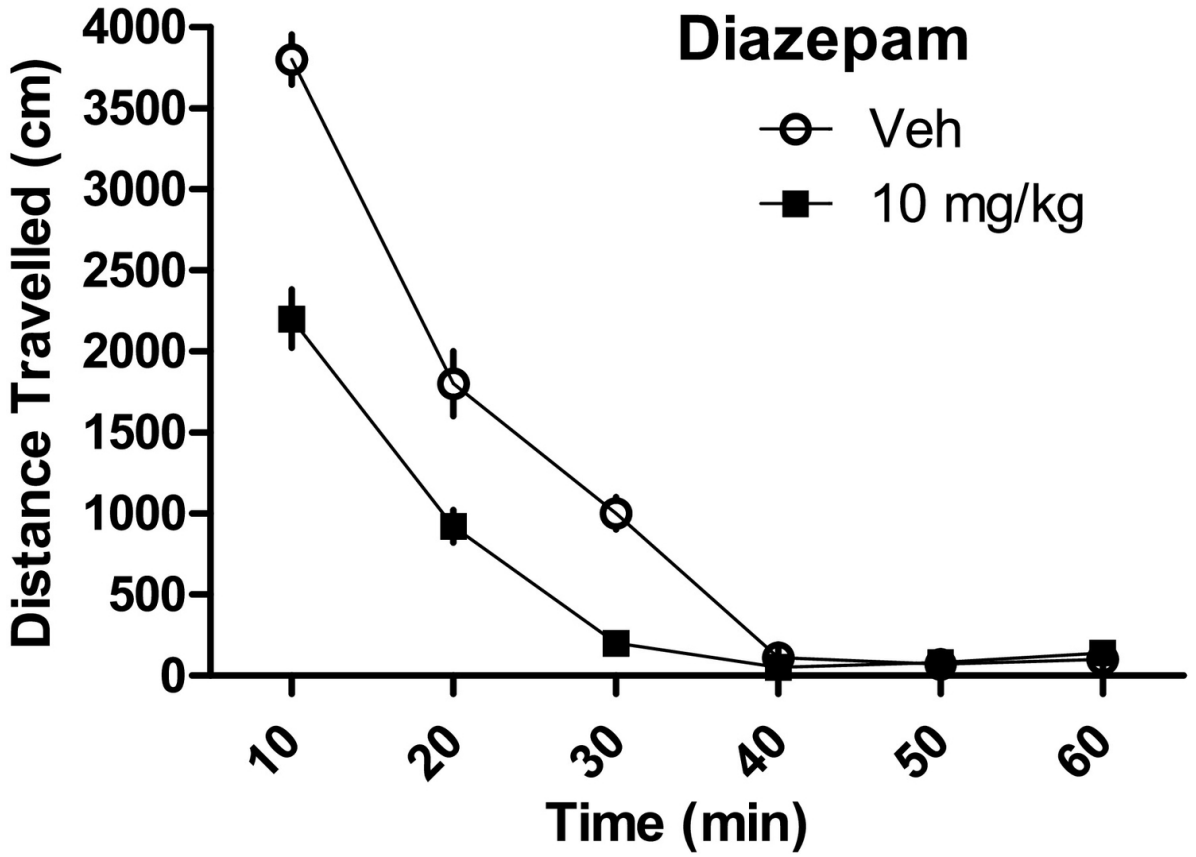

Diazepam
Veh
10 mg/kg
Distance Travelled (cm)
4000
3500
3000
2500
2000
1500
1000
500
0
10
20
30
40
50
60
Time (min)

Figure 7