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Comparison of the Novel Subtype-Selective $GABA_A$ Receptor-Positive Allosteric Modulator NS11394 [3′-[5-(1-Hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile] with Diazepam, Zolpidem, Bretazenil, and Gaboxadol in Rat Models of Inflammatory and Neuropathic Pain

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ABSTRACT

Spinal administration of $GABA_A$ receptor modulators, such as the benzodiazepine drug diazepam, partially alleviates neuropathic hypersensitivity that manifests as spontaneous pain, allodynia, and hyperalgesia. However, benzodiazepines are hindered by sedative impairments and other side effect issues occurring mainly as a consequence of binding to $GABA_A$ receptors containing the α_1 subunit. Here, we report on the novel subtype-selective $GABA_A$ receptor-positive modulator NS11394 [3′-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile], which possesses a functional efficacy selectivity profile of $\alpha_5 > \alpha_3 > \alpha_2 > \alpha_1$ at $GABA_A$ α subunit-containing receptors. Oral administration of NS11394 (1–30 mg/kg) to rats attenuated spontaneous nociceptive behaviors in response to hindpaw injection of formalin and capsaicin, effects that were blocked by the benzodiazepine site antagonist flumazenil. Ongoing inflammatory nociception, observed as hindpaw weight-bearing deficits after Freund's adjuvant injection, was also completely reversed by NS11394. Likewise, hindpaw mechanical allodynia was fully reversed by NS11394 in two rat models of peripheral neuropathic pain. Importantly, NS11394-mediated antinociception occurred at doses 20 to 40-fold lower than those inducing minor sedative or ataxic impairments. In contrast, putative antinociception associated with administration of either diazepam, zolpidem, or gaboxadol only occurred at doses producing intolerable side effects, whereas bretazenil was completely inactive despite minor influences on motoric function. In electrophysiological studies, NS11394 selectively attenuated spinal nociceptive reflexes and C-fiber-mediated wind-up in vitro pointing to involvement of a spinal site of action. The robust therapeutic window seen with NS11394 in animals suggests that compounds with this in vitro selectivity profile could have potential benefit in clinical treatment of pain in humans.

Within the mammalian spinal cord, GABA is the principal inhibitory transmitter and is localized both presynaptically in primary afferents and postsynaptically in dorsal horn interneurones (Malcangio and Bowery, 1996). Immunohistochemical studies suggest that all major classes of $GABA_A$ receptors, e.g., those containing α_1, α_2, α_3, and α_5 subunits (hereafter referred to as $GABA_A$-α_1, α_2, α_3, and α_5, respectively), are variously expressed within the spinal dorsal horn, although $GABA_A$-α_2 receptors predominate within superficial layers that receive nociceptive input from primary afferents (Bohlhalter et al., 1996; Knabl et al., 2008). Loss of GABA function contributes to central hyperexcitability associated with tissue injury, a process referred to as spinal disinhibition. After partial or complete nerve injury, primary afferent evoked inhibitory postsynaptic potentials in dorsal horn neurones are substantially reduced in frequency, magnitude, and duration, probably as a consequence of reduced GABA release (Moore et al., 2002). Nerve injury also modulates levels of GABA, the GABA-synthesizing enzyme glu-

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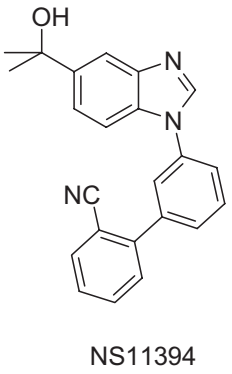
ABBREVIATIONS: L-838,417, 7-*tert*-butyl-6-(2-ethyl-2*H*-1,2,4-triazol-3-ylmethoxy)-3-(2-fluoro-phenyl)-1,2,4-triazolo[4,3-b]pyridazine; CFA, Complete Freund's adjuvant; ACSF, artificial cerebrospinal fluid; CCI, chronic constriction injury; CNS, central nervous system; DR-VRr, dorsal root-ventral root reflex; MA, mechanical allodynia; MH mechanical hyperalgesia; MPE, maximal possible effect; SNI, spared nerve injury; TF, tail-flick; TRPA1, transient receptor potential A1; TRPV1, transient receptor potential V1; WB, weight bearing; ANOVA, analysis of variance

tamic acid decarboxylase and $GABA_A$ receptor expression within the spinal dorsal horn (Castro-Lopes et al., 1993, 1995; Moore et al., 2002). At the behavioral level, $GABA_A$ receptor agonists (e.g. muscimol) and positive allosteric modulators such as diazepam, attenuate nociceptive transmission in animal models of persistent and neuropathic pain associated with central sensitization (Hwang and Yaksh, 1997; Kaneko and Hammond, 1997; Malan et al., 2002).

Why then, have encouraging preclinical observations on the functional importance of $GABA_A$ receptors in the mammalian spinal cord failed to translate into successful clinical treatments for pain? The majority of $GABA_A$ receptors are sensitive to allosteric modulation by benzodiazepine drugs and typically contain the α subunits α_1, α_2, α_3, or α_5, together with a β subunit and a γ_2 subunit in a 2:2:1 stoichiometry (Sieghart 1995). Pharmacological studies, recently combined with the use of genetically engineered mice, have led to a general consensus that $GABA_A$-α_1 receptors mediate sedation and $GABA_A$-α_2/$GABA_A$-α_3 receptors are involved in anxiety, whereas $GABA_A$-α_5 receptors are relevant to memory function (Squires et al., 1979; Griebel et al., 1999a,b; Paronis et al., 2001; Rudolph and Möhler, 2006). Recently, using a combination of molecular and pharmacological techniques, Knabl et al. (2008) reported that $GABA_A$-α_2/$GABA_A$-α_3 receptors are the principal contributors to spinal disinhibition occurring after injury. Importantly, the prototype modulator tested (L-838,417) was analgesic in a range of rat models of persistent pain and was devoid of sedative or motor impairing qualities, albeit no therapeutic window was provided. Thus, development of subtype-selective $GABA_A$ receptor modulators designed to normalize spinal inhibition after injury might offer a novel mechanistic approach for treating neuropathic pain.

To investigate further, we decided to test for antinociceptive actions of the subtype-selective $GABA_A$ receptor-positive modulator NS11394 (Fig. 1) in a range of animal models of chronic pain. NS11394 binds potently and nonselectively (K_i, ~0.5 nM) to $GABA_A$ receptors containing either α_1, α_2, α_3, or α_5 subunits combined with β_2 and γ_2s subunits, whereas affinity at $GABA_A$-α_4 or $GABA_A$-α_6 containing receptors is 600 to 2000-fold lower. Despite this lack of affinity selectivity, in electrophysiological studies, NS11394 differentially modulates these receptors, such that it possesses a functional selectivity profile of $\alpha_5 > \alpha_3 > \alpha_2 > \alpha_1$. This in vitro profile translates to potent (0.1–0.3 mg/kg p.o.) anxiolytic-like effects in rodent shock/nonshock-based models of anxiety, probably attributable to its efficacy at $GABA_A$-α_3 and/or $GABA_A$-α_2 receptors (Mirza et al., 2008). By contrast, at doses 200–600-fold higher, NS11394 minimally impairs motor performance, probably attributable to low efficacy at $GABA_A$-α_1 receptors and poor affinity for $GABA_A$-α_4 and $GABA_A$-α_6 receptors.

To obtain a comprehensive picture of the role of $GABA_A$ receptors in mediating pain-like behaviors after injury, we compared NS11394 to other known allosteric modulators: the full nonselective modulator diazepam, the partial nonselective modulator bretazenil, and the $GABA_A$-α_1-selective full modulator zolpidem. Furthermore, we included the GABA site partial/full agonist gaboxadol to compare the actions of modulators to those of direct receptor activation (Ebert et al., 2006). This comparative pharmacological approach allows us to delineate pharmacologically which $GABA_A$ receptors



NS11394

Ki (nM), rat cortex	0.423
Ki (nM), α1, α2, α3, & α5	0.4, 0.8, 0.5 & 0.1
Ki (nM), α4 & α6	320 & 1000
Emax (%), α1	7.8
Emax (%), α2	26
Emax (%), α3	52
Emax (%), α5	78
T½ (h), rat	2.8
F%, rat	~80%
Brain/plasma, rat	~1
ED_{50} (mg/kg) in-vivo binding, rat cortex	1.3
$MED_{anxiety}$, rat	0.3 mg/kg

Fig. 1. Chemical structure of NS11394 and summary of key data from Mirza et al. (2008). The table shows i) the K_i (in nM) for NS11394 to inhibit [^{3}H]flunitrazepam binding to rat cortical tissue (row 1), [^{3}H]Ro 15-1788 (flumanezil) binding to human $GABA_A$ receptors containing the α_1, α_2, α_3, or α_5 subunits together with β_3 and γ_2s stably expressed in HEK-293 cells (row 2), and human $GABA_A$ receptors containing the α_4 or α_6 subunits together with β_3 and γ_2s stably expressed in HEK-293 cells (row 3); ii) the maximal potentiation (E_{max}, %), relative to diazepam (0.5 μM), by NS11394 of GABA (EC_5–EC_{25}) evoked currents recorded from oocytes containing human $GABA_A$ receptors containing the α_1, α_2, α_3, or α_5 subunits together with β_2 and γ_2s (rows 4–7); iii) half-life ($T½$), bioavailability (F%), and calculated brain/plasma ratio of NS11394 in rat based on dosing animals with 3 mg/kg p.o. and 3 mg/kg i.v. (rows 8–10); iv) ED_{50} of NS11394 for displacing [^{3}H]flunitrazepam binding to rat cortex in vivo (row 11) and minimal effective dose (MED) for anxiolytic-like effect in the rat-conditioned emotional response test (row 12).

might be relevant to pain and, therefore, complements recent findings in transgenic mice (Knabl et al., 2008). However, the approach here is necessary per se to understand pharmacological nuances not easily discernable using a transgenic mouse approach, e.g., level of functional efficacy in addition to selectivity necessary for in vivo efficacy. We also compare the motor side effects of the various $GABA_A$ compounds, thereby deriving therapeutic indices, a fundamental cornerstone in the development of novel therapeutics. Finally, we report on mechanistic studies to determine the effects of NS11394 on the spinal nociceptive circuits that mediate withdrawal reflexes.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (Harlan Scandinavia, Alleroed, Denmark) were used except where stated. They were housed in Macrolon III cages (20 × 14 × 18 cm or 20 × 40 × 18 cm; in groups of 2–5 per cage according to weight) containing wood-chip bedding material (3 × 1 × 4 mm) and in a temperature-controlled environment with a light/dark cycle of 13:11 h (lights on at 6:00 AM and off at 7:00 PM). Food (Altromin GmbH & Co., Lage, Germany) and water were available ad libitum. The animals were allowed to habituate to the housing facilities for at least 1 week before surgery or behavioral testing. Neuropathic animals were subsequently housed on soft bedding material. All behavioral experiments were performed according to the Ethical Guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and licensed by the Animals Experiments Inspectorate, The Danish Ministry of Justice.

Behavioral Tests

Explorative Motility and Rotarod Test. Nonhabituated male rats (body weight 90–120 g) were administered test drug or vehicle and returned to their home cage for 30 to 60 min depending on the drug administered. They were then placed individually in transparent cages (30 × 20 × 25 cm; TSE Systems, Bad Homburg, Germany)

equipped with 12 infrared sensors (6 × 2). Locomotor activity, measured as distance traveled (meters), was monitored automatically in the chambers via the interruption of two consecutive infrared sensors. Interruptions were detected by a control unit and recorded via a computer running ActiMot software (TSE Systems). Raw data obtained via 3-min sampling intervals was summed for the 30-min duration of the experiment and expressed as a percentage of the vehicle response according to eq. 1,

$$\% \text{ Vehicle} = (\text{Post-treatment value/vehicle value}) \times 100 \quad (1)$$

In normal, uninjured rats (body weight 250–350 g), the effects of $GABA_A$ receptor modulators on activity-induced motor function were evaluated using an accelerating rotarod (Ugo Basile, Comero, Italy). The rotarod (6 cm diameter) speed was increased from 3 to 30 rpm over a 180-s period, with the minimal time possible to spend on the rod designated as 0 s and the maximal cut-off time set at 180 s. Rats received two training trials (separated by 3–4 h) on 2 separate days before drug testing for acclimatisation purposes. On the day of drug testing, a baseline response was obtained, and rats were subsequently administered drug or vehicle with the time course of motor performance tested at 30 min after injection for $GABA_A$ receptor modulators and agonists and 60 min after injection of gabapentin. Raw data were subsequently expressed as a percentage of the corresponding baseline response according to eq. 2,

$$\% \text{ Baseline} = (\text{Post-treatment value/baseline value}) \times 100 \quad (2)$$

Hot Plate. For the hot plate test, noninjured rats (body weight 100–120 g) were placed individually on a hot plate (Ugo Basile), maintained at a constant preset temperature of 52.5°C with a cut-off time of 40 s. The baseline latency response (seconds) for the rat to lift either hindpaw induced by the thermal stimulus was then measured, at which point the animal was immediately removed from the hot plate by the investigator to prevent any tissue damage to the hindpaws. Animals were then administered drug or vehicle, and post-treatment latency responses were determined at 30 to 75 min after injection depending on the drug administered, enabling the change in latency response (seconds) to be calculated.

For all experiments involving hot plate measurements, raw data obtained at the time points indicated was converted to a maximal possible effect value according to eq. 3,

$$\% \text{ MPE} = (\text{Post-treatment value} - \text{pretreatment value}) \times 100/(\text{Ceiling value of assay} - \text{pretreatment value}) \quad (3)$$

Formalin Test and Capsaicin-Induced Sensitization. Assessment of formalin-induced and capsaicin-induced flinching behavior in normal, uninjured rats (body weight 180–265 g) was made with the use of an Automated Nociception Analyzer (University of California, San Diego, CA). In brief, this involved placing a small C-shaped metal band (10 mm wide × 27 mm long) around the hindpaw of the rat to be tested. Each rat (four rats were included in each testing session) was administered drug or vehicle according to the experimental paradigm being followed and then placed in a cylindrical acrylic observation chamber (30.5 cm diameter × height 15 cm). For formalin experiments, individual rats were then gently restrained, and formalin (5% in saline, 50 μl s.c.) was injected into the dorsal surface of the hindpaw using a 27-gauge needle. For capsaicin-induced sensitization experiments, individual rats were gently restrained, and capsaicin (10 μg in 10 μl s.c. of 10% Tween 80) was injected into the plantar surface of the hindpaw using a 0.3-ml insulin syringe with a 29-gauge needle (Terumo Europe, Belgium). All rats were immediately returned to their separate observation chambers, each of which was situated upon an enclosed detection device consisting of two electromagnetic coils designed to produce an electromagnetic field in which movement of the metal band could be detected. The analog signal was then digitized, and a software algorithm was applied to enable discrimination of flinching behavior from other paw movements. A sampling interval of 1 min was used. On the basis of the resulting response patterns, for formalin experiments, three phases of nociceptive behavior were identified and scored: first phase (0–5 min), interphase (6–15 min), and second phase (16–40 min) (Munro et al., 2008). For capsaicin-induced sensitization experiments, two phases of nociceptive behavior were identified and scored: first phase (0–5 min) and second phase (6–30 min). For both formalin and capsaicin experiments, raw data from the 1-min sampling intervals were summed for each phase to obtain the total number of flinches occurring during that phase. This value was then expressed as a percentage of the vehicle response according to eq. 1.

Complete Freund's Adjuvant-Induced Inflammatory Nociception. Rats (body weight 300–400 g) were given a subcutaneous injection of CFA (50% in saline, 100 μl; Sigma-Aldrich (Munich, Germany) into the plantar surface of the hindpaw under brief halothane anesthesia. Nociceptive behaviors were routinely assessed for 2 to 3 days before and 24 h after CFA injection. Changes in hindpaw weight bearing were assessed using an incapacitance tester (Linton Instrumentation, Norfolk, UK), which incorporates a dual channel scale used to separately assess the weight distributed to each hindpaw of the rat. Normally, uninjured rats distribute their weight evenly between the two hindpaws (50:50). However, after tissue injury, the rat preferentially favors the noninjured hindpaw, such that the weight-bearing difference can be used as an index of spontaneous nociception. A rat was placed in the supplied Perspex chamber, which is designed so that each hindpaw must be placed on separate transducer pads. The testing duration was set to 5 s, and the digital readout for each hindpaw was taken as the distributed body weight on each paw (grams). Three readings were obtained to ensure that consistent responses were measured. These were averaged for each hindpaw, and the weight-bearing difference was calculated as the difference between the two hindpaws.

Tail-flick responses were also measured in the same CFA rats. A radiant heat source (Ugo Basile) was focused on the underside of the rat's tail 3 cm from its distal end, with the apparatus calibrated to give a tail-flick latency of approximately 4 to 6 s (cut-off time = 15 s). This enabled increases or decreases in tail-flick latency to be measured to the nearest 0.1 s. Two baseline measurements (two measurements each separated by 5 min) were made before CFA injection to familiarize the rats with the testing procedure. Two further baseline latency measurements were obtained on the day of drug testing to ensure that consistent reflex responses were present. Animals were then administered drug or vehicle according to the experimental paradigm, with weight-bearing responses determined at 30, 60, or 120 min after injection, and tail-flick latency responses determined at 60 to 90 min depending on the drug administered. Weight-bearing differences after drug treatment were expressed as a percentage of the corresponding baseline response according to eq. 2. Tail-flick latencies were converted to a maximal possible effect value according to eq. 3.

Peripheral Nerve Injury. A chronic constriction injury (CCI) or spared nerve injury (SNI) was performed in rats (body weight 180–220 g at the time of surgery) as described previously (Bennett and Xie, 1988; Decosterd and Woolf, 2000). Anesthesia was induced and maintained by 2% isoflurane (Baxter A/S, Alleroed, Denmark), combined with oxygen (30%) and nitrous oxide (68%). For CCI rats, the sciatic nerve was exposed at the mid-thigh level proximal to the sciatic trifurcation. Four chromic gut ligatures (4/0) (Ethicon, New Brunswick, NJ) were tied loosely around the nerve, 1–2 mm apart, such that the vascular supply was not overtly compromised. For SNI rats, the skin of the lateral left thigh was incised, and the cranial and caudal parts of the biceps femoris muscle were separated and held apart by a retractor to expose the sciatic nerve and its three terminal branches: the sural, common peroneal, and tibial nerves. The tibial and common peroneal nerves were tightly ligated with 4/0 silk, and 2 to 3 mm of the nerve distal to the ligation was removed. Any stretching or contact with the intact sural nerve was avoided. For

both CCI and SNI rats, the overlying muscle was closed in layers with 4/0 synthetic absorbable surgical suture. The skin was closed and sutured with 4/0 silk thread.

Behavioral Testing of Nerve-Injured Rats. Nerve-injured rats were routinely tested for the presence of hindpaw mechanical hypersensitivity according to previously described methods (Munro et al., 2008). Before testing, individual rats were removed from their home cage and allowed to habituate for 15 min in an openly ventilated 15 × 20-cm white Plexiglas testing cage placed upon an elevated metal grid allowing access to the plantar surface of the injured hindpaw. The presence of mechanical allodynia was assessed using a series of calibrated von Frey hairs (lower limit = 0.06 and upper limit = 13.5 g; Stoelting Co., Wood Dale IL), which were applied to the plantar surface of the hindpaw (lateral aspect in SNI rats given that this is the area of the paw innervated by the intact sural nerve) with increasing force until an individual filament used just started to bend. The filament was applied for a period of 1 to 2 s and was repeated five times at 1 to 2-s intervals. The filament that induced a reflex paw withdrawal in three out of five applications was considered to represent the threshold level for a mechanical allodynic response to occur. The presence of mechanical hyperalgesia was determined in SNI rats by pressing the plantar surface of the hindpaw, with the point of a safety pin, at an intensity sufficient to produce a reflex withdrawal response in normal, unoperated animals but at an intensity that was insufficient to penetrate the skin. A cut-off time of 15 s was applied to long withdrawals often seen for the nerve-injured paw. For both von Frey hair and pin prick measurements, raw data values obtained from 30 to 180 min after drug administration were expressed as a %MPE response according to eq. 3 described above.

Only those animals showing distinct neuropathic behaviors from between 10 and 30 days after surgery were included in drug-testing experiments. During an individual testing session, which used six to eight nerve-injured rats, nociceptive measurements were performed with the observer blinded to treatment. By strictly adhering to a minimum 2 to 3-day drug washout period between experiments, an escalating dose crossover paradigm could be used. Thus, although the majority of SNI and CCI rats typically received more than one drug treatment, no animal received more than i) one injection of vehicle plus injection of the same drug at two different doses or i) one injection of vehicle plus two injections of two mechanistically distinct drugs.

In Vitro Electrophysiology

The Isolated Rat Spinal Cord Preparation. The preparation and in vitro maintenance of the spinal cord followed procedures described previously (Hedo and Lopez-Garcia, 2001). In brief, Wistar rat pups (7–11 days old) were anesthetized with urethane (2 g/kg i.p.), and their spinal cords were extracted after a dorsal laminectomy. With the cord in cold artificial cerebrospinal fluid (ACSF), the outer meninges were removed, and the cord was hemisected and then pinned down to a Sylgard-based recording chamber (NeuroSearch A/S) with the medial side upwards. The preparation was maintained with oxygenated (95% O_2, 5% CO_2) ACSF at room temperature (23 ± 1°C). Flow rate was 5 ± 2 ml/min. The composition of the ACSF was 128 mM NaCl, 1.9 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose, pH 7.4. All drugs tested in vitro were dissolved in ACSF at the desired concentrations and applied to the whole preparation.

Dorsal Root Recordings. The basal potential of the L_4 or the L_5 dorsal root was recorded by means of a suction electrode coupled to a Cyber-Amp amplifier (Molecular Devices, Sunnyvale, CA) set in DC mode (further details in Rivera-Arconada and Lopez-Garcia, 2006). The spontaneous activity was quantified in terms of mean amplitude and frequency in 10-min periods. The spinal cord was challenged by three increasing concentrations of exogenous GABA applied at 10-min intervals. Depolarization in response to GABA applications were quantified as the area of depolarization (in millivolts · seconds). The sequence of three GABA boluses was applied at 45-min intervals. Three control responses were obtained before NS11394 application. NS11394 was applied for 15 min, and responses to subsequent applications of GABA were continued for periods up to 4 h.

Ventral Root Recordings. The L_5 dorsal root and the corresponding ventral root were placed in tight fitting glass suction electrodes. Electrical stimuli sufficient to activate C-fibers (200 μA and 200 μs) were applied to the dorsal root. The stimulation protocol consisted of a single stimulus followed by a 60-s rest and a train of 20 stimuli at 1 Hz. Ventral root responses to stimulation of the dorsal root were recorded using simultaneous AC-DC recording procedures as described previously (Hedo and Lopez-Garcia, 2001). In brief, ventral root signals were split and amplified in different channels of a Cyber-Amp set to DC and AC modes, respectively. The AC channel was band-pass filtered between 300 and 1500 Hz.

The stimulation protocol was applied at 20-min intervals. Three basal responses were obtained before 15-min superfusion with NS11394. Afterward, stimulation was applied during drug application and up to 4 h of washout. To fully characterize the ventral root responses and the effects of the drugs, the following variables were quantified: for DC signals from single stimuli, the amplitude of the monosynaptic reflex (in millivolts) and the area of depolarization measured in a time window between 100 ms and 4 s from stimulus artifact (in millivolts · seconds); for AC signals from trains of stimuli, the number of spikes overshooting a threshold set 5 μV over the mean noise level. Under the present recording conditions, AC recordings reflect fast events, such as action potentials fired by motor neurons, whereas DC recordings reflect slow depolarization of motor neurons.

Compounds, Administration Protocols, and Dose Selection. NS11394 [3′-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile] and zolpidem (tartrate salt) were synthesized in the Medicinal Chemistry Department (NeuroSearch A/S). Gaboxadol (chloride salt) was purchased from Sigma-Aldrich. Gabapentin was purchased from Actavis Nordic A/S (Gentofte, Denmark). Morphine hydrochloride and diazepam were purchased from Nomeco A/S (Copenhagen, Denmark). Bretazenil and flumazenil were gifts from Roche Diagnostics (Basel, Switzerland).

For behavioral studies, NS11394 was dissolved in 5% Tween 80/Milli-Q water (Millipore Corporation, Billerica, MA) and administered orally in a dosing volume of 5 ml/kg. Diazepam, bretazenil, and flumazenil were dissolved in 5% Cremophor (BASF, Ludwigshafen, Germany). Gabapentin was dissolved in 5% glucose/Milli-Q water. These four drugs were administered intraperitoneally in a dosing volume of 2 ml/kg. Gaboxadol and morphine were dissolved in 0.9% saline and zolpidem in 5% glucose/Milli-Q water. These three drugs were administered subcutaneously in a dosing volume of 1 ml/kg. All doses are expressed as either milligram weight salt or milligram weight-free base per kilogram body weight, respectively.

For behavioral studies, we tried to ensure that, over the dose range of each drug tested and using our dosing regime, we had CNS penetration resulting in virtually full occupancy of $GABA_A$ receptors. Thus, all four $GABA_A$ modulators showed good CNS penetration as measured by in vivo [^{3}H]flunitrazepam binding to rat cortex [>90% for diazepam (10 mg/kg), bretazenil (5 mg/kg), and NS11394 (5 mg/kg) and ~70% for zolpidem (20 mg/kg)] (unpublished data; see also Mirza and Nielsen, 2006; Mirza et al., 2008). Although zolpidem appeared at the bottom end of the desired range, this is slightly misleading due to its affinity selectivity for $GABA_A$-α_1 receptors (Sanger and Benavides, 1993). Despite this lower level of in vivo receptor occupancy with zolpidem, it is very clear from the data described under *Results* that major CNS-mediated effects are seen at doses between 1 and 10 mg/kg; therefore, it was not meaningful to dose higher. With respects to gaboxadol, pharmacokinetic data in the rat show clear CNS penetration and CNS-related effects at the doses used here (Cremers and Ebert, 2007).

For electrophysiology studies, NS11394 was diluted in dimethyl

sulfoxide at 10 mM and stored at −20°C. NS11394 was diluted down to 1 μM in ACSF just before use and superfused to the entire preparation for 15-min periods to ensure a complete tissue equilibration. Application of 0.01% dimethyl sulfoxide alone did not have any observable effect on spinal reflexes ($n = 2$). GABA was dissolved in ultrapure water at 10 mM stored at −20°C, diluted down to its final concentration in ACSF just before use where it was delivered to the preparation as a 1-min bolus.

Data Analysis. Comparison of drug effects between injured and noninjured rats across the range of behavioral tests employed was made via conversion of raw data to either percentage of vehicle, baseline or maximal possible effect values. Analysis of data was performed using SigmaStat 2.03 (SPSS Inc., Chicago, IL). All data are presented as mean ± S.E.M. Unless stated otherwise, ANOVA was used to analyze the overall effects of treatments. When the F value was significant, this was followed by Bonferroni's all pairwise comparison. $p < 0.05$ was considered to be statistically significant.

For electrophysiology studies, statistical analysis was performed on raw data using GraphPad Prism (version 3.02). Repeated measures ANOVA was used to analyze responses to trains of stimuli. Results are expressed as mean ± S.E.M. $p < 0.05$ was considered to be statistically significant.

Results

Explorative Motility and Rotarod Test. To determine whether the analgesic properties of $GABA_A$ receptor modulators described below were specific and not confounded by any extraneous properties, we evaluated their central nervous system side effect profile in studies of explorative motility and activity-induced motor function in noninjured rats (Fig. 2A). Rats administered vehicle displayed typical explorative behavior of a novel environment throughout a 30-min test (range of five experiments 52 ± 10 to 98 ± 3 m). Although administration of NS11394 (3–120 mg/kg), diazepam (1–5 mg/kg), zolpidem (1–10 mg/kg), gaboxadol (1–6 mg/kg), and bretazenil (10–60 mg/kg) all attenuated explorative motility behavior [$F(5,55) = 2.474$, $p < 0.05$; $F(3,27) = 29.277$, $p < 0.001$; $F(3,27) = 35.822$, $p < 0.001$; and $F(3,23) = 31.608$, $p < 0.001$; $F(3,27) = 3.69$, $p < 0.05$, respectively], it was clear from further analysis that these compounds differed in their potency and magnitude of effect (Fig. 2A), with NS11394 and bretazenil having minor effects relative to the other drugs.

In the rotarod test (Fig. 2B), NS11394 (1–120 mg/kg) and bretazenil (60–240 mg/kg) engendered a modest but nevertheless significant ataxia [$F(6,71) = 3.303$, $p < 0.01$ and $F(3,28) = 4.193$, $p < 0.05$]. As in the explorative motility experiment, only exceptionally high doses of NS11394 and bretazenil significantly attenuated motor function compared with corresponding vehicle responses (−1.9 ± 1.1 and −15 ± 8.5 s; both $p < 0.05$, Fig. 2B). In contrast, the modulators diazepam (1–5 mg/kg) and zolpidem (1–10 mg/kg) as well as the agonist gaboxadol (1–6 mg/kg) all produced marked dose-dependent deficits in motor function [$F(3,31) = 48.084$, $p < 0.001$; $F(3,31) = 89.960$, $p < 0.001$; and $F(3,30) = 28.138$, $p < 0.001$]. Finally, as expected over the dose range studied (100–400 mg/kg), the antiepileptic drug gabapentin, routinely used in the clinical treatment of neuropathic pain, dose-dependently impaired motor function compared with vehicle treatment [$F(3,31) = 10.215$, $p < 0.001$].

These findings are wholly consistent with the literature and confirm that partial allosteric modulators (NS11394, bretazenil) contrary to full allosteric modulators (diazepam, zolpidem), and GABA site agonists (gaboxadol) display a very benign CNS side effect profile (Haefely et al., 1990; Ebert et al., 2006). However, it is important to emphasize that, in our experience and observations here, the influence of zolpidem on motoric integrity in the rat is more profound than that seen with diazepam, despite seemingly similar impacts on explorative motility and rotarod ataxia (Fig. 2).

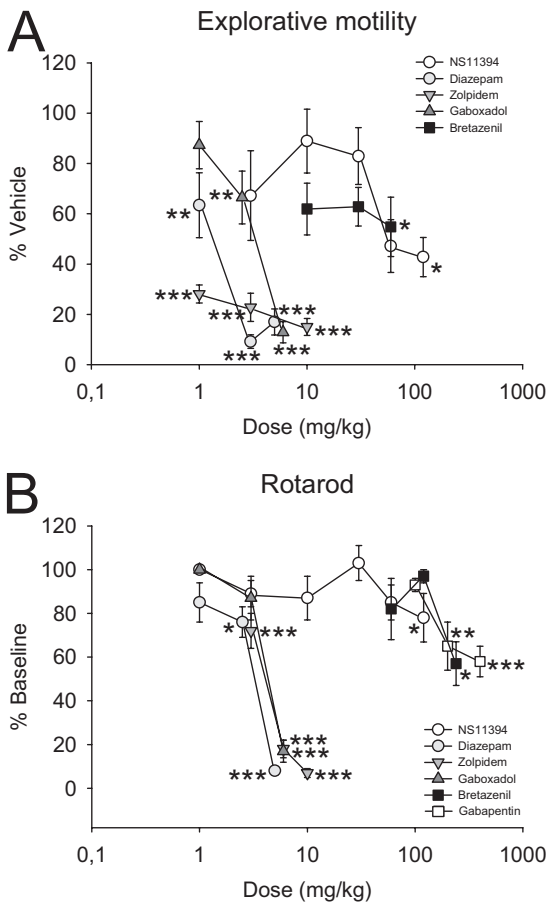


Fig. 2. The effects of NS11394 and $GABA_A$ receptor modulators on motor function. A, normal, uninjured rats were administered either drug or vehicle and the effects on explorative motility determined either 60 min later for NS11394 and gaboxadol or 30 min later in the case of diazepam, zolpidem, and bretazenil. Rats were individually placed in cages for 30 min with automatic recording of activity measured as distance traveled (in meters; represented as % Vehicle). All groups $n = 6–7$ rats. B, normal, uninjured rats were administered either drug or vehicle immediately after a baseline response had been obtained and the maximal effects on activity-induced motor performance (represented as % Baseline) determined 30 min later, with the exception of gabapentin, which was determined 60 min after injection. All groups ($n = 6–7$ rats), except NS11394 vehicle group ($n = 24$ rats). Data are presented as mean ± S.E.M. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus corresponding vehicle group (one-way ANOVA followed by Bonferroni's test).

Hot Plate Test. To test for possible analgesic actions on acute nociceptive processing, $GABA_A$ receptor modulators were administered to normal, uninjured rats in the hot plate test (Fig. 3). Vehicle responses were −2.0 ± 1.0, −2.7 ± 1.3, −3.8 ± 1.1, 0.8 ± 0.5, −0.8 ± 0.5, and −3.0 ± 1.3 s for NS11394, diazepam, zolpidem, gaboxadol, bretazenil, and morphine experiments, respectively. One-way ANOVA failed to reveal any effect of NS11394 (3–30 mg/kg), diazepam (2–20 mg/kg), zolpidem (1–10 mg/kg), or bretazenil (3–30 mg/kg) treatment on the latency to respond to noxious thermal stimulation of either hindpaw [$F(3,31) = 2.616$, $p = 0.071$; $F(3,31) = 0.639$, $p = 0.596$; $F(3,31) = 2.870$, $p = 0.054$; and

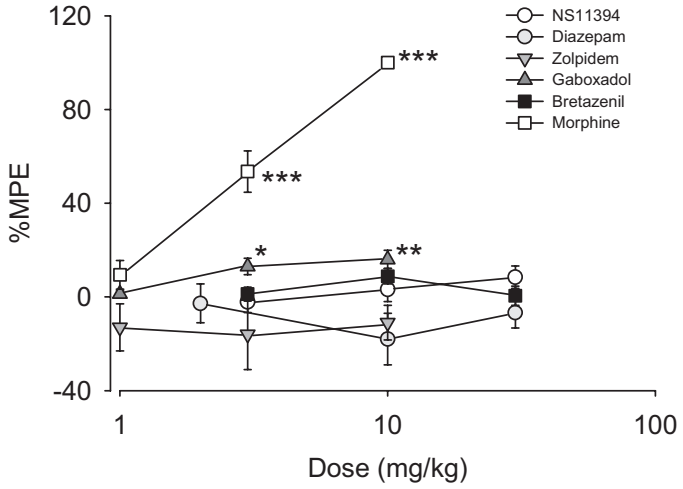


Fig. 3. Effects of NS11394 and $GABA_A$ receptor modulators on acute nociception. Hot plate %MPE values were measured at 30 min after administration for all compounds except for gaboxadol, which was measured at 75 min after administration. All groups (n = 7–8). Data are presented as mean ± S.E.M. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus corresponding vehicle group (one-way ANOVA followed by Bonferroni's t test).

$F(3,30) = 2.581$, $p = 0.074$]. In contrast, gaboxadol (1–10 mg/kg) exhibited a modest but significant analgesic-like profile in this test [$F(3,31) = 7.575$, $p < 0.001$], whereas the μ-opioid receptor agonist morphine (1–10 mg/kg) was as expected, fully analgesic under the conditions tested [$F(3,31) = 79.978$, $p < 0.001$].

Formalin Test and Capsaicin-Induced Sensitization. Injection of formalin into the rat hindpaw selectively activates TRPA1 containing C-fibers and initiates spontaneous nociceptive behaviors consisting of flinching, licking, and/or biting of the injected paw (McNamara et al., 2007). The first phase can be attributed to direct chemical stimulation of nociceptors, interphase can be attributed to activation of noxious inhibitory controls, and the second phase can be attributed to peripheral inflammatory processes and subsequent sensitization of nociceptive spinal neurones (Coderre et al., 1993; Henry et al., 1999). Injection of capsaicin into the rat hindpaw selectively activates and sensitizes TRPV1 containing C-fiber primary afferents and manifests as flinching, licking, and/or biting of the injected paw.

NS11394 (3–30 mg/kg) significantly reduced flinching behavior during interphase [$F(3,30) = 4.139$, $p < 0.05$] and the second phase [$F(3,30) = 11.033$, $p < 0.001$] of the formalin test compared with vehicle treatment indicative of a selective effect on injury-induced nociceptive transmission (Fig. 4A and Table 1). Similarly, diazepam (1–10 mg/kg) and zolpidem (1–10 mg/kg) appeared to selectively attenuate second phase flinching behavior [$F(3,31) = 6.880$, $p < 0.01$; and $F(3,29) = 7.234$, $p < 0.01$, respectively; Table 1]. Gaboxadol (3–10 mg/kg) was the only compound tested that reduced flinching throughout all three phases of the test [$F(3,31) = 14.797$, $p < 0.001$; $F(3,31) = 20.484$, $p < 0.001$; and $F(3,31) = 45.495$, $p < 0.001$ for first phase, interphase, and second phase, respectively], indicative of a nonselective effect on pathological nociceptive transmission in this model. Interestingly, formalin-induced flinching was completely unaffected by administration of bretazenil (3–30 mg/kg). Finally, as expected, gabapentin (50–200 mg/kg) attenuated second-phase flinching

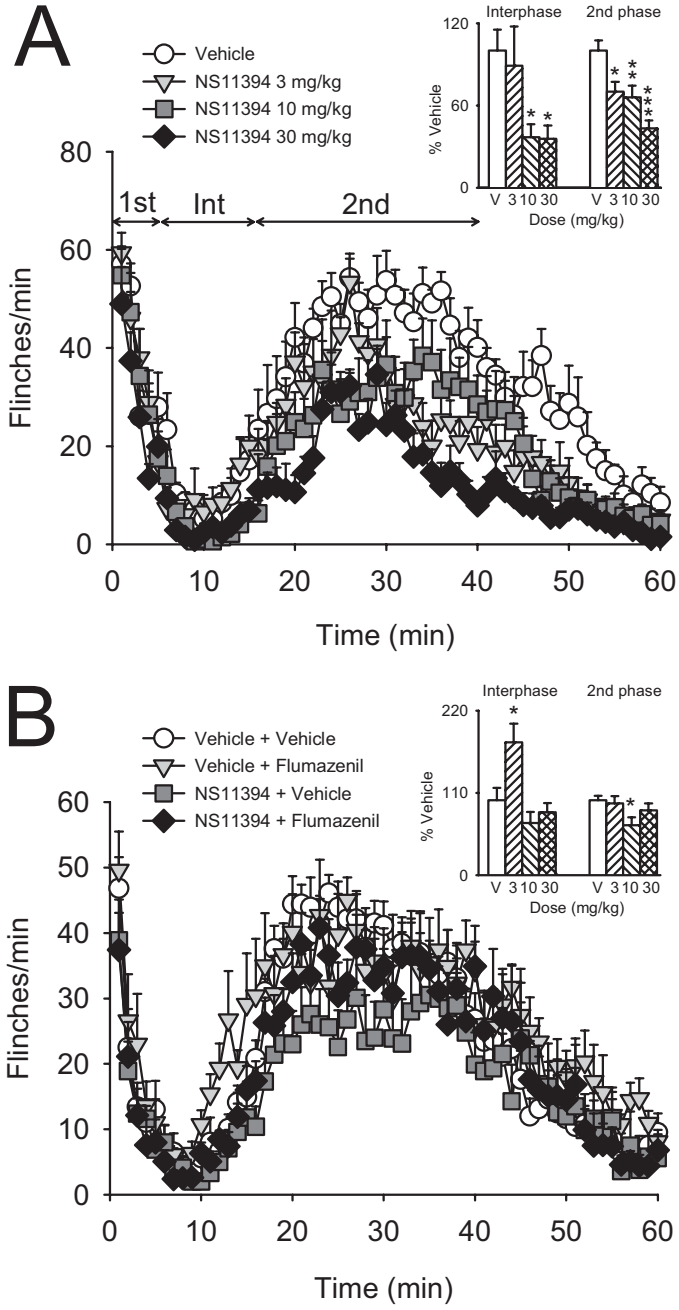


Fig. 4. NS11394 selectively attenuates injury-induced nociceptive behaviors in the formalin test. Normal, uninjured rats were administered either NS11394 (3–30 mg/kg p.o.) or vehicle 30 min before hindpaw formalin (5% in saline, 50 μl s.c.) injection. A, time course of flinching behavior during first phase (1st 0–5 min), interphase (Int 6–15 min), and second phase (2nd 16–40 min) after administration of NS11394. Inset, % Vehicle values are shown for interphase and second phase of the test; all groups (n = 7–8 rats). B, time course of flinching behavior after coadministration of the benzodiazepine site antagonist flumazenil (30 mg/kg i.p.) and NS11394 (10 mg/kg p.o.). Inset, % Vehicle values are shown for interphase and second phase of the test; all groups (n = 7–8 rats). Data are presented as mean ± S.E.M. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus corresponding vehicle group (one-way ANOVA followed by Bonferroni's test).

behavior [$F(3,31) = 5.320$, $p < 0.01$]. To verify that the antinociceptive actions of NS11394 were mediated selectively via $GABA_A$ receptors, the benzodiazepine site antagonist flumazenil was administered in combination with NS11394. Administration of NS11394 (10 mg/kg) alone produced a 33%

TABLE 1

Effects of $GABA_A$ receptor modulators in the formalin test

Rats were administered either drug or vehicle before hindpaw formalin (5% in saline, 50 μl s.c.) injection enabling the number of formalin-induced flinches to be measured. Preadministration times were 30 min for NS11394 (orally), diazepam (intraperitoneally), bretazenil (intraperitoneally), zolpidem (subcutaneously), and gaboxadol (subcutaneously) and 60 min for gabapentin (intraperitoneally). All groups $n = 7–8$ rats. Data are expressed as % Vehicle (where vehicle is 100%); data in parentheses represent raw untransformed data for vehicle-treated animals. All data are presented as mean ± S.E.M. and are shown for the first phase (0–5 min), interphase (6–15 min), or second phase (16–40 min) of the test. Although diazepam, gaboxadol, and zolpidem all apparently attenuated flinching behavior, these effects were observed at doses that also markedly attenuated explorative motility behavior and thus are not considered as a direct antinociceptive action.

Drug	Dose	Formalin Test % Vehicle		
		1st Phase	Interphase	2nd Phase
	mg/kg			
NS11394	Vehicle	100 ± 11	100 ± 15	100 ± 7.5
		(190 ± 21)	(109 ± 17)	(1094 ± 82)
	3	100 ± 8.9	89 ± 29	70 ± 7.4*
	10	95 ± 17	37 ± 9.4*	66 ± 8.6**
	30	77 ± 8.9	36 ± 9.6*	43 ± 5.6***
Diazepam	Vehicle	100 ± 21	100 ± 35	100 ± 11
		(143 ± 30)	(133 ± 46)	(838 ± 94)
	1	67 ± 25	55 ± 19	88 ± 9.5
	5	92 ± 18	38 ± 7.3	81 ± 6.6
	10	57 ± 16	35 ± 10	45 ± 11***
Zolpidem	Vehicle	100 ± 13	100 ± 25	100 ± 7.2
		(150 ± 19)	(108 ± 27)	(961 ± 69)
	1	101 ± 12	95 ± 22	109 ± 6.7
	3	108 ± 14	67 ± 11	95 ± 8.9
	10	87 ± 7.5	121 ± 17	58 ± 13**
Gaboxadol	Vehicle	100 ± 9.7	100 ± 16	100 ± 8.6
		(142 ± 14)	(195 ± 31)	(884 ± 76)
	3	100 ± 8.4	72 ± 15	111 ± 6.8
	6	39 ± 11***	5.6 ± 2.3***	38 ± 10***
	10	40 ± 26***	8.3 ± 11***	9.5 ± 3.4***
Bretazenil	Vehicle	100 ± 12	100 ± 11	100 ± 13
		(203 ± 24)	(166 ± 19)	(832 ± 104)
	3	78 ± 15	90 ± 20	104 ± 9.3
	10	66 ± 16	68 ± 16	116 ± 6.6
	30	80 ± 8	77 ± 13	105 ± 8.5
Gabapentin	Vehicle	100 ± 16	100 ± 19	100 ± 13
		(162 ± 26)	(156 ± 30)	(834 ± 104)
	50	76 ± 16	72 ± 32	72 ± 14
	100	72 ± 12	79 ± 25	66 ± 11
	200	46 ± 16	23 ± 9.4	42 ± 6**

** $P < 0.01$, *** $P < 0.001$ vs. corresponding vehicle group (one-way ANOVA followed by Bonferroni's t test).

reduction in flinching during the second phase of the test ($p < 0.05$ versus vehicle; Fig. 4B), and this effect was blocked by coadministration of flumazenil (30 mg/kg). Intriguingly, although flumazenil alone had no effect on second-phase flinching, an increase in the number of flinches during interphase compared with vehicle was observed ($p < 0.05$). Therefore we reinvestigated various doses of flumazenil (3–30 mg/kg s.c., $n = 8$ rats per group) in a separate study and found only a small and nonsignificant (20 ± 17%) increase in flinching during interphase at 30 mg/kg (data not shown), indicating that this was not a robust finding.

Compared with vehicle treatment (156 ± 22 total flinches), the hindpaw flinching observed within 5 min after capsaicin injection was unaffected by NS11394 (0.3–30 mg/kg; Fig. 5). Over the next 25 min, which would be expected to coincide with a more persistent and selective activation of TRPV1 containing C-fibers, vehicle-treated rats continued to exhibit a robust flinching response (400 ± 44 total flinches). During this period, NS11394 treatment significantly reduced flinching behavior [$F(5,57) = 8.701$, $p < 0.001$; Fig. 5]. Administration of diazepam (1–10 mg/kg), zolpidem (1–6 mg/kg), and gaboxadol (3–10 mg/kg) also dose-dependently attenuated flinching from 6 to 30 min [$F(3,31) = 9.294$, $p < 0.001$; $F(3,31) = 4.017$, $p < 0.05$; and $F(3,27) = 34.448$, $p < 0.001$, respectively; Fig. 5B] after capsaicin injection. However, in addition, diazepam and gaboxadol both dose-dependently attenuated flinching from 0 to 5 min [$F(3,31) = 6.539$, $p < 0.01$ and $F(3,27) = 10.626$, $p < 0.001$, respectively; data not shown]. Capsaicin-induced flinching was completely unaffected by administration of bretazenil (10–60 mg/kg).

Complete Freund's Adjuvant-Induced Inflammatory Nociception. Peripheral inflammation increases inhibitory synaptic transmission mediated by $GABA_A$ receptors within the spinal dorsal horn (Poisbeau et al., 2005). At the behavioral level, gait deficits associated with established monoarthritis are completely prevented by continuous administration of muscimol (Simjee et al., 2004). Thus, we compared antinociceptive actions of NS11394 and diazepam relative to morphine in the CFA model of inflammatory pain. Twenty-four hours after injection of CFA into the hindpaw, a marked alteration in hindpaw weight bearing (indicative of spontaneous nonevoked pain) was observed compared with weight bearing before injection (35 ± 1.9 g versus −0.1 ± 3.2 g, $n = 95$, $p < 0.001$, Student's t test).

Injection of NS11394 (1–10 mg/kg) markedly attenuated the deficit in hindpaw weight bearing [$F(4,61) = 7.569$, $p < 0.001$] in CFA rats (Fig. 6A). In contrast, the tail-flick response in the same CFA rats was completely unaffected by NS11394 [$F(3,30) = 0.215$, $p = 0.885$]. One-way ANOVA also revealed a significant antinociceptive effect of diazepam (0.5–2.5 mg/kg) treatment against hindpaw weight-bearing deficits in CFA-treated rats [$F(4,63) = 7.672$, $p < 0.001$]. How-

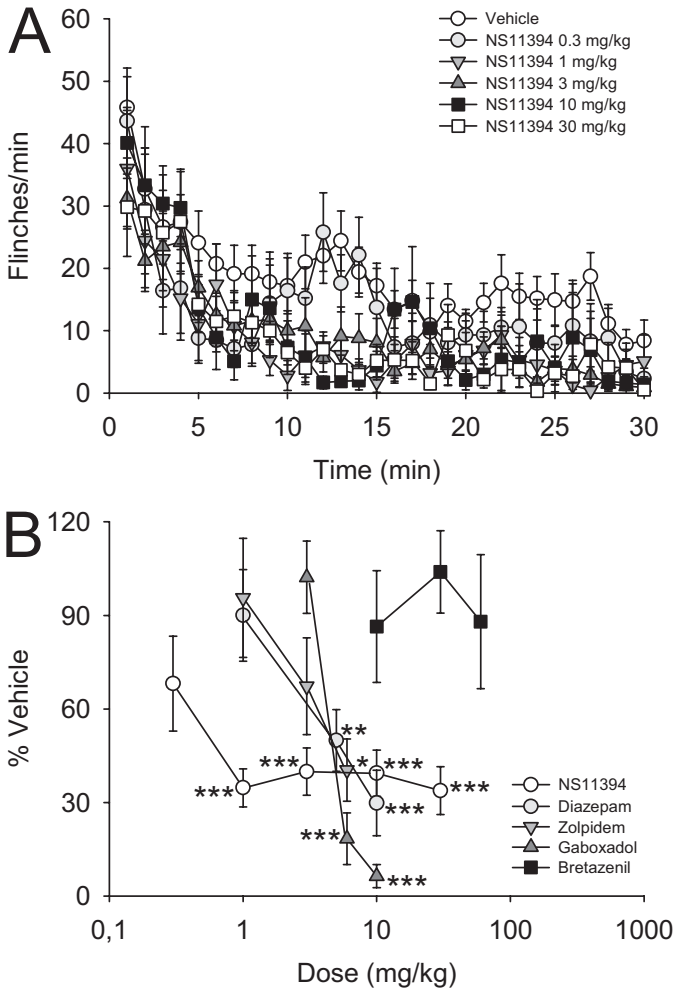


Fig. 5. NS11394 selectively attenuates VR1-containing C-fiber-mediated nociceptive behaviors. A, normal, uninjured rats were administered either NS11394 (0.3–30 mg/kg p.o.) or vehicle 30 min before hindpaw capsaicin (10 μg in 10 μl of 10% Tween 80) injection and the time course of flinching behavior followed. B, % Vehicle (where vehicle is 100%) values are shown for the second phase (6–30 min) of the test. Preadministration times were 30 min for diazepam (intraperitoneally), bretazenil (intraperitoneally), zolpidem (subcutaneously), and gaboxadol (subcutaneously). All groups (n = 6–8 rats). Data are presented as mean ± S.E.M. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus corresponding vehicle group (one-way ANOVA followed by Bonferroni's test).

ever, in contrast to NS11394, diazepam also significantly affected the tail-flick response in CFA-treated rats [$F(3,31) = 6.740$, $p < 0.01$; Fig. 6B]. As expected, morphine (3–10 mg/kg) significantly attenuated weight-bearing deficits [$F(4,63) = 21.896$, $p < 0.001$] and increased tail-flick latency responses in CFA rats [$F(3,31) = 91.094$, $p < 0.001$; Fig. 6C].

Peripheral Nerve Injury—SNI and CCI. To evaluate drug effects upon the reflex sensory component of the nociceptive response associated with evoked neuropathic pain behaviors, $GABA_A$ receptor modulators/agonists and gabapentin were tested for antiallodynic effects in the CCI model of peripheral nerve injury. NS11394 and gabapentin were also assessed for antiallodynic and antihyperalgesic effects in the SNI model of neuropathic pain. After surgery, both CCI (n = 103) and SNI (n = 34) rats developed behavioral signs of mechanical allodynia (observed as a decrease in the paw withdrawal threshold in response to von Frey hair stimulation) of the ipsilateral hindpaw (1.1 ± 0.1 and 0.5 ± 0 g,

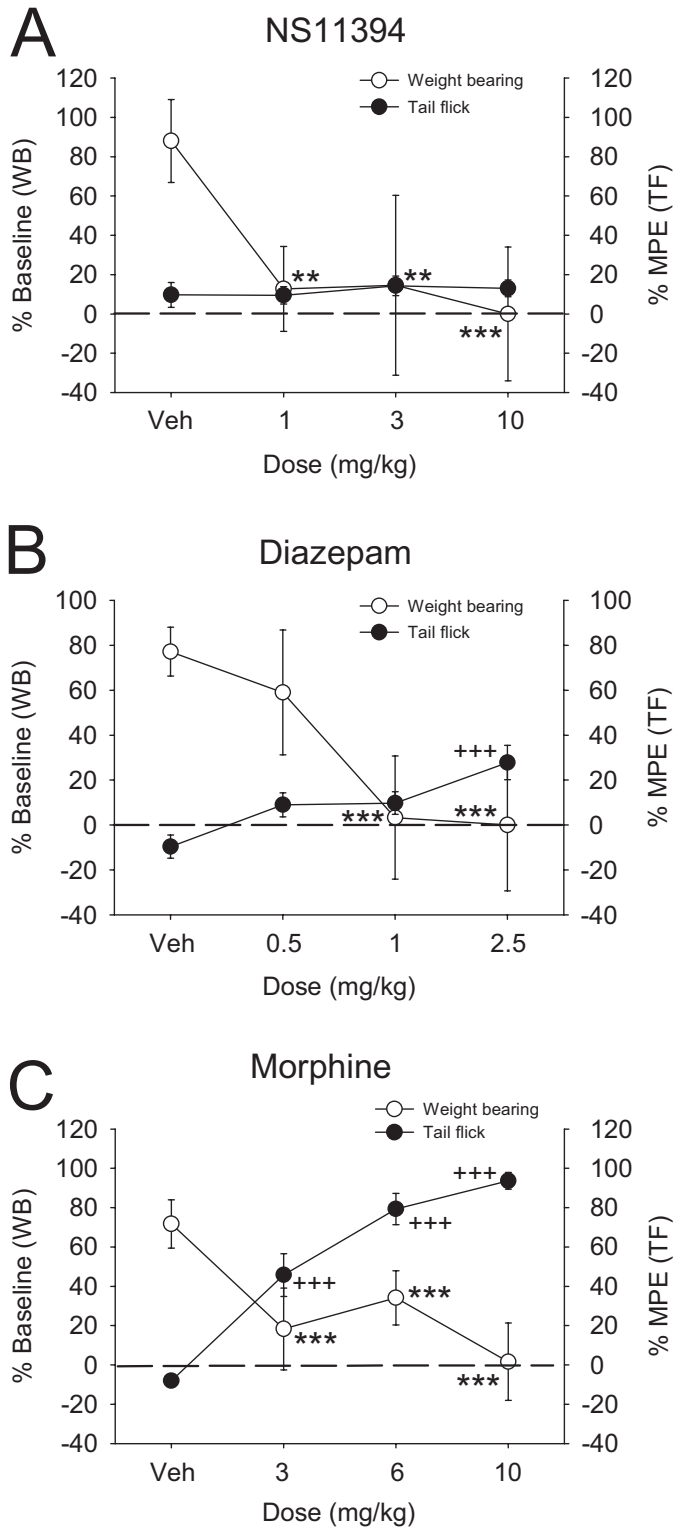


Fig. 6. NS11394 selectively attenuates ongoing nociceptive behaviors in the CFA model of inflammatory pain. Rats were given a subcutaneous injection of Complete Freund's adjuvant into the hindpaw and both evoked (tail-flick latency) and nonevoked (weight-bearing difference) nociceptive behaviors measured. Twenty-four hours later, two baseline responses were obtained, and rats were then immediately administered either NS11394 (1–10 mg/kg p.o.) (A), diazepam (0.5–2.5 mg/kg i.p.) (B), morphine (3–10 mg/kg s.c.) or vehicle (C). Weight bearing (WB) values are expressed as % Baseline and tail-flick (TF) values as %MPE. All values shown represent maximal response obtained from 30 to 60 min after drug administration. Data are presented as mean ± S.E.M. All groups (n = 7–8 rats). **, $p < 0.01$, ***, $p < 0.001$ versus corresponding baseline; +++, $p < 0.001$ versus corresponding vehicle (one-way ANOVA followed by Bonferroni's test).

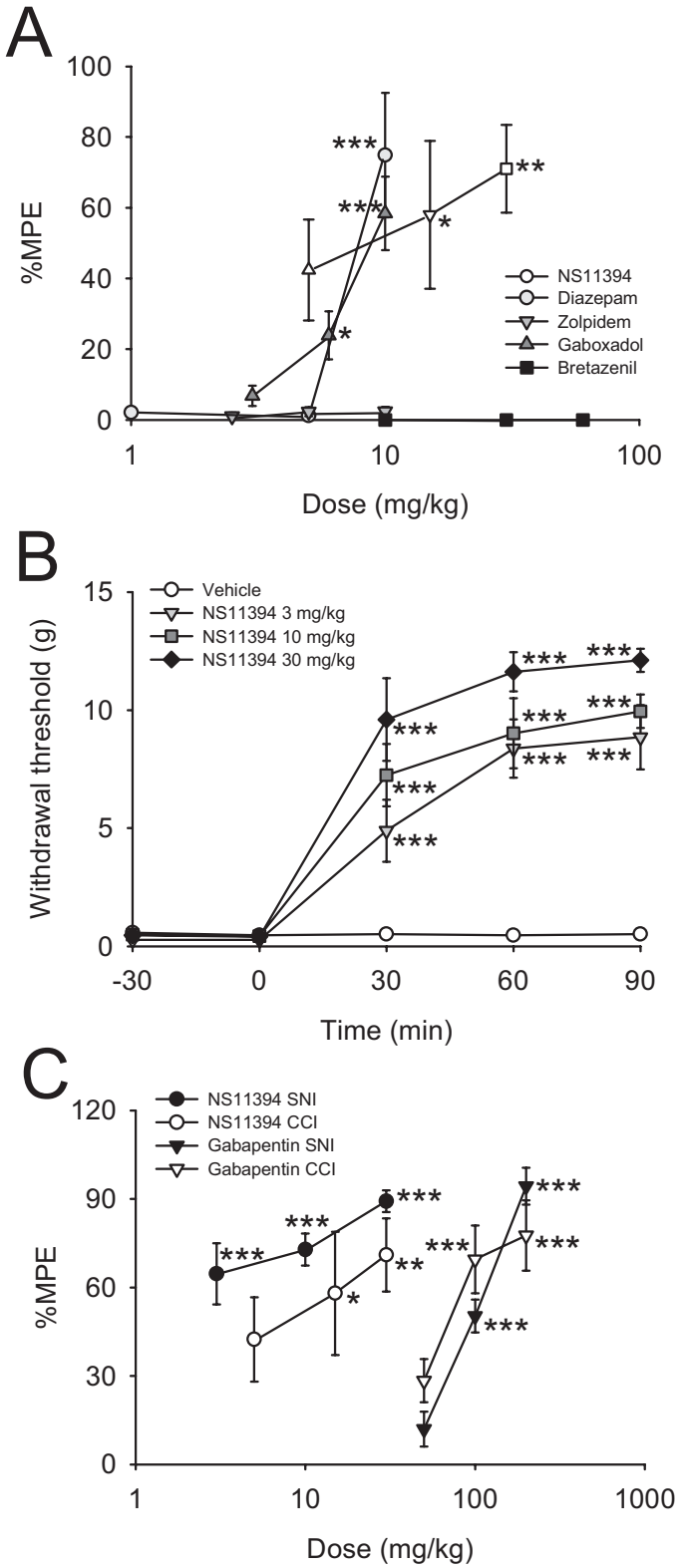


Fig. 7. NS11394 attenuates mechanical allodynia in rats with peripheral nerve injury. A, comparison of maximal antiallodynic effects (expressed as %MPE) of $GABA_A$ receptor modulators and gaboxadol in CCI rats. %MPE responses were obtained at 60 min after administration of NS11394 and gaboxadol and 30 min after administration of diazepam, zolpidem, and bretazenil. B, time course of antiallodynic actions of NS11394. Immediately after a second baseline response had been obtained (0 min), SNI rats were administered NS11394 (3–30 mg/kg p.o.) or vehicle, and effects on ipsilateral hindpaw withdrawal threshold (grams) were measured. C, comparison of maximal antiallodynic effects (expressed as %MPE) of NS11394 and gabapentin (50–200 mg/kg i.p.) in SNI and CCI rats. In SNI and CCI rats, %MPE responses were obtained at 90 and 60 min, respectively, after administration of NS11394 and at 120 min after administration of gabapentin. All groups ($n = 6$ rats). Data are presented as mean ± S.E.M. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus corresponding vehicle group (one-way ANOVA followed by Bonferroni's test).

respectively) compared to presurgery levels that typically measured 13.5 g. Mechanical hyperalgesia (observed as an increase in the paw withdrawal duration in response to pin prick stimulation) of the ipsilateral hindpaw was also observed in both nerve injury models (15 ± 0 and 14 ± 0.3 s for CCI and SNI rats, respectively) compared to presurgery levels that were <0.5 s.

In CCI rats, mechanical allodynia was dose-dependently reversed [$F(3,23) = 5.021$, $p < 0.01$] by administration of NS11394 (5–30 mg/kg; Fig. 7A). Likewise, gaboxadol (3–10 mg/kg) dose-dependently reversed hindpaw mechanical allodynia in CCI rats [$F(3,23) = 20.244$, $p < 0.001$]. Although diazepam also reversed mechanical allodynia [$F(3,23) = 20.732$, $p < 0.001$] in CCI rats, the attenuation in hindpaw hypersensitivity was significant only for the highest dose of diazepam tested ($p < 0.001$; 10 mg/kg versus vehicle). By contrast, both zolpidem (3–10 mg/kg) and bretazenil (10–60 mg/kg) were completely ineffective at reversing hindpaw mechanical allodynia in CCI rats.

In SNI rats, two-way repeated measures ANOVA revealed a clear interaction between NS11394 (3–30 mg/kg) treatment with respect to time on hindpaw withdrawal threshold [$F(9,95) = 12.537$, $p < 0.001$; Fig. 7B]. In terms of comparative antiallodynic efficacy, the magnitude of the reversal obtained with 30 mg/kg NS11394 in SNI and CCI rats (89 and 71%, respectively) was equivalent to that obtained with the antiepileptic drug gabapentin (200 mg/kg) in the same injury models (94 and 78% for SNI and CCI, respectively; Fig. 7C). Likewise, both NS11394 and gabapentin dose-dependently reversed hindpaw mechanical hyperalgesia in SNI rats [$F(3,23) = 11.745$, $p < 0.001$; and $F(3,23) = 17.804$, $p < 0.001$]. Again, the magnitude of reversal obtained was comparable in SNI rats administered either 30 mg/kg NS11394 or 200 mg/kg gabapentin (86 versus 90%, respectively; data not shown).

In Vitro Electrophysiology. In the light of a spinal action of diazepam (Siarey et al., 1992, 1994), we performed electrophysiological experiments on the isolated spinal cord to learn about possible mechanisms of action of NS11934 in the spinal circuits that mediate withdrawal reflexes. To this end, we first checked the effects of NS11394 on native spinal cord $GABA_A$ receptors localized to the central terminals of primary afferents. NS11394 ($n = 3$) applied at 1 μM produced a long-lasting potentiation of GABA-induced primary afferent depolarization for all three concentrations of exogenously applied GABA (Fig. 8A). The mean integrated area of depolarization produced by GABA applied at 30 μM increased from 19 ± 1.2 mV · s in control ACSF to 84 ± 2.9 mV · s after superfusion of NS11394, corresponding to a 454 ± 44% increase. In addition, application of NS11394 produced an increase in amplitude (133 ± 7.5% control) and frequency (167 ± 12%) of spontaneous dorsal root potentials in all three preparations.

To evaluate the effects of NS11394 on the overall spinal integration of afferent signals, we recorded dorsal root-ventral

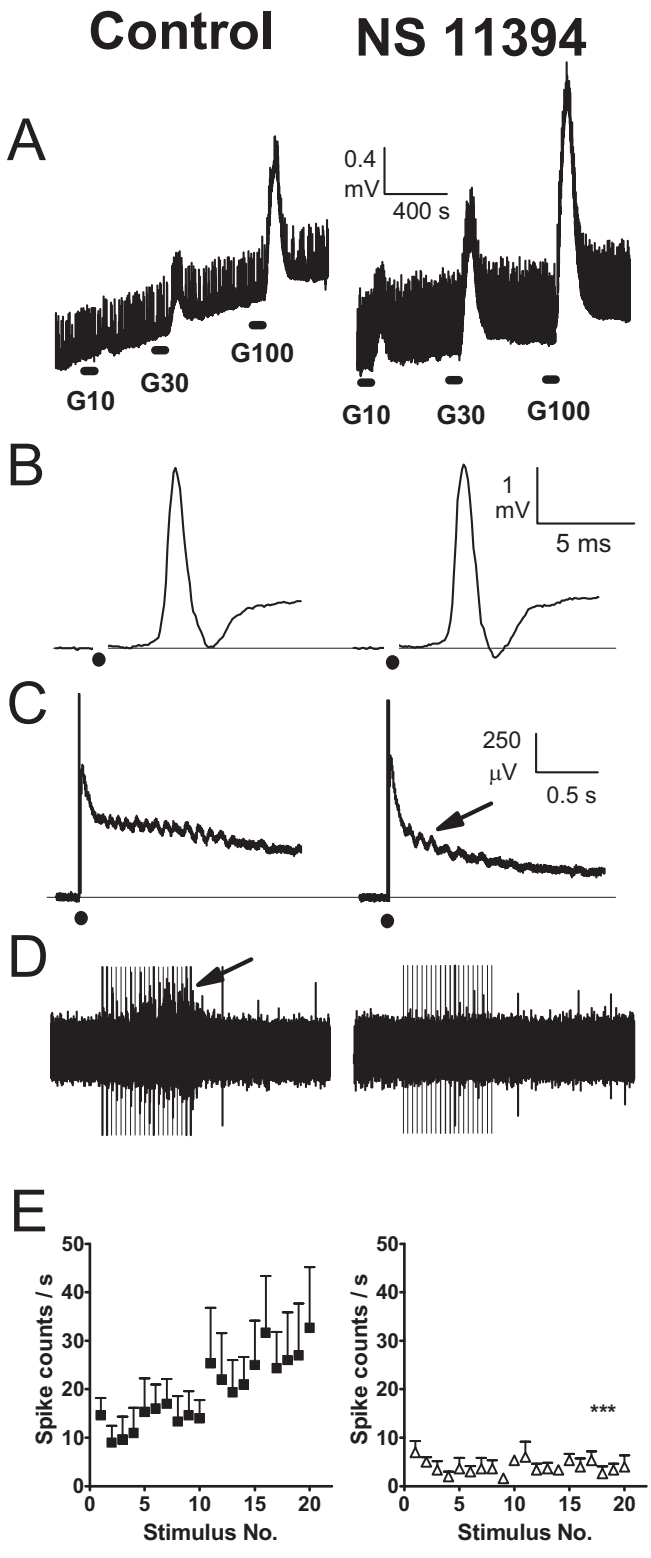


Fig. 8. NS11394 attenuates nociceptive transmission through spinal circuits. Panels A to D show original recordings obtained under control conditions (left) and after superfusion of 1 μM NS11394 (right). A, dorsal root recordings. Responses to application of three concentrations of GABA (as marked in micromolar) produced depolarizations lasting less than 4 min. Spontaneous activity appears as rapid upward deflections of the baseline potential. Note the increased responses to GABA and spontaneous activity after application of NS11394. B, monosynaptic reflexes showing no effect of NS11394 (electrical artifacts are hidden for clarity and marked with a black circle). C, reduction of the long latency components of the ventral root response to dorsal root stimulation as obtained in the same experiment as B. The arrow signals reduced long latency components of the response. D, ventral root responses to repetitive stimulation showing wind-up of action potentials. Large vertical lines correspond to stimulus artifacts. Wind-up of action potentials under control ACSF corresponds to the thickening of the basal recordings (marked by an arrow). Note how wind-up disappears after superfusion with NS11394. E, mean data for spike counts in response to repetitive stimulation. All observations ($n = 3$). Data are presented as mean ± S.E.M. ***, $p < 0.001$ control versus NS11394 (ANOVA).

root reflexes (DR-VRr) in the absence and presence of NS11394. The analysis of responses to single stimuli showed that the monosynaptic reflex, mediated by the activation of thin myelinated fibers, was unaltered by NS11394 ($n = 3$). In contrast, longer latency components of the DR-VRr, mediated by activation of nociceptive afferents, were strongly and systematically depressed by NS11394 (Fig. 8, B and C). The integrated area of the response decreased to 60 ± 1.5% control after application of NS11394. This reduction was due to a fall in the amplitude of the depolarization most clearly seen at latencies exceeding 300 ms from stimulus artifact (Fig. 8C).

Repetitive stimulation of the dorsal root produced a typical wind-up of action potentials, which depends on C-fiber activation. The slope of the action potential wind-up was eliminated by superfusion with NS11394 ($n = 3$), and the total count of action potentials to this repetitive stimuli fell to 9 ± 4% control (Fig. 8, D and E).

Discussion

NS11394 is a positive allosteric modulator at $GABA_A$ receptors with a subtype-selectivity profile that translates to marked anxiolytic properties at doses devoid of motoric impairment in rodents (summarized in Fig. 1) (Mirza et al., 2008). Recent studies in gene-targeted animals suggest a pivotal role for $GABA_A$-α_2/$GABA_A$-3 receptors in normalizing spinal disinhibition after injury. Therefore, we have tested orally administered NS11394 against a range of injury-induced nociceptive behaviors in rat models associated with central sensitization.

Persistent and Inflammatory Nociception. In the rat formalin test, spinal administration of $GABA_A$ receptor agonists and modulators have been reported to produce conflicting effects on nociceptive behaviors (Dirig and Yaksh, 1995; Kaneko and Hammond, 1997). In our experiments, NS11394 had no effect on first-phase flinching after injection of formalin or capsaicin, consistent with its lack of effect on acute nociceptive behaviors (see below). It is crucial that NS11394 reduced second-phase formalin- and capsaicin-induced flinching. Here, the efficacy of NS11394 in the formalin test was equivalent to that of gabapentin. Given that persistent nociceptive transmission mediated by TRPA1- (formalin) and TRPV1- (capsaicin) containing C-fiber afferents was similarly attenuated by NS11394, this indicates a common site of integrative antinociceptive action, perhaps on postsynaptically located $GABA_A$ receptor-containing neurones within the spinal dorsal horn. However, supraspinal sites of action cannot be excluded, and indeed there is a close correlation between the minimal effective dose of NS11394 for second-phase antinociception (1–3 mg/kg) with the ED_{50} to displace cortical [^{3}H]flunitrazepam binding (1.3 mg/kg) in vivo (Mirza et al., 2008). Regardless, we can ascribe NS11394-mediated antinociception in the formalin test to binding to $GABA_A$ receptors in vivo given that flumazenil reversed the effect of NS11394. Diazepam, zolpidem, and gaboxadol, but

not bretazenil, also had antinociceptive effects in the formalin and capsaicin models with efficacy essentially on par with NS11394, albeit at doses associated with motoric impairments.

In noninjured rats, spinal administration of the $GABA_A$ agonist isoguvacine produces a bicuculline-reversible increase in acute nociceptive threshold. To investigate whether NS11394 would selectively attenuate behavioral sequelae associated with injury-induced central sensitization, its influence on spontaneous nociceptive behaviors in the CFA model of inflammatory pain was compared with its actions on acute nociceptive reflexes. Hindpaw weight bearing deficits induced by CFA were exquisitely sensitive to treatment with NS11394, which displayed full efficacy at all of the doses tested. However, NS11394 had no effect on tail-flick latency in CFA rats and did not affect hot plate latency responses in noninjured rats, indicating no influence on acute nociceptive responses. By contrast, diazepam increased acute nociceptive threshold in CFA-treated animals in addition to altering hindpaw weight bearing deficits.

Neuropathic Injury. Whereas spinal administration of bicuculline induces mechanical allodynia in normal rats (Malan et al., 2002), spinal administration of muscimol reverses mechanical allodynia in the spinal nerve ligation model of neuropathic pain (Hwang and Yaksh, 1997; Malan et al., 2002). Importantly, these effects on allodynia occur at spinal doses that minimally affect motor function, implying that robust and selective antinociception is possible by specifically targeting $GABA_A$ receptors localized within central pain pathways (Hwang and Yaksh, 1997; Knabl et al., 2008).

Our data support this hypothesis. NS11394 reversed mechanical allodynia in SNI and CCI rats to a level comparable with gabapentin, which is routinely used in the clinical treatment of neuropathic pain. Whereas similar antiallodynic efficacy was achieved with diazepam and gaboxadol in CCI rats, effective doses of both drugs were associated with overt sedation and ataxia (Table 2). By contrast, bretazenil was inactive in the CCI model and, indeed, all models of pain. Interestingly, despite doses of zolpidem tested in CCI rats leading to clear motoric impairment, it was inactive in this model of neuropathic pain.

TABLE 2

Summary of antinociceptive potency of NS11394 and $GABA_A$ receptor modulators in uninjured and injured rats

For each compound and associated behavioral test, the indicated value represents the minimal effective dose (MED; mg/kg) required to significantly modulate behavior. All values were obtained from corresponding behavioral experiments represented in Table 1 and Figs. 2 through 7.

	MED				
	NS11394	Diazepam	Zolpidem	Gaboxadol	Bretazenil
	mg/kg				
Acute nociception					
Tail flick	>30	10	n.t.	n.t.	n.t.
Hot plate	>30	>20	>10	6	>30
Persistent pain					
Capsaicin	3	5	6	6	>60
Formalin	3	10	10	6	>30
Inflammatory pain					
CFA	1	1	n.t.	n.t.	n.t.
Neuropathic pain					
SNI (MA)	3	n.t.	n.t.	n.t.	n.t.
SNI (MH)	3	n.t.	n.t.	n.t.	n.t.
CCI (MA)	15	10	>10	6	>60
Motor effects					
Exploratory motility	60	1	1	3	60
Rotarod	120	1	3	6	240

MA, mechanical allodynia; MH, mechanical hyperalgesia; n.t., not tested.

What Are the Pharmacological Determinants of Antinociceptive Actions of NS11394? Diazepam has full efficacy at α_1, α_2, α_3, and α_5 containing $GABA_A$ receptors and is effective across the range of pain models used here. Because effective doses of diazepam in these models were associated with CNS-related motor side effects, one might reasonably speculate that the antinociceptive effects of diazepam are secondary to side effects. However, we do not believe that the explanation is so straightforward because broad spectrum antinociception was not seen with zolpidem, which severely affected motor function. From our data with NS11394, we conclude that robust antinociception with minimal influence on basic motor function can be obtained with only partial allosteric modulation of $GABA_A$ receptors. However, one key finding in our dataset is the complete lack of efficacy of bretazenil across all models of pain, despite this compound being very potent ($\leq$ 0.3 mg/kg) and efficacious in animal models of anxiety (Haefely et al., 1990). A logical conclusion from this is that bretazenil simply has insufficient efficacy at $GABA_A$ receptors (Sieghart, 1995) to produce antinociception in animal models, which implies that efficacy requirements for antianxiety and antinociceptive effects differ.

Another interesting finding is the inconsistent efficacy of zolpidem across the range of pain models. Because zolpidem has ~10-fold higher affinity for $GABA_A$-α_1 compared to $GABA_A$-α_2 and $GABA_A$-α_3 receptors (Sieghart, 1995), this indicates that $GABA_A$-α_1 receptors are not involved in antinociception, consistent with NS11394 and L-838,417 data (Knabl et al., 2008). However, zolpidem is still a full efficacy modulator of $GABA_A$-α_2 and $GABA_A$-α_3 receptors, so why does it not work in CCI rats? We believe that, although zolpidem might have sufficient activity at $GABA_A$-α_2 and $GABA_A$-α_3 receptors to engender antinociceptive activity, this is masked in some models by severe motor side effects. An alternative and intriguing explanation is that there is a role for $GABA_A$-α_5 receptors. Thus, whereas efficacy of zolpidem at $GABA_A$-α_2- and $GABA_A$-α_3-containing receptors is sufficient to mediate antinociceptive actions in formalin/capsaicin models, its poor affinity for $GABA_A$-α_5 receptors (Sieghart, 1995) precludes efficacy in CCI rats. Although speculative, this explanation is consistent with our data as diazepam, NS11394, and L-838,417 all have efficacy at $GABA_A$-α_5 receptors. Furthermore, $GABA_A$-α_5-containing receptors are distributed through numerous spinal lamina, and α_5 gene expression is modulated in the dorsal root ganglia and dorsal horn in neuropathic pain models (Xiao et al., 2002; Yang et al., 2004).

Limited studies with α_4 knockout mice implicate $GABA_A$-α_4 receptors in gaboxadol's antinociceptive properties (Chandra et al., 2006). However, this receptor is unlikely to be relevant for NS11394 and diazepam-induced antinociception, because both bind poorly to $GABA_A$-α_4 receptors (Mirza et al., 2008). Indeed, although at the doses used here it has been suggested that gaboxadol has a higher activity level at extrasynaptic $GABA_A$-α_4, -α_5, and -α_6 receptors (Ebert et al., 2006; Cremers and Ebert, 2007), we would urge future studies to investigate the possibilities that antiociceptive actions of gaboxadol might be mediated i) via $GABA_A$-α_3 and/or $GABA_A$-α_2 receptors; or ii) possibly via $GABA_A$-α_5 receptors, in reference to the discussion on zolpidem above.

Possible Mechanisms of Action of NS11934. Our exploratory spinal cord electrophysiology studies confirm that NS11394 enhances the inhibitory effect of native $GABA_A$ receptors and profoundly depresses the activity of spinal circuits that mediate nociceptive withdrawal reflexes. Primary afferent depolarization, mediated predominantly by $GABA_A$ receptors, constitutes a classical mechanism for presynaptic inhibition (Rudomin and Schmidt, 1999). Here we show that NS11394 potentiates primary afferent depolarization induced by exogenous GABA and spontaneous activity recorded from primary afferents—both phenomena known to be sensitive to picrotoxin (Rivera-Arconada and Lopez-Garcia, 2006). The actions of NS11394 are specific because it did not alter the monosynaptic reflex, which in the rat is sensitive to baclofen but not to bicuculline (Akagi and Yanagisawa, 1987). These observations indicate that the native spinal GABAergic system is modulated by NS11394 as predicted from studies performed on recombinant $GABA_A$ receptors (Mirza et al., 2008). Furthermore, these results indicate that NS11394 modulates sensory processing in the dorsal horn.

Our studies also indicate that NS11394 selectively depresses spinal nociceptive transmission arising from activation of unmyelinated afferent fibers. In fact, our indices of nociceptive processing, including the long-latency components of the DR-VRr and the wind-up of motor neurons, were systematically decreased by NS11394. In contrast, our index of non-nociceptive transmission derived from activation of thick myelinated afferents, i.e., monosynaptic reflexes, was not modified by NS11394.

The strong depression of spinal nociceptive processing of NS11394 is commensurate with its effects in neuropathic pain models and suggests that the spinal cord is a likely site through which NS11394 produces antinociception. However, other studies have shown that activation of $GABA_A$ receptors in the amygdala or the anterior cingulate cortex reverses both sensory and affective pain-like behaviors in neuropathic rats (LaGraize and Fuchs, 2007; Pedersen et al., 2007). In addition, preliminary work in our laboratory suggests that supraspinal mechanisms might also be relevant to antinociceptive actions of NS11394.

Conclusions

The subtype-selective $GABA_A$ receptor modulator NS11394 has powerful and selective antinociceptive actions in animal models associated with injury-induced central sensitization with minimal motor-impairing side effects. Based on our comparative pharmacological approach, we conclude that NS11394 selectivity for and level of efficacy at $GABA_A$-α_3 and/or $GABA_A$-α_2 receptors mediate its antinociceptive actions. Thus, molecules being developed for anxiety with a $GABA_A$-α_2/$GABA_A$-α_3 over $GABA_A$-α_1 selectivity profile might not necessarily have the appropriate in vitro profile for efficacy in pain. At this point in time, a potential role for $GABA_A$-α_5-containing receptors in pain should not be dismissed because all compounds active across the full range of pain models in this study also possess in vitro efficacy at this subtype. Finally, we suggest that antinociceptive effects of NS11394 are mediated at least partly via presynaptic and postsynaptic spinal mechanisms.

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