JPET Fast Forward. Published on September 12, 2008 as DOI: 10.1124/jpet.108.144568




# Comparison of the novel subtype selective $GABA_A$ receptor positive allosteric modulator NS11394 with diazepam, zolpidem, bretazenil and gaboxadol in rat models of inflammatory and neuropathic pain

Munro G, Lopez-Garcia JA, Rivera-Arconada I, Erichsen HK, Nielsen EØ, Larsen JS, Ahring PK, Mirza NR*

*NeuroSearch A/S, 93 Pederstrupvej, DK-2750, Ballerup, Denmark (G.M., H.K.E., E.Ø.N, P.K.A, J.S.L., N.R.M.); Department of Physiology, University of Alcalá, Madrid, E-28871 Spain (I.R.-A., J.A.L.-G.)*

**Running title:** Antinociceptive actions of NS11394 in rat pain models

***Corresponding author:** Dr Naheed Mirza

Department of Pharmacology

NeuroSearch A/S

93 Pederstrupvej

DK-2750, Ballerup, Denmark

E-mail - max@neurosearch.dk

Telephone - +45 44608271; Fax; +45 44608080

**Number of text pages: 46**

**Number of tables: 2**

**Number of figures: 8**

**Number of references: 38**

**Number of words in Abstract: 250**

**Number of words in Introduction: 749**

**Number of words in Discussion: 1549**

**Abbreviations:** ACSF, artificial cerebrospinal fluid; CCI, chronic constriction injury; CFA, complete Freund's adjuvant; CNS, central nervous system; DR-VRr, dorsal root-ventral root reflex; GAD, glutamic acid decarboxylase; GABA, gamma-amino-butyric acid; IPSP, inhibitory postsynaptic potential; MA, mechanical allodynia; MED, minimum effective dose; MH mechanical hyperalgesia; MPE, maximum possible effect; SNI, spared nerve injury; TF, tail flick; TRPA1, transient receptor potential A1; TRPV1, transient receptor potential V1; WB, weight bearing

JPET Fast Forward. Published on September 12, 2008 as DOI: 10.1124/jpet.108.144568
This article has not been copyedited and formatted. The final version may differ from this version.



**Recommended section assignment:** Neuropharmacology

## 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 α1 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 α5 > α3 > α2 > α1 at $GABA_A$ α subunit-containing receptors. Oral administration of NS11394 (1-30 mg/kg) to rats attenuated spontaneous nociceptive behaviours 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 Freunds adjuvant injection was also completely reversed by NS11394. Similarly, hindpaw mechanical allodynia was fully reversed by NS11394 in two rat models of peripheral neuropathic pain. Importantly, NS11394-mediated antinociception occurred at doses 20-40 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-fibre 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.

## Introduction

Within the mammalian spinal cord, GABA is the principal inhibitory transmitter and is localized both pre-synaptically in primary afferents and post-synaptically in dorsal horn interneurones (Malcangio and Bowery, 1996). Immunohistochemical studies suggest all major classes of $GABA_A$ receptors, e.g. those containing α1, α2, α3 and α5 subunits (hereafter referred to as $GABA_A$-α*x*), are variously expressed within the spinal dorsal horn, although $GABA_A$-α2 receptors predominate within superficial layers which 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 IPSPs in dorsal horn neurones are substantially reduced in frequency, magnitude and duration, likely as a consequence of reduced GABA release (Moore *et al.*, 2002). Nerve injury also modulates levels of GABA, the GABA synthesizing enzyme GAD and $GABA_A$ receptor expression within the spinal dorsal horn (Castro-Lopes *et al.*, 1993; Castro-Lopes *et al.*, 1995; Moore *et al.*, 2002). At the behavioural level, $GABA_A$ receptor agonists such as muscimol and positive allosteric modulators such as diazepam attenuate nociceptive transmission in animal models of persistent and neuropathic pain associated with central sensitization (Kaneko and Hammond, 1997; Hwang and Yaksh, 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 α1, α2, α3 or

α5, together with a β subunit and a γ2 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 $GABA_A$-α1 receptors mediate sedation, $GABA_A$-α2/$GABA_A$-α3 receptors are involved in anxiety, whilst $GABA_A$-α5 receptors are relevant to memory function (Squires *et al*., 1979; Griebel *et al*., 1999a; 1999b; Paronis *et al*., 2001; Rudolph and Möhler, 2006). Recently, using a combination of molecular and pharmacological techniques Knabl *et al*. reported that $GABA_A$-α2/ $GABA_A$-α3 receptors are the principal contributors to spinal disinhibition occurring after injury (Knabl *et al*., 2008). 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 (Figure 1) in a range of animal models of chronic pain. NS11394 binds potently and non-selectively (Ki, ~0.5 nM) to $GABA_A$ receptors containing either α1, α2, α3 or α5 subunits combined with β2 and γ2S subunits, whereas affinity at $GABA_A$-α4 or $GABA_A$-α6 containing receptors is 600-2000 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 α5 > α3 > α2 > α1. This *in vitro* profile translates to potent (0.1-0.3 mg/kg, p.o.) anxiolytic-like effects in rodent shock/non-shock based

models of anxiety, likely attributable to its efficacy at $GABA_A$-α3 and/or $GABA_A$-α2 receptors (Mirza *et al.*, 2008). By contrast, at doses 200-600 fold higher NS11394 minimally impairs motor performance, likely attributable to low efficacy at $GABA_A$-α1 receptors and poor affinity for $GABA_A$-α4 and $GABA_A$-α6 receptors.

To obtain a comprehensive picture of the role of $GABA_A$ receptors in mediating pain-like behaviours after injury, we compared NS11394 to other known allosteric modulators: the full non-selective modulator diazepam, the partial non-selective modulator bretazenil, and the $GABA_A$-α1 selective full modulator zolpidem. Furthermore, we included the GABA site partial/full agonist gaboxadol to compare the actions of modulators to that of direct receptor activation (Ebert *et al.*, 2006). This comparative pharmacological approach allows us to delineate pharmacologically which $GABA_A$ receptors 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 which mediate withdrawal reflexes.

# Methods

## Animals

Adult male Sprague-Dawley rats (Harlan Scandinavia, Alleroed, Denmark) were used except where stated. They were housed in Macrolon III cages (20 x 14 x 18 cm or 20 x 40 x18 cm; in groups of 2-5 per cage according to weight) containing wood-chip bedding material (3 x 1 x 4 mm), and in a temperature-controlled environment with a light-dark cycle of 13:11 h (lights on at 06.00 h and off at 19.00 h). Food (Altromin®) and water were available *ad libitum*. The animals were allowed to habituate to the housing facilities for at least one week prior to surgery or behavioural testing. Neuropathic animals were subsequently housed on soft bedding material. All behavioural 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.

## Behavioural tests

*Explorative motility and Rotarod test*

Non-habituated male rats (body weight 90-120 g) were administered test drug or vehicle and returned to their home cage for 30-60 min depending on the drug administered. They were then placed individually in transparent cages (30 x 20 x 25 cm, TSE Systems, Bad Homburg, Germany) equipped with 12 infrared sensors (6 x 2). Locomotor activity, measured as distance travelled (m), 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 % of the vehicle response according to the equation,

(1) % Vehicle = (Post-treatment value/Vehicle value) × 100.

In normal, uninjured rats (body weight 250-350 g) the effects of $GABA_A$ receptor modulators on activity-induced motor function was evaluated using an accelerating rotarod (Ugo Basile, Comero, Italy). The rotarod (6 cm diameter) speed was increased from 3-30 rpm over a 180 s period, with the minimum time possible to spend on the rod designated as 0 s and the maximum cut-off time set at 180 s. Rats received two training trials (separated by 3-4 h) on two separate days prior to drug testing for acclimatisation purposes. On the day of drug testing, a baseline response was obtained, and rats 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 was subsequently expressed as a % of the corresponding baseline response according to the equation,

(2) % Baseline = (Post treatment value/Baseline value) × 100.

*Hot plate*

For the hot plate test, non-injured rats (body weight 100-120 g) were placed individually on a hot plate (Ugo Basile) maintained at a constant pre-set temperature of 52.5°C with a cut-off time of 40 s. The baseline latency response (s) 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 30-75 min after injection depending on the drug administered, enabling the change in latency response (s) 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 the equation,

(3) %MPE = (Post-treatment value - Pre-treatment value) × 100/(Ceiling value of assay - Pre-treatment value).

*Formalin test and capsaicin-induced sensitization*

Assessment of formalin-induced and capsaicin-induced flinching behaviour 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). Briefly, this involved placing a small C-shaped metal band (10 mm wide x 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 (diameter 30.5 cm x 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 27G needle. For capsaicin-induced sensitization experiments, individual rats were gently restrained and capsaicin (10 µg in 10 µl 10% Tween 80, s.c.) was injected into the plantar surface of the hindpaw using a 0.3 ml insulin syringe with a 29G 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 analogue signal was then digitised and a software algorithm applied to enable discrimination of flinching behaviour 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 behaviour 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 behaviour 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 was summed for each phase to obtain the total number of flinches occurring during that phase. This value was then expressed as a % of the vehicle response according to the equation,

(4) % Vehicle = (Post-treatment value)/(Vehicle value) × 100.

*Complete Freunds adjuvant-induced inflammatory nociception*

Rats (body weight 300–400 g) were given a s.c. injection of CFA (50% in saline, 100 µl, Sigma) into the plantar surface of the hindpaw under brief halothane anaesthesia. Nociceptive behaviours were routinely assessed for 2-3 days prior to, and 24 h following CFA injection. Changes in hindpaw weight bearing were assessed using an Incapacitance tester (Linton Instrumentation, U.K.), 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 favours the non-injured 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 read out for each hindpaw was taken as the distributed body weight on each paw (g). Three readings were obtained to ensure that consistent responses were measured. These were averaged for each hindpaw and the weight bearing difference 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 4-6 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 (2 measurements each separated by 5 min) were made prior to CFA injection, to familiarise the rats with the testing procedure. Two further baseline latency measurements were obtained on the day of drug testing to ensure 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 post-injection and tail flick latency responses determined at 60-90 min depending on the drug administered. Weight bearing differences after drug treatment were expressed as a % of the corresponding baseline response according to the equation,

(5) % Baseline = (Post-treatment value)/(Baseline value) × 100.

Tail flick latencies were converted to a maximal possible effect value according to the equation,

(6) %MPE = (Post-treatment value - Pre-treatment value) × 100/(ceiling value of assay - Pre-treatment value).

*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). Anaesthesia 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 2-3 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.

*Behavioural 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). Prior to testing individual rats were removed from their home cage and allowed to habituate for 15 min in an openly ventilated 15 x 20 cm white Plexiglass 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 since 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 1-2 s and was repeated 5 times at 1-2 s intervals. The filament that induced a reflex paw withdrawal in 3 out of 5 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 which 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 30-180 min after drug administration were expressed as a %MPE response according to equation (6) described above.

Only those animals showing distinct neuropathic behaviours from between 10-30 days post-surgery were included in drug testing experiments. During an individual testing

session which used 6-8 nerve-injured rats nociceptive measurements were performed with the observer blinded to treatment. By strictly adhering to a minimum 2-3 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 (ii) one injection of vehicle plus two injections of 2 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). Briefly, Wistar rat pups (7–11 days old) were anaesthetised with urethane (2 g/kg, i.p.) and their spinal cords extracted following a dorsal laminectomy. With the cord in cold artificial cerebrospinal fluid (ACSF), the outer meninges were removed, the cord was hemisected and then pinned down to a Sylgard based recording chamber 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 (in mM) NaCl (128); KCl (1.9); $KH_2PO_4$ (1.2); $MgSO_4$ (1.3); $CaCl_2$ (2.4); $NaHCO_3$ (26); glucose (10); (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 L4 or the L5 dorsal root was recorded by means of a suction electrode coupled to a Cyber-Amp amplifier (Axon Instruments, USA) 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 mV*s). The sequence of three GABA boluses was applied at 45 min intervals. Three control responses were obtained prior to 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 L5 dorsal root and the corresponding ventral root were placed in tight fitting glass suction electrodes. Electrical stimuli sufficient to activate C-fibres (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). Briefly, 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 Hz and 1500 Hz.

The stimulation protocol was applied at 20 min intervals. Three basal responses were obtained prior to 15 min superfusion with NS11394. Afterwards, 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 mono-synaptic reflex (in mV) and the area of depolarisation measured in a time window between 100 ms and 4 s from stimulus artefact (in mV*s); 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 synthesised in the Medicinal Chemistry Department, NeuroSearch A/S. Gaboxadol (chloride salt) was purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). 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 behavioural studies, NS11394 was dissolved in 5% Tween80/milliQ water and administered p.o. 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/milliQ water. These four drugs were administered i.p. in a dosing volume of 2 ml/kg. Gaboxadol and morphine were dissolved in 0.9% saline and zolpidem in 5% glucose/milliQ water. These three drugs were administered s.c. in a

dosing volume of 1 ml/kg. All doses are expressed as either mg weight salt or mg weight free base per kg body weight, respectively.

For behavioural 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 $GABA_A$-α1 receptors (Sanger and Benavides, 1993). Despite this lower level of *in-vivo* receptor occupancy with zolpidem, it is very clear from the data described in the Results section that major CNS mediated effects are seen at doses between 1-10 mg/kg, and it was therefore 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 utilised here (Cremers and Ebert, 2007).

For electrophysiology studies, NS11394 was diluted in DMSO at 10 mM and stored at -20ºC. NS11394 was diluted down to 1 μM in ACSF just prior to use and superfused to the entire preparation for 15 min periods to ensure a complete tissue equilibration. Application of 0.01% DMSO alone did not have any observable effect on spinal reflexes (n = 2). GABA was dissolved in ultrapure water at 10 mM stored at -20ºC,

diluted down to its final concentration in ACSF just prior to use where it was delivered to the preparation as a 1 min bolus.

*Data analysis*

Comparison of drug effects between injured and non-injured rats across the range of behavioural tests employed was made via conversion of raw data to either % of vehicle, baseline or maximal possible effect values. Analysis of data was performed using Sigmastat 2.03 (SPSS Inc., Chicago, ILL.). All data are presented as mean ± SEM. 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 (v3.02). Repeated measures ANOVA was used to analyze responses to trains of stimuli. Results are expressed as mean ± SEM. $p<0.05$ was considered to be statistically significant.

## Results

*Explorative motility and Rotarod test*

To determine if 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 non-injured rats (Figure 2A). Rats administered vehicle displayed typical explorative behaviour of a novel environment throughout a 30 min test (range of 5 experiments 52 ± 10 – 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 behaviour ($F[5,55]=2.474$, $p<0.05$; $F[3,27]=29.277$, $p<0.001$; $F[3,27]=35.822$, $p<0.001$ 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 (Figure 2A), with NS11394 and bretazenil having minor effects relative to the other drugs.

In the rotarod test (Figure 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 s and -15 ± 8.5 s; both $p<0.05$, Figure 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 ($F[3,31]=48.084$, $p<0.001$; $F[3,31]=89.960$, $p<0.001$; $F[3,30]=28.138$, $p<0.001$). 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, zolpidem's influence on motoric integrity in the rat is more profound than that seen with diazepam, despite seemingly similar impacts on explorative motility and rotarod ataxia (Figure 2).

*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 (Figure 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 ($F[3,31]=2.616$, $p=0.071$; $F[3,31]=0.639$, $p=0.596$; $F[3,31]=2.870$, $p=0.054$; $F[3,30]=2.581$, $p=0.074$). In contrast, gaboxadol (1-10 mg/kg) exhibited a modest but significant analgesic-like profile in this test ($F[3,31]=7.575$, $p<0.001$), whilst 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-fibres and initiates spontaneous nociceptive behaviours consisting of either flinching, and 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 to activation of noxious inhibitory controls and the second phase 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-fibre primary afferents and manifests as flinching, and licking and/or biting of the injected paw.

NS11394 (3-30 mg/kg) significantly reduced flinching behaviour 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 (Figure 4A and Table 1). Similarly, diazepam (1-10 mg/kg) and zolpidem (1-10 mg/kg) appeared to selectively attenuate second phase flinching behaviour ($F[3,31]=6.880$, $p<0.01$ 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 ($F[3,31]=14.797$, $p<0.001$; $F[3,31]=20.484$, $p<0.001$; $F[3,31]=45.495$, $p<0.001$ for first phase, interphase and second phase, respectively), indicative of a non-selective 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 behaviour ($F[3,31]=5.320$, $p<0.01$). 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% reduction in flinching during the second phase of the test ($p<0.05$ *vs* vehicle; Figure 4B), and this effect was blocked by co-administration 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 re-investigated 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 non-significant (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, Figure 5). Over the following 25 min which would be expected to coincide with a more persistent and selective activation of TRPV1 containing C-fibres, vehicle-treated rats continued to exhibit a robust flinching response (400 ± 44 total flinches). During this period, NS11394 treatment significantly reduced flinching behaviour ($F[5,57]=8.701$, $p<0.001$, Figure 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 6-30 min ($F[3,31]=9.294$, $p<0.001$, $F[3,31]=4.017$, $p<0.05$, $F[3,27]=34.448$, $p<0.001$, respectively; Figure 5B) after capsaicin injection. However,

in addition diazepam and gaboxadol both dose-dependently attenuated flinching from 0-5 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 Freunds 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 behavioural 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 non-evoked pain was observed compared with weight bearing prior to injection (35 ± 1.9 g *vs* -0.1 ± 3.2 g, n=95, $p<0.001$, Students *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 (Figure 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 ($F[4,63]=7.672$, $p<0.001$). However, in contrast to NS11394, diazepam also significantly affected the tail flick response in CFA treated rats ($F[3,31]=6.740$, $p<0.01$, Figure 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, $p<0.001$; Figure 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 behaviours, $GABA_A$ 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. Following surgery, both CCI ($n$=103) and SNI ($n$=34) rats developed behavioural 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 g and 0.5 ± 0 g, respectively), compared to pre-surgery 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 s and 14 ± 0.3 for CCI and SNI rats, respectively) compared to pre-surgery 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, Figure 7A). Similarly, 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 *vs* 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 ($F[9,95]=12.537$, $p<0.001$, Figure 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, Figure 7C). Similarly, 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 vs 90%, respectively; data not shown).

*In vitro* electrophysiology

In the light of a spinal action of diazepam (Siarey *et al*., 1992; Siarey *et al*., 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 (Figure 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% of 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 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 fibres, 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 (Figures 8B and C). The integrated area of the response decreased to 60 ± 1.5 % of 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 artefact (Figure 8C).

Repetitive stimulation of the dorsal root produced a typical wind-up of action potentials which depends on C-fibre 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% of control (Figures 8D 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 (Mirza *et al.*, 2008, summarised in Figure 1). Recent studies in gene targeted animals suggest a pivotal role for $GABA_A$-α2/$GABA_A$-3 receptors in normalising spinal disinhibition after injury. Therefore we have tested orally administered NS11394 against a range of injury-induced nociceptive behaviours 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 behaviours (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 behaviours (see below). Crucially, NS11394 reduced second phase formalin- and capsaicin- induced flinching. 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-fibre 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 minimum 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* since 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 a par with NS11394, albeit at doses associated with motoric impairments.

In non-injured rats, spinal administration of the $GABA_A$ agonist isoguvacine produces a bicuculline-reversible increase in acute nociceptive threshold. To investigate if NS11394 would selectively attenuate behavioural sequelae associated with injury-induced central sensitization, its influence on spontaneous nociceptive behaviours 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 doses tested. However, NS11394 had no effect on tail flick latency in CFA rats and did not affect hot plate latency responses in non-injured 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 localised 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.

*What are the pharmacological determinants of NS11394's antinociceptive actions?*

Diazepam has full efficacy at $\alpha1$, $\alpha2$, $\alpha3$ and $\alpha5$ containing $GABA_A$ receptors and is effective across the range of pain models utilised here. Since 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 the explanation is so straightforward, since 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 anti-anxiety and antinociceptive effects differ.

Another interesting finding is the inconsistent efficacy of zolpidem across the range of pain models. Since zolpidem has ~10-fold higher affinity for $GABA_A$-α1 compared to $GABA_A$-α2 and $GABA_A$-α3 receptors (Sieghart, 1995) this indicates that $GABA_A$-α1 receptors are not involved in antinociception, consistent with NS11394 and L838,417 data (Knabl *et al*., 2008). However, zolpidem is still a full efficacy modulator of $GABA_A$-α2 and $GABA_A$-α3 receptors so why does it not work in CCI? We believe that although zolpidem might have sufficient activity at $GABA_A$-α2 and $GABA_A$-α3 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 $GABA_A$-α5 receptors. Thus, whereas zolpidem's efficacy at $GABA_A$-α2 and $GABA_A$-α3 containing receptors is sufficient to mediate antinociceptive actions in formalin/capsaicin models, its poor affinity for $GABA_A$-α5 receptors (Sieghart, 1995) precludes efficacy in CCI rats. Although speculative this explanation is consistent with our data as diazepam, NS11394 and L838,417 all have efficacy at $GABA_A$-α5 receptors. $GABA_A$-α5 containing receptors are distributed through numerous spinal lamina, and α5 gene expression is modulated in the DRG and dorsal horn in neuropathic pain models (Xiao *et al*. 2002; Yang *et al*., 2004).

Limited studies with α4 knockout mice implicate $GABA_A$-α4 receptors in gaboxadols antinociceptive properties (Chandra *et al.*, 2006). However, this receptor is unlikely to be relevant for NS11394 and diazepam induced antinociception since both bind poorly to $GABA_A$-α4 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 $GABA_A$-α4, α5 and α6 receptors (Ebert *et al*., 2006; Cremers and Ebert, 2007), we would urge future studies to investigate the possibilities that gaboxadol's antiociceptive actions might be mediated, (i) via $GABA_A$-α3 and/or $GABA_A$-α2 receptors; or (ii) possibly via $GABA_A$-α5 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 which mediate nociceptive withdrawal reflexes. Primary afferent depolarization (PAD), mediated predominantly by $GABA_A$ receptors, constitutes a classical mechanism for pre-synaptic inhibition (Rudomin and Schmidt, 1999). Here we show that NS11394 potentiates PAD 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). NS11394's actions are specific since it did not alter the monosynaptic reflex, which in the rat is sensitive to baclofen but not to bicuculline (Akagi *et al*., 1987). These observations indicate that the native spinal GABA-ergic 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 fibres. 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.

NS11394's strong depression of spinal nociceptive processing is commensurate with its effects in neuropathic pain models, and suggests that the spinal cord is a likely site through which NS11394 produces antinociception. Work from other laboratories shows 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 (Pedersen *et al.*, 2007; LaGraize and Fuchs, 2007). In addition, preliminary work in our laboratory suggests that supraspinal mechanisms might also be relevant to NS11394's antinociceptive actions.

*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 would conclude that NS11394's *selectivity* for and *level of efficacy* at $GABA_A$-α3 and/or $GABA_A$-α2 receptors mediates its antinociceptive actions. Thus, molecules being developed for anxiety with a $GABA_A$-α2/$GABA_A$-α3 over $GABA_A$-α1 selectivity profile might not necessarily have the appropriate *in vitro*

JPET Fast Forward. Published on September 12, 2008 as DOI: 10.1124/jpet.108.144568
This article has not been copyedited and formatted. The final version may differ from this version.



profile for efficacy in pain. At this point in time, a potential role for $GABA_A$-α5 containing receptors in pain should not be dismissed since 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 NS11394's antinociceptive effects are mediated at least partly via pre- and postsynaptic spinal mechanisms.

## Acknowledgements

The expert technical assistance of Helene Dyhr and Margit Nelboe Jeppesen is greatly appreciated.

## References

Akagi H and Yanagisawa M (1987) GABAergic modulation of a substance P-mediated reflex of slow time course in the isolated rat spinal cord. *Br J Pharmacol* 91:189-197.

Bennett GJ and Xie YK (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 33:87-107.

Bohlhalter S, Weinmann O, Mohler H and Fritschy JM (1996) Laminar compartmentalization of GABAA-receptor subtypes in the spinal cord: an immunohistochemical study. *J Neurosci* 16:283-297.

Castro-Lopes JM, Malcangio M, Pan BH and Bowery NG (1995) Complex changes of GABAA and GABAB receptor binding in the spinal cord dorsal horn following peripheral inflammation or neurectomy. *Brain Res* 679:289-297.

Castro-Lopes JM, Tavares I and Coimbra A (1993) GABA decreases in the spinal cord dorsal horn after peripheral neurectomy. *Brain Res* 620:287-291.

Coderre TJ, Katz J, Vaccarino AL and Melzack R (1993) Contribution of central neuroplasticity to pathological pain: review of clinical and experimental evidence. *Pain* 52:259-285.

Cremers T and Ebert B (2007) Plasma and CNS concentrations of Gaboxadol in rats following subcutaneous administration. *Eur J Pharmacol* 562:47-52.

Decosterd I and Woolf CJ (2000) Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* 87:149-158.

Dirig DM and Yaksh TL (1995) Intrathecal baclofen and muscimol, but not midazolam, are antinociceptive using the rat-formalin model. *J Pharmacol Exp Ther* 275:219-227.

Ebert B, Wafford KA and Deacon S (2006) Treating insomnia: Current and investigational pharmacological approaches. *Pharmacol Therap* 112:612-629.

Griebel G, Perrault G, Letang V, Granger P, Avenet P, Schoemaker H, and Sanger DJ (1999a) New evidence that the pharmacological effects of benzodiazepine receptor ligands can be associated with activities at different BZ (omega) receptor subtypes. *Psychopharmacology (Berl*) 146:205–213.

Griebel G, Perrault G, and Sanger DJ (1999b) Study of the modulatory activity of BZ (omega) receptor ligands on defensive behaviors in mice: evaluation of the importance of intrinsic efficacy and receptor subtype selectivity. *Prog Neuropsychopharmacol Biol Psychiatry* 23:81–98.

Haefely W, Martin JR and Schoch P (1990) Novel anxiolytics that act as partial agonists at benzodiazepine receptors. *Trends Pharmacol Sci* 11:452-456.

Hedo G and Lopez-Garcia JA (2001) Alpha-1A adrenoceptors modulate potentiation of spinal nociceptive pathways in the rat spinal cord *in-vitro*. *Neuropharmacology* 41:862-869.

Henry JL, Yashpal K, Pitcher GM and Coderre TJ (1999) Physiological evidence that the 'interphase' in the formalin test is due to active inhibition. *Pain* 82:57-63.

Hwang JH and Yaksh TL (1997). The effect of spinal GABA receptor agonists on tactile allodynia in a surgically-induced neuropathic pain model in the rat. *Pain* 70:15-22.

Kaneko M and Hammond DL (1997) Role of spinal gamma-aminobutyric acidA receptors in formalin-induced nociception in the rat. *J Pharmacol Exp Ther* 282:928-938.

Knabl J, Witschi R, Hösl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy JM, Rudolph U, Möhler H and Zeilhofer HU (2008) Reversal of pathological pain through specific spinal GABAA receptor subtypes. *Nature* 451:330-334.

LaGraize SC and Fuchs PN (2007) GABAA but not GABAB receptors in the rostral anterior cingulate cortex selectively modulate pain-induced escape/avoidance behavior. *Exp Neurol* 204:182-194.

Malan TP, Mata HP and Porreca F (2002) Spinal GABA(A) and GABA(B) receptor pharmacology in a rat model of neuropathic pain. *Anesthesiology* 96:1161-1167.

Malcangio M and Bowery NG (1996) GABA and its receptors in the spinal cord. *Trends Pharmacol Sci* 17:457-462.

McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, Hayward NJ, Chong JA, Julius D, Moran MM and Fanger CM (2007) TRPA1 mediates formalin-induced pain. *Proc Natl Acad Sci U S A* 104:13525-13530.

Mirza NR and Nielsen EØ (2006) Do subtype-selective gamma-aminobutyric acid A receptor modulators have a reduced propensity to induce physical dependence in mice? *J Pharmacol Exp Ther* 316:1378-1385.

Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H and Woolf CJ (2002) Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. *J Neurosci* 22:6724-6731.

Munro G, Bæk C, Erichsen HK, Nielsen AN, Nielsen EØ, Scheel-Krüger J, Weikop P and Peters D (2008) The novel compound (+/-)-1-[10-((E)-3-Phenyl-allyl)-3,10-diaza-bicyclo[4.3.1]dec-3-yl]-propan-1-one (NS7051) attenuates nociceptive transmission in animal models of experimental pain; a pharmacological comparison with the combined mu-opioid receptor agonist and monoamine reuptake inhibitor tramadol. *Neuropharmacology* 54:331-343.

Paronis CA, Cox ED, Cook JM, and Bergman J (2001) Different types of GABA(A) receptors may mediate the anticonflict and response rate-decreasing effects of zaleplon, zolpidem and midazolam in squirrel monkeys. *Psychopharmacology (Berl*) 156:461-468.

Pedersen LH, Scheel-Krüger J and Blackburn-Munro G (2007) Amygdala GABA-A receptor involvement in mediating sensory-discriminative and affective-motivational pain responses in a rat model of peripheral nerve injury. *Pain* 127:17-26.

Poisbeau P, Patte-Mensah C, Keller AF, Barrot M, Breton JD, Luis-Delgado OE, Freund-Mercier MJ, Mensah-Nyagan AG and Schlichter R (2005) Inflammatory pain

upregulates spinal inhibition via endogenous neurosteroid production. *J Neurosci* 25:11768-11776.

Rivera-Arconada I and Lopez-Garcia JA (2006) Retigabine-induced population primary afferent hyperpolarisation *in-vitro*. *Neuropharmacology* 51:756-763.

Rudolph U and Möhler H (2006) GABA-based therapeutic approaches: GABAA receptor subtype functions. *Curr Opin Pharmacol* 6:18-23.

Rudomin P and Schmidt RF (1999) Presynaptic inhibition in the vertebrate spinal cord revisited. *Exp Brain Res* 129:1-37.

Siarey RJ, Long SK and Evans RH (1992) The effects of centrally acting myorelaxants on NMDA receptor-mediated synaptic transmission in the immature rat spinal cord *in-vitro*. *Br J Pharmacol* 107:628-633.

Siarey RJ, Long SK, Tulp MTH and Evans RH (1994) The effects of central myorelaxants on synaptically-evoked primary afferent depolarization in the immature rat spinal cord *in-vitro*. *Br J Pharmacol* 111:497-502.

Sanger DJ and Benavides J (1993) Discriminative stimulus effects of omega (BZ) receptor ligands: correlation with *in-vivo* inhibition of [3H]-flumazenil binding in different regions of the rat central nervous system. *Psychopharmacology (Berl*) 111:315–322.

Sieghart W (1995) Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. *Pharmacol Rev* 47:181–234.

Simjee SU, Pleuvry BJ and Coulthard P (2004) Modulation of the gait deficit in arthritic rats by infusions of muscimol and bicuculline. *Pain* 109:453-460.

Squires RF, Benson DI, Braestrup C, Coupet J, Klepner CA, Myers V, and Beer B (1979) Some properties of brain specific benzodiazepine receptors: new evidence for multiple receptors. *Pharmacol Biochem Behav* 10:825-830.

Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 16:109-110.

## Legends for Figures

**Figure 1.** Chemical structure of NS11394: [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]biphenyl-2-carbonitrile] and summary of key data from Mirza *et al*. (2008). The table shows, (i) the $K_i$ (nM) for NS11394 to inhibit [$^3$H]flunitrazepam binding to rat cortical tissue (row 1), [$^3$H]Ro 15-1788 binding to hGABA$_A$ receptors containing the α1, α2, α3 or α5 subunits together with β3 and γ2s stably expressed in HEK-293 cells (row 2), and hGABA$_A$ receptors containing the α4 or α6 subunits together with β3 and γ2s stably expressed in HEK-293 cells (row 3); (ii) the maximum potentiation (Emax, %), relative to diazepam (0.5 μM), by NS11394 of GABA ($EC_5$-$EC_{25}$) evoked currents recorded from oocytes containing hGABA$_A$ receptors containing the α1, α2, α3 or α5 subunits together with β2 and γ2s (rows 4-7); (iii) NS11394's half-life (T½), bioavailability (F%) and calculated brain/plasma ratio in rat based on dosing animals with 3 mg/kg, p.o. and 3 mg/kg, i.v. (rows 8-10); (iv) NS11394's $ED_{50}$ for displacing [$^3$H]flunitrazepam binding to rat cortex *in vivo* (row 11) and MED for anxiolytic-like effect in the rat conditioned emotional response test (row 12).

**Figure 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 travelled (m; 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, except for 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 *vs* corresponding vehicle group (one way ANOVA followed by Bonferroni's test).

**Figure 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 ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001 *vs* corresponding vehicle group (one way ANOVA followed by Bonferroni's t test).

**Figure 4.** NS11394 selectively attenuates injury-induced nociceptive behaviours in the formalin test. Normal, uninjured rats were administered either NS11394 (3-30 mg/kg, p.o.) or vehicle 30 min prior to hindpaw formalin (5% in saline, 50 µl, s.c.) injection. (A) time-course of flinching behaviour 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 behaviour after co-administration 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 *vs* corresponding vehicle group (one way ANOVA followed by Bonferroni's test).

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

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

**Figure 7.** NS11394 attenuates mechanical allodynia in rats with peripheral nerve injury. (A) Comparison of maximum 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 NS1194. 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 (g) measured. (C) Comparison of maximum 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 *vs* corresponding vehicle group (one way ANOVA followed by Bonferroni's test).

**Figure 8.** NS11394 attenuates nociceptive transmission through spinal circuits. Panels A to D show original recordings obtained under control conditions (left) and after superfusion of 1μM NS11394 (right). (A) Dorsal root recordings. Responses to application of three concentrations of GABA (as marked in μM) 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 artefacts 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 artefacts. 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 *vs* NS11394 (ANOVA).

JPET Fast Forward. Published on September 12, 2008 as DOI: 10.1124/jpet.108.144568
This article has not been copyedited and formatted. The final version may differ from this version.



**TABLE 1.** Effects of $GABA_A$ receptor modulators in the formalin test.

| **Drug** | **Dose (mg/kg)** | **1st phase** | **Formalin test % Vehicle Interphase** | **2nd phase** |
|---|---|---|---|---|
| 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** |

Rats were administered either drug or vehicle prior to hindpaw formalin (5% in saline, 50 μl, s.c.) injection enabling the number of formalin-induced flinches to be measured. Pre-administration times were 30 min for NS11394 (p.o.), diazepam (i.p.) bretazenil, (i.p.), zolpidem (s.c.), and gaboxadol (s.c.) and 60 min for gabapentin (i.p.). 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 behaviour, these effects were observed at doses that also markedly attenuated explorative motility behaviour and so are not considered as a direct antinociceptive action. $^{**}p<0.01$, $^{***}p<0.001$ *vs* corresponding vehicle group (one way ANOVA followed by Bonferroni's t test).

JPET Fast Forward. Published on September 12, 2008 as DOI: 10.1124/jpet.108.144568
This article has not been copyedited and formatted. The final version may differ from this version.

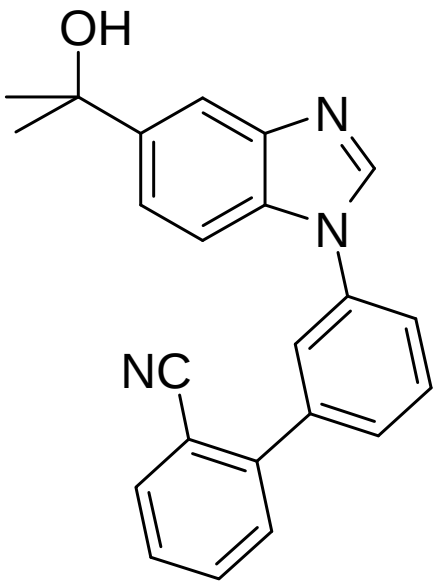


**TABLE 2.** Summary of antinociceptive potency of NS11394 and $GABA_A$ receptor modulators in uninjured and injured rats.

| | | | **MED (mg/kg)** | | |
|---|---|---|---|---|---|
| | **NS11394** | **Diazepam** | **Zolpidem** | **Gaboxadol** | **Bretazenil** |
| *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 |

For each compound and associated behavioural test the indicated value represents the minimum effective dose (MED; mg/kg) required to significantly modulate behaviour. All values were obtained from corresponding behavioural experiments represented in Table 1 and Figures 2-7. CCI, chronic constriction injury; CFA, complete Freunds adjuvant; MA, mechanical allodynia; MH, mechanical hyperalgesia; SNI, spared nerve injury; n.t., not tested.

JPET Fast Forward. Published on September 12, 2008 as DOI: 10.1124/jpet.108.144568
This article has not been copyedited and formatted. The final version may differ from this version.




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 |



Figure 1

JPET Fast Forward. Published on September 12, 2008 as DOI: 10.1124/jpet.108.144568
This article has not been copyedited and formatted. The final version may differ from this version.

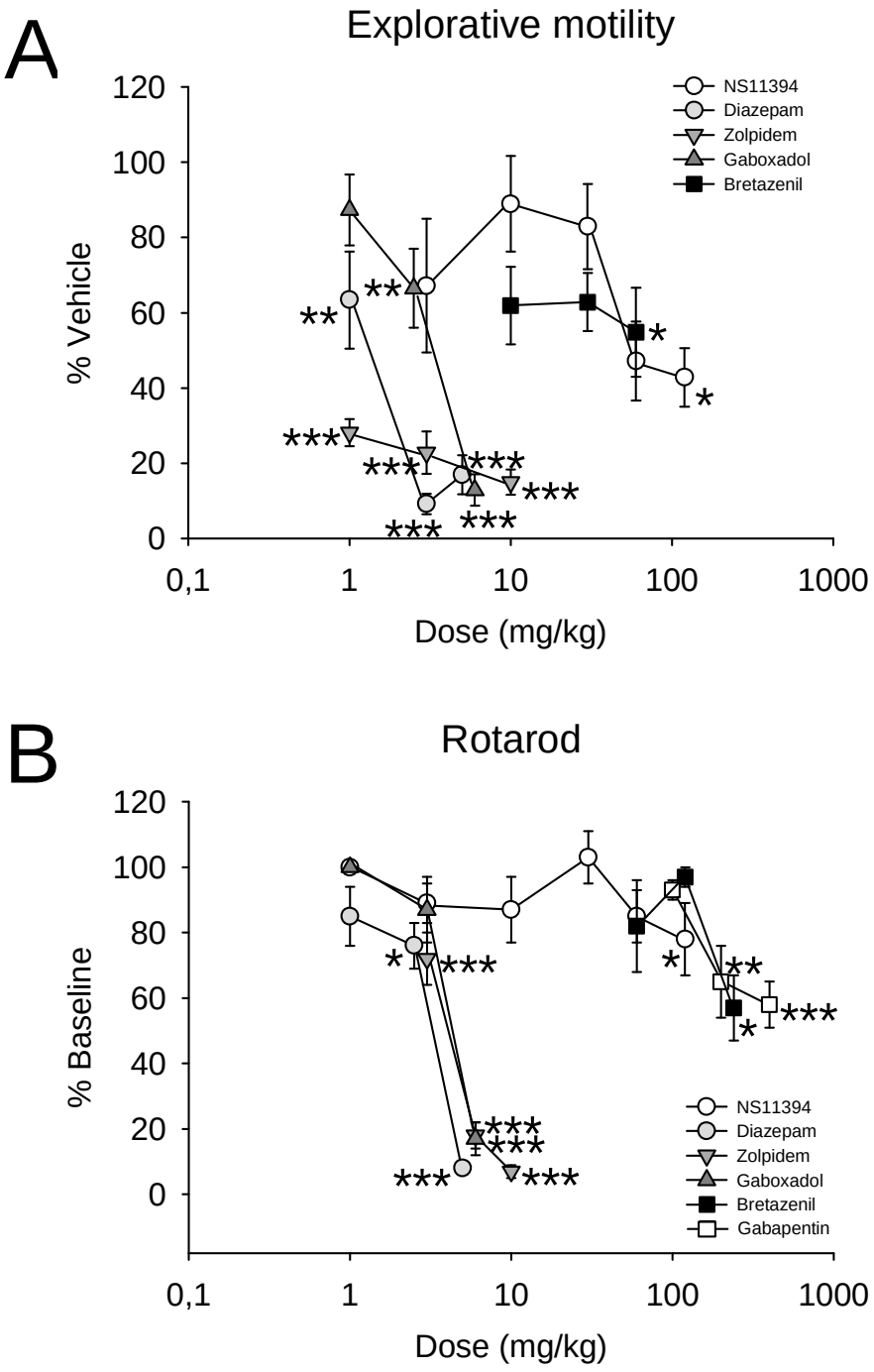



Figure 2

JPET Fast Forward. Published on September 12, 2008 as DOI: 10.1124/jpet.108.144568
This article has not been copyedited and formatted. The final version may differ from this version.

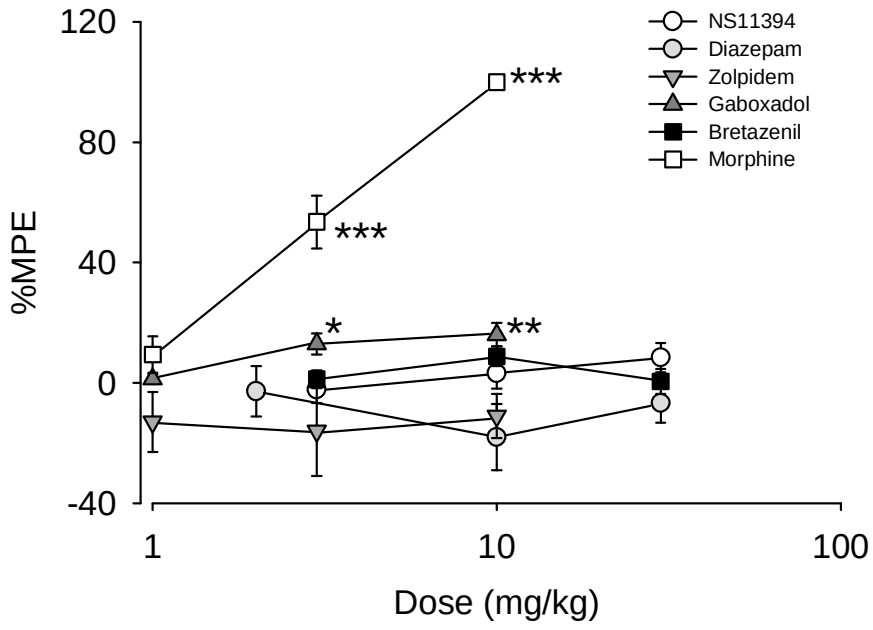



Figure 3

JPET Fast Forward. Published on September 12, 2008 as DOI: 10.1124/jpet.108.144568
This article has not been copyedited and formatted. The final version may differ from this version.

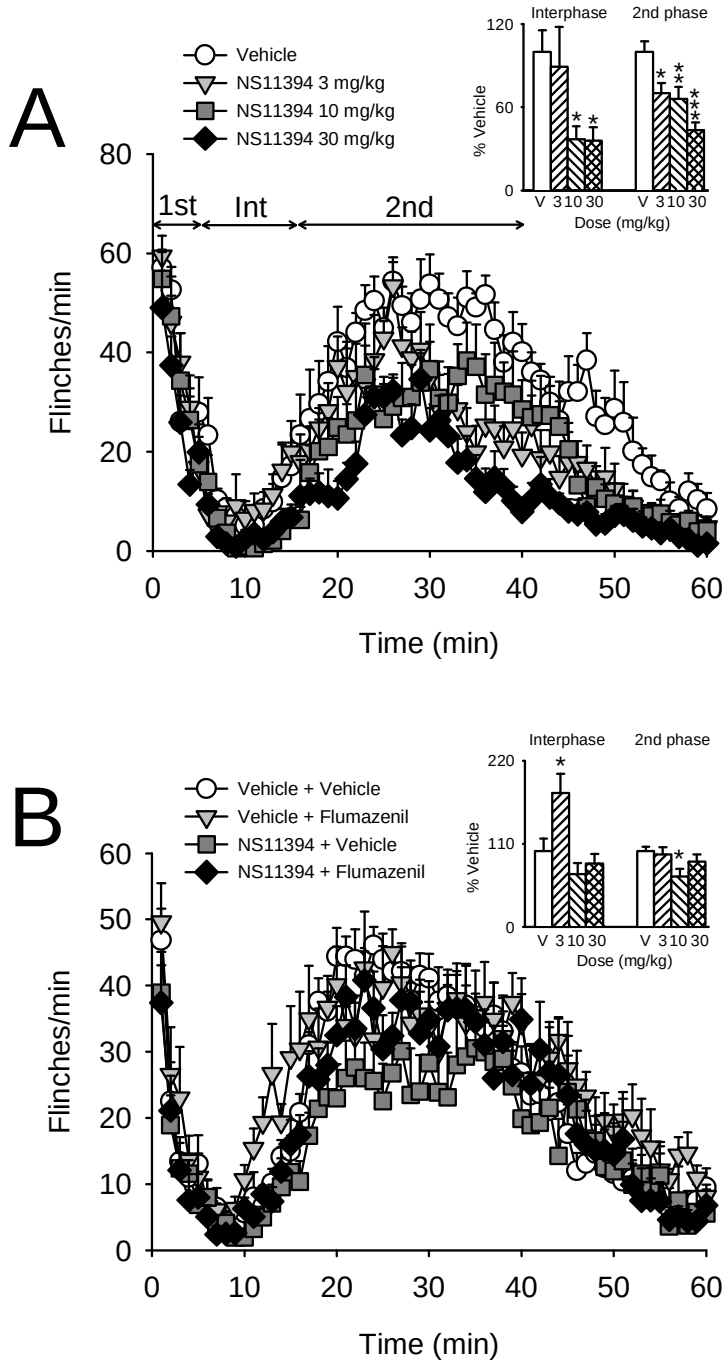



Figure 4

JPET Fast Forward. Published on September 12, 2008 as DOI: 10.1124/jpet.108.144568
This article has not been copyedited and formatted. The final version may differ from this version.

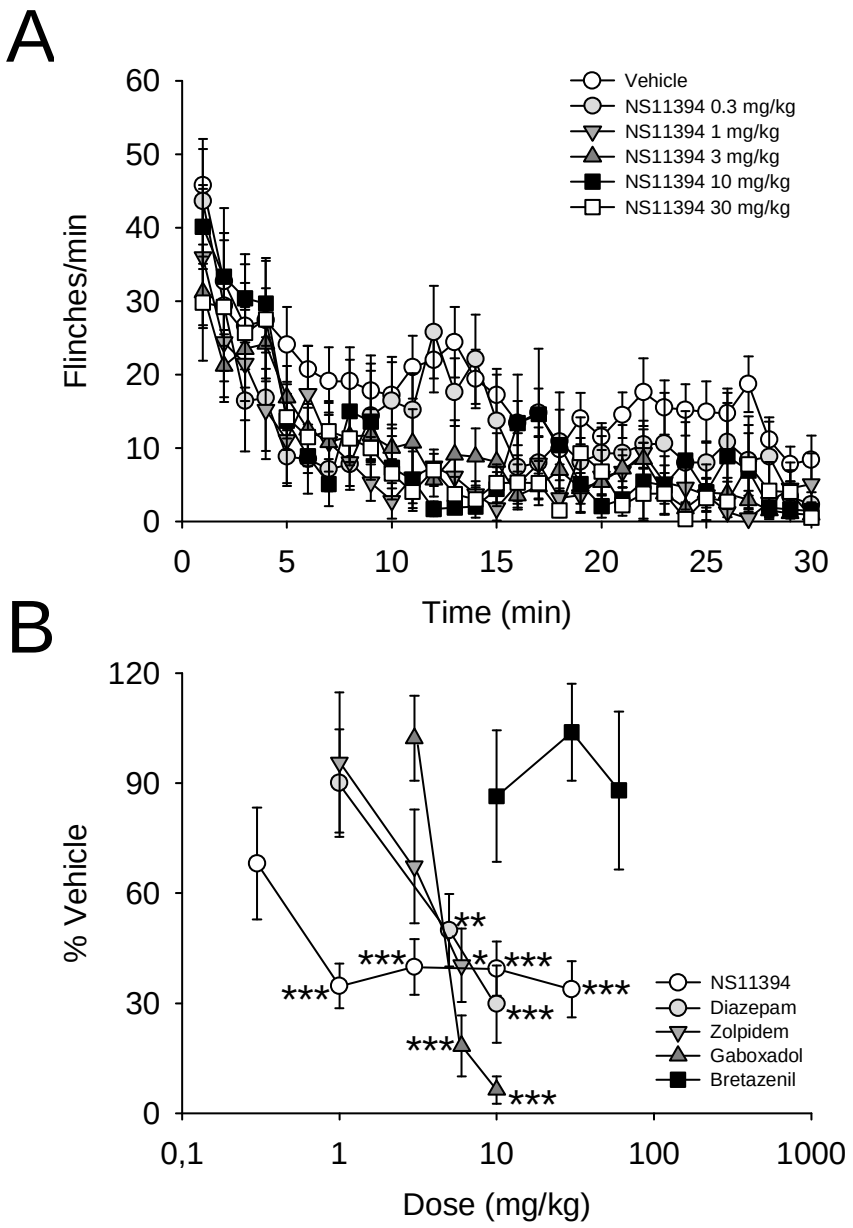



Figure 5

JPET Fast Forward. Published on September 12, 2008 as DOI: 10.1124/jpet.108.144568
This article has not been copyedited and formatted. The final version may differ from this version.

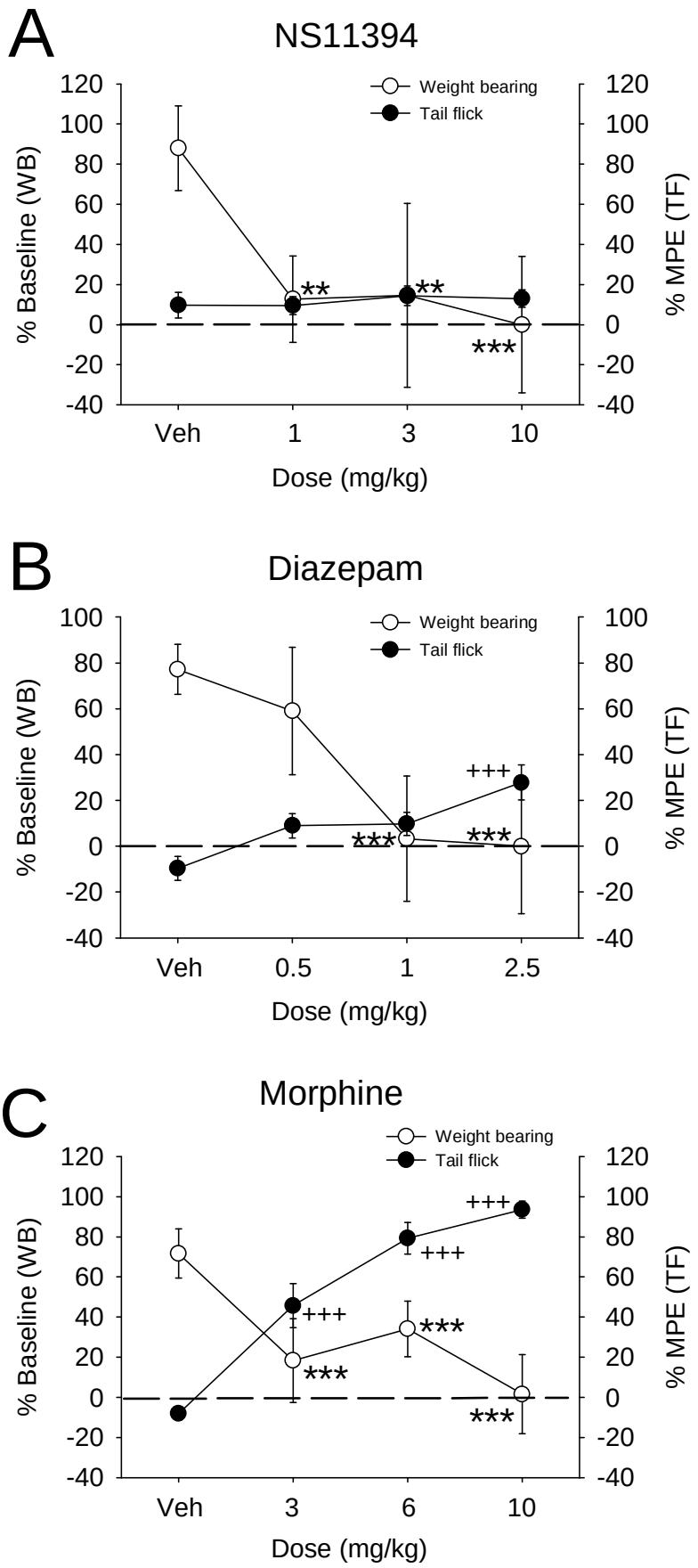



Figure 6

JPET Fast Forward. Published on September 12, 2008 as DOI: 10.1124/jpet.108.144568
This article has not been copyedited and formatted. The final version may differ from this version.

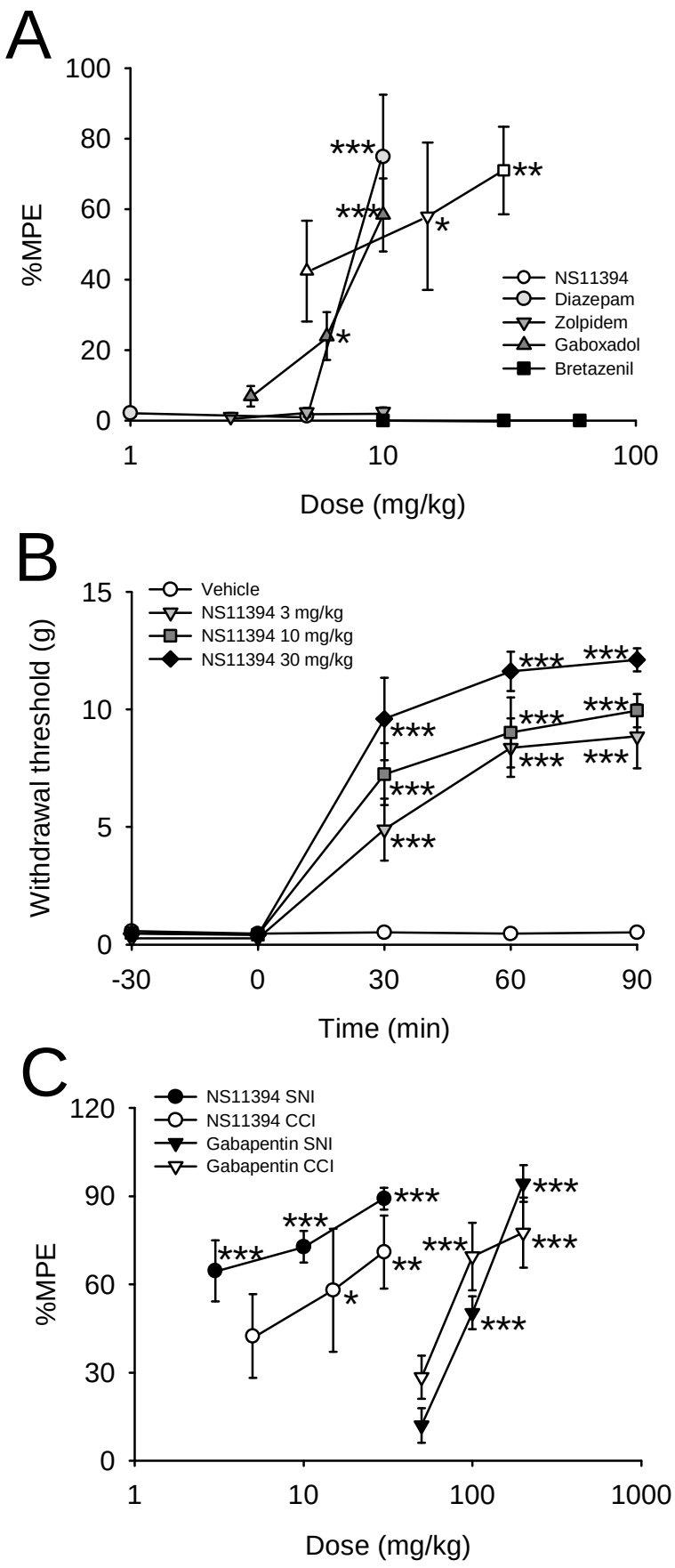



Figure 7

JPET Fast Forward. Published on September 12, 2008 as DOI: 10.1124/jpet.108.144568
This article has not been copyedited and formatted. The final version may differ from this version.

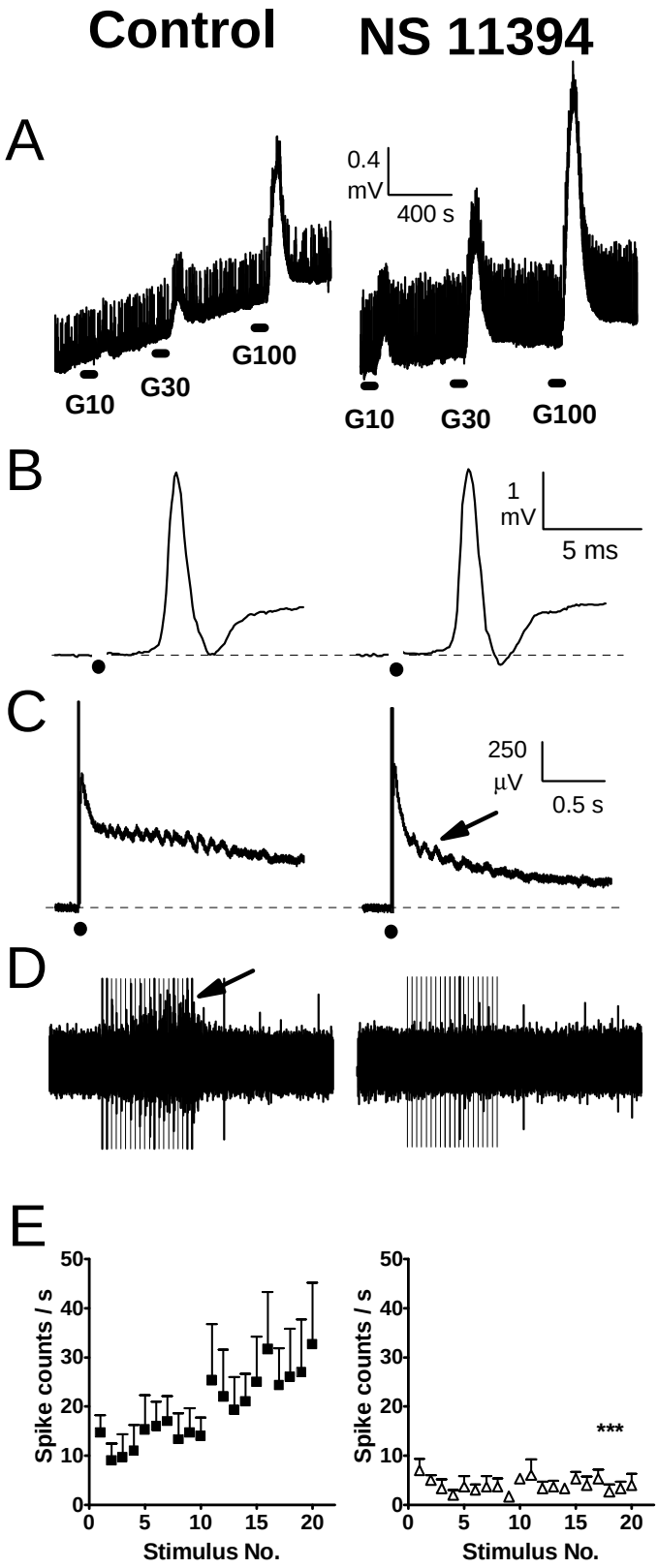



Figure 8