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# $GABA_A$ α5 subunit-containing receptors do not contribute to reversal of inflammatory-induced spinal sensitization as indicated by the unique selectivity profile of the $GABA_A$ receptor allosteric modulator NS16085

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ABSTRACT

$GABA_A$ receptor positive allosteric modulators (PAMs) mediate robust analgesia in animal models of pathological pain. Restoration of diminished spinal $GABA_A$-α2 and -α3 subunit-containing receptor function is a principal contributor to this analgesia, albeit involvement of $GABA_A$-α5-receptors has not been excluded. Thus, we compared NS11394 and TPA023 (PAMs with selectivity/efficacy at $GABA_A$-α2/α3/α5 receptors) with TP003 (a reportedly $GABA_A$-α3 selective PAM) against spinal sensitization. However, in-house electrophysiology studies designed to confirm the selectivity of TPA023 and TP003 for human $GABA_A$ receptors did not corroborate published data, with TP003 displaying considerable $GABA_A$-α5 receptor efficacy. Therefore, we identified a novel PAM, NS16085, which possesses negligible efficacy at $GABA_A$-α5 receptors, but with $GABA_A$-α2/α3 efficacy equivalent to NS11394. At the $GABA_A$-α1 receptor the compound gives low level of negative modulation further separating it from the other compounds. Rat pups with carrageenan-induced hindpaw inflammatory hyperalgesia were used to make *ex vivo* spinal dorsal root-evoked ventral root recordings. Some spontaneous activity and large numbers of spikes to repetitive stimulation of dorsal roots at C-fibre intensity, indicative of wind-up and sensitization were observed. Equimolar concentrations of NS11394, TP003 and NS16085 all attenuated wind-up to a similar degree; TPA023 was clearly less effective. In adult rats, NS16085 (3–30 mg/kg, p.o.) dose-dependently reduced formalin-induced hindpaw flinching with efficacy comparable to NS11394. Thus, potentiation of $GABA_A$-α2 and-α3 receptors is sufficient to depress spinal sensitization and mediate analgesia after inflammatory injury. Positive modulation at $GABA_A$-α5-receptors is apparently dispensable for this process, an important consideration given the role of this receptor subtype in cognitive function.



## 1. Introduction

$GABA_A$ receptors are distributed throughout the CNS and contribute to a spectrum of disease pathologies. These ligand-gated chloride channels typically consist of two α, two β and one γ subunit, and have specific binding sites through which a range of compounds interact, including the endogenous neurotransmitter GABA, muscimol, barbiturates, neurosteroids, anaesthetics and benzodiazepines such as diazepam [1]. The benzodiazepines and other related compounds function as positive allosteric modulators (PAMs) to enhance GABA-gated currents at receptors containing an α1, α2, α3, or α5 subunit.

Within the spinal dorsal horn $GABA_A$ receptors are localized both pre- and post-synaptically [2,3], enabling GABA to profoundly influence synaptic transmission. Studies using transgenic mice with selected individual point mutations in the four $GABA_A$ α-subunits have indicated a robust contribution from $GABA_A$ receptors containing α2 and α3 subunits to intrathecal diazepam-mediated

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analgesia in pathological pain [4,5]. This is complimented by pharmacological studies performed in rodent pain models with systemically administered subtype-selective $GABA_A$ receptor PAMs such as L838,417, TPA023, HZ166 and NS11394 [4,6–10]. Whilst both of the above strategies have generally ruled out a contribution of the α1 subunit to PAM-mediated analgesia in these models, the contribution of α5 subunit-containing $GABA_A$ receptors is less clear, a finding which could have repercussions for developing novel $GABA_A$ receptor based analgesics [11], as this subtype has been implicated in mediating the memory impairing effects of benzodiazepines [12].

To obtain a clearer picture of the contribution played by α5 subunit-containing $GABA_A$ receptors to PAM-mediated analgesia, we decided to compare the efficacy of various subtype-selective PAMs on the electrical excitability of spinal cord pain circuits using tissue sourced from rats with carrageenan-induced inflammatory hyperalgesia [13]. When establishing the contribution of individual $GABA_A$ subunits to pain signalling, an *ex vivo* preparation as typified by the hemisected cord preparation used here can have several advantages. Firstly, the uncertainty in ensuring the desired target concentration of a compound is reached in living animals is circumvented. Moreover, the issue of whether a particular compound has active metabolites with different selectivity profiles from the parent is also mitigated. Finally, the associated plasticity changes occurring within spinal pain networks induced in this preparation, termed wind-up, reflect aspects of central sensitization, a process which in itself is intimately linked to a loss of $GABA_A$ receptor-mediated neurotransmission after injury [14,15].

For the current studies, we compared the α2/α3/α5 subtype-selective PAM NS11394 [16], with the α2/α3 subtype-selective PAM TPA023 [17,18], and the α3 subtype-selective PAM TP003 [19] on spinal wind-up. Aware that it can be difficult to assign absolute values to efficacy selectivity data obtained across labs due to the use of *e.g.* different cell lines, receptor combinations and/or reference compounds amongst a number of other variables [20], we first characterized the selectivity profiles of these compounds in house, and in doing so realized that they would be sub-optimal for the task at hand. Accordingly, we identified another $GABA_A$-α2/α3 selective compound (NS16085) with a highly unique selectivity profile exhibiting negligible activity at $GABA_A$-α5. Our data show that $GABA_A$ receptor PAM-mediated reversal of spinal wind up in rats, requires a certain threshold level of receptor modulation at $GABA_A$ α2/α3-containing receptors. Efficacy at $GABA_A$ α5-containing receptors does not appear to be necessary for mediating analgesia after inflammatory injury, a key finding which has important implications for developing $GABA_A$ receptor based analgesics.

## 2. Materials and methods

### 2.1. Synthesis and structure of NS16085

NS16085 (4-chloro-3-{6-[5-(2-hydroxypropan-2-yl)-1H-1,3-benzodiazol-1-yl]pyridin-2-yl}benzonitrile) was developed as part of an ongoing internal drug discovery effort within the $GABA_A$ receptor PAM field. The structure of NS16085 is shown in Fig. 1 together with TPA023 (5-({[7-tert-butyl-3-(2-fluorophenyl)-[1,2,4]triazolo[4,3-b]pyridazin-6-yl]oxy}methyl)-1-ethyl-1H-1,2,4-triazole), TP003 (2-{3-[8-fluoro-7-(2-hydroxypropan-2-yl)imidazo[1,2-a]pyridin-3-yl]phenyl}benzonitrile) and NS11394 (2-{3-[5-(2-hydroxypropan-2-yl)-1H-1,3-benzodiazol-1-yl]phenyl}-benzonitrile) which were also tested in this study. All four compounds were synthesized internally within the Department of Medicinal Chemistry at NeuroSearch A/S.

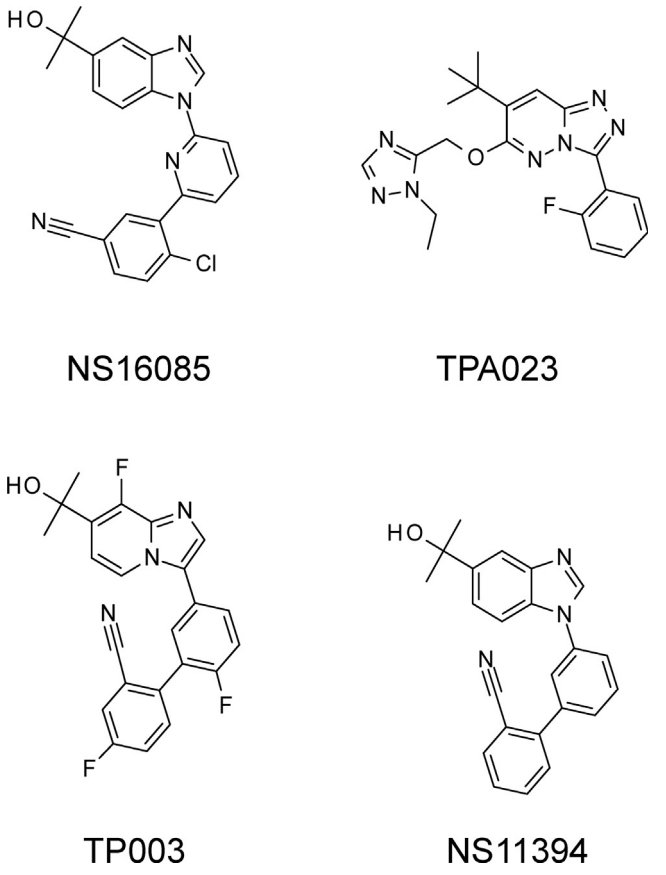


**Fig. 1.** Chemical structure of NS16085. For comparison with NS16085 the structures of TPA023, TP003 and NS11394 are also shown.

### 2.2. Cloning of cDNA and cRNA preparation

cDNA for human $GABA_A$ receptor α1-, α2-, α3-, α5-, β2- and γ2s subunits was cloned and inserted into plasmids as described previously [16]. For cRNA production plasmids were linearized using a unique downstream polylinker enzyme (NotI, XhoI or XbaI). cRNA was prepared using the mMESSAGE mMACHINE T7 Transcription kit (Ambion) according to manufactures instructions. RNA was purified using the Rneasy mini kit (Qiagen), adjusted to a concentration of 0.5 μg/μl and stored at −80 °C until use.

### 2.3. Isolation and injection of Xenopus laevis oocytes

Oocyte preparation was performed as described previously [16]. Briefly, to obtain isolated oocytes lobes from ovaries of female adult *Xenopus laevis* were removed and defolliculated using collagenase. Oocytes were injected with a total of ~25 ng cRNA encoding human $GABA_A$ receptor subunits α(x), β2, and γ2s in the ratio of 1:1:2 in a concentration of 0.5 μg/μl and incubated for 2–7 days at 15–18 °C in modified Barth's solution (90 mM NaCl, 1.0 mM KCl, 0.66 mM $NaNO_3$, 2.4 mM $NaHCO_3$, 10 mM HEPES, 2.5 mM sodium pyruvate, 0.74 mM $CaCl_2$, 0.82 mM $MgCl_2$, 100 μg/ml Gentamycin and pH adjusted to 7.5).

### 2.4. Two-electrode voltage clamp experiments from oocytes

Oocytes were subjected to two-electrode voltage-clamp electrophysiological testing using a custom-built system as described previously [16]. All compounds were dissolved in OR2 (90 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl2, 1.0 mM MgCl2, 5.0 mM

HEPES, and pH adjusted to 7.5), and solutions were applied directly to the oocytes *via* a glass capillary tube placed in the vicinity of the cell to ensure rapid solution exchange (few seconds). $GABA_{control}$ concentrations selected to approximately constitute $EC_{10}$ at the $\alpha1$, $\alpha2$, $\alpha3$ and $\alpha5$ containing receptors were 1, 3, 3 and 0.5 μM, respectively, and modulatory actions were assessed by recording $GABA_{control}$-evoked currents in absence and presence of compounds. For each application lasting ~50 s, responses were monitored for a 1.5 min period followed by a 0.5 min washout period. For calculating modulatory actions of compounds (PAMs), baseline subtracted peak current amplitudes for $GABA_{control}$ + PAM was first normalized to the amplitude of $GABA_{control}$ alone and next indexed to the modulatory effect of 0.5 μM diazepam in the same oocyte. NS16085 actions at $\alpha1$ receptors were an exception to this as the negative modulation was only normalized to the $GABA_{control}$ current.

### 2.5. Additional biochemical profiling of NS16085

The putative activity of NS16085 against a selected set of G protein coupled receptors, ion channels and transporters was performed by MDS Pharma Services (now incorporated within Eurofins; www.eurofins.com). NS16085 was tested in 10 μM concentrations with the standard lead profiling screen performed according to MDS Pharma Specifications.

### 2.6. Animals and behavioural testing

For spinal cord electrophysiology experiments, Wistar rat pups (7–11 day old) were generated in the in house breeding facility at University of Alcala. They were housed together with their respective mothers and maintained under a light dark cycle of 12 h:12 h with 55 ± 15% humidity. Prior to inclusion in electrophysiology experiments, individual pups were tested for mechanical withdrawal thresholds and then received an intraplantar injection of carrageenan (3% in saline; 50 μl). Twenty hours later were tested for the presence of mechanical hypersensitivity. The pups were lightly restrained in one hand, and the mechanical withdrawal threshold was measured using von Frey filaments (4–103 mN). The filaments were applied every 30 s alternately to the dorsum of both hindpaws a maximum of five times per filament. Threshold force for each hindpaw was taken as the minimum force at which withdrawal was evoked in over 50% of the applications. The paw diameter was also measured with a hand held micrometre.

For formalin test experiments adult male Sprague-Dawley rats (Harlan Scandinavia, Alleroed, Denmark) were used. They were housed in Macrolon III cages (20 cm × 14 cm × 18 cm or 20 cm × 40 cm × 18 cm; in groups of 3–5 per cage according to weight) containing wood-chip bedding material (3 mm × 1 mm × 4 mm). The environment was temperature (20 ± 2 °C) and humidity (55 ± 15%) controlled and consisted of 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 rats were allowed to habituate to the housing facilities for at least one week prior to formalin testing. At the end of the experiment rats were euthanized by cervical dislocation. All experiments were performed according to the Ethical Guidelines of the International Association for the Study of Pain [21] and the Danish Committee for Experiments on Animals.

### 2.7. The isolated rat spinal cord preparation

Spinal cord extraction was performed immediately after behavioural testing following previously described procedures [22,23]. Briefly, Wistar rat pups were anaesthetized with urethane (2 g/kg, i.p.) and their spinal cords extracted. With the cord in cold artificial cerebrospinal fluid (ACSF), the outer meninges were removed enabling the cords to be hemisected and pinned to a Sylgard based recording chamber with the medial side facing down. The preparation was maintained with oxygenated (95% $O_2$, 5% $CO_2$) ACSF at room temperature (23 ± 2 °C). Flow rate was 6 ± 2 ml/min. The composition of the ACSF was (in mM) NaCl (127); KCl (1.9); $KH_2PO_4$ (1.5); $MgSO_4$ (1.3); $CaCl_2$ (2); $NaHCO_3$ (22); glucose (10); (pH 7.4). The hemi-cord used was ipsilateral to the site of carrageenan injection.

### 2.8. Ventral root recordings from hemisected spinal cord

A lumbar dorsal root (L4 or L5) and the corresponding ventral root were placed in tight-fitting glass suction electrodes. Electrical stimulation was then applied to the dorsal root and responses were recorded from the corresponding ventral root. The electrical stimulation test consisted of one C-fibre intensity stimulus (300 μA, 200 μs) followed by a train of 20 consecutive C-fibre intensity stimuli applied at 1 Hz (300 μA, 200 μs) to produce wind-up responses. Stimuli were applied at 45 s intervals and the electrical stimulation tests were done at 30 min intervals.

The signal coming from the ventral suction electrode was amplified using a Dugan EX4-400 AC/DC amplifier to record fast compound spikes produced by the action potential firing of motor neurones. Signals were digitized at 5 kHz using a CED 1401 interface and stored for offline computer-aided analysis using Spike 2 software (Cambridge Electronic Design Ltd, Cambridge, UK). AC recordings were analysed based on threshold criteria to count spikes to each stimulus when a train of stimuli was applied. Spike counting was performed for each stimulus of the train in a window between 20 and 950 ms from the corresponding stimulus artefact. The amplitude of the monosynaptic reflex in DC recordings was assessed in responses to single stimuli. This protocol allowed us to evaluate drug effects on both non-nociceptive motor circuits (monosynaptic reflex) and nociceptive circuits (spike wind-up to trains of stimuli). After two to four repetitions of the stimulation test to obtain stable baseline responses, compounds were superfused. Timing was arranged so as to perform the electrical stimulation test at the end of the superfusion of the compound.

### 2.9. Formalin test

Assessment of formalin-induced flinching behaviour in normal, uninjured rats (body weight 180–300 g) was made with the use of an Automated Nociception Analyser (University of California, San Diego, CA), [24]. 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 and had been randomly assigned into treatment groups) was administered test compound or vehicle, and then placed in a cylindrical acrylic observation chamber (diameter 15 cm and height 30.5 cm). 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. They were then returned to their separate observation chambers, each of which were in turn 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 digitized and a software algorithm applied to enable discrimination of flinching behaviour from other paw movements prior to binning into 1 min sampling intervals. On the basis of the resulting response patterns three phases of pain-like behaviour were identified; first phase (0–5 min), interphase (6–15 min) and second phase (16–40 min) [8]. Raw data from the 1 min sampling intervals was summed for each phase to obtain the total number of flinches occurring during that period. For purposes of statistical analysis this value was then expressed as a % of the

vehicle response according to the equation, % Vehicle = (Post-treatment value)/(Vehicle value) × 100.

### 2.10. Compound preparation

NS11394, TPA023, TP003 and NS16085 were diluted in DMSO as a concentrated stock solution and stored at −20 °C. For hemisected spinal cord experiments compounds were diluted down to the final concentration in ACSF (all components of which were obtained from Sigma-Aldrich, Madrid, Spain) just prior to use and superfused to the entire preparation for 15 min periods to ensure a complete tissue equilibration. Compounds were applied following a cumulative protocol (NS 11934, TP003 and NS16085: 0.01, 0.1 and 1 μM; TPA023: 0.001, 0.01 and 1 μM). Each preparation received applications of only one of the compounds at all three concentrations. For *in vivo* experiments NS16085 was dissolved in 5% Tween 80 in milliQ water and administered p.o. in a dosing volume of 10 ml/kg 60 minutes prior to evaluation of efficacy in the formalin test. All doses are expressed as mg weight free base per kg body weight.

### 2.11. Statistical analysis

All data are presented as mean ± standard error of mean (S.E.M.) unless otherwise stated. For carrageenan inflammation experiments statistical analysis was performed with GraphPad Prism software. For spinal electrophysiology data comparisons were made on raw data by one- or two-way ANOVA followed by Bonferonni's test. Data on paw diameter and mechanical thresholds were compared with the Wilcoxon matched pairs test. For formalin test data statistical analysis was performed with SigmaPlot 11.2.0.5 (Systat Software, Inc., Chicago IL, USA) software and analysed by one way ANOVA followed by Bonferonni's test. $P < 0.05$ was considered significant and used where appropriate to indicate the minimum effective dose (MED) of compounds.

## 3. Results

### 3.1. In vitro *efficacy of $GABA_A$ PAMs in oocytes selectively expressing human $GABA_A$ receptors*

NS16085 was identified as a positive modulator of $GABA_A$ receptors as part of an ongoing internal drug discovery effort within this field (Fig. 1). The modulation of GABA-evoked currents at human α1-, α2-, α3- or α5-containing receptors expressed in combination with β2 and γ2s subunits in *X. laevis* oocytes by all four PAMs shown in Fig. 1 can be seen in Fig. 2, with modulatory effects of diazepam shown within the inset of each panel. For these experiments, a $GABA_{control}$ concentration giving rise to approximately 10% of the maximal GABA-evoked current ($EC_{10}$) was chosen for each receptor combination and full concentration response relationships of the four compounds were obtained by co-applications with $GABA_{control}$. The depicted percent modulations are indexed relative to the potentiating effects of 0.5 μM diazepam on the same oocytes except for that of NS16085 at the α1-containing receptor.

Fig. 2a shows that TPA023 modulated GABA responses at all four receptor combinations. The greatest efficacy was observed at α3- and α5-containing receptors where it produced a maximum 19% and 17% potentiation relative to diazepam. However, efficacy at α2- and α1-containing receptors was only marginally lower where it maximally potentiated by 14 and 8% respectively, compared to diazepam. Thus, under the conditions tested herein TPA023 is not particularly selective in contrast to previous reports [17,18]. This efficacy profile is clearly different to that previously reported for NS11394 [16] and shown in Fig. 2b for comparison. Although NS11394 produces a maximal potentiation of just 8% relative to diazepam at α1-containing receptors, the maximal potentiation compared to TPA023 is 2–4 fold higher at the remaining receptor combinations (α2β2γ2 = 26%, α3β2γ2 = 52%, and α5β2γ2 = 78%). Clearly, the greatest magnitude difference between TPA023 and NS11394 relates to the efficacy obtained at α5-containing receptors. To gain possible insight into the contributory role of this subtype to putative effects mediated by NS11394 and TPA023 on sensitized pain circuits [25,26] we proceeded to test the compound TP003 which has been reported to be highly selective for α3-containing receptors [19]. As can be seen in Fig. 2c, under the current testing conditions, TP003 somewhat unexpectedly potentiated GABA responses at all four receptor combinations with greatest efficacy at α3- and α5-containing receptors (43% and 41% relative to diazepam respectively). However potentiation at α2- and α1-containing receptors was still considerable (19% and 21% respectively). To give some context to the latter statement, the non-selective partial agonist/low efficacy PAM bretazenil under our test conditions potentiates α1-containing receptors by 20% relative to diazepam, and is highly sedative in man [27]. Therefore, we identified and tested the novel molecule NS16085, the efficacy profile of which is shown in Fig. 2d. Whereas NS16085 produced a maximal potentiation of 27% and 47% relative to diazepam at α2- and α3-containing receptors, by comparison the potentiation at α5 containing receptors was essentially negligible. Further, NS16085 exhibited weak negative modulation at α1-containing receptors, thereby separating this compound completely from the other three with respect to this subtype. Thus, NS16085 was used in subsequent spinal cord electrophysiology experiments as a pharmacological tool to dissect out a putative role for α5-containing receptors to $GABA_A$ PAM-mediated inhibition of spinal sensitization associated with inflammatory injury.

### 3.2. Additional biochemical profiling of NS16085

To further confirm the selectivity of NS16085 for $GABA_A$ receptors it was also tested at a concentration of 10 μM in a standard lead profiling screen provided by MDS Pharma Services. In binding assays against a panel of 68 G protein coupled receptors, ion channels and transporters screened, NS16085 bound weakly to a range of targets. Using an arbitrary cut off threshold, greater than 50% displacement was only seen for the rat $GABA_A$ benzodiazepine site (100%) as expected, and for the human adenosine A3 receptor (56%). Notably, these binding assays do not inform as to whether the effect at a given target is that of an agonist or antagonist.

### 3.3. Efficacy of $GABA_A$ PAMs on spinal nociceptive reflexes and wind-up

Electrophysiology experiments designed to assess the efficacy of $GABA_A$ PAMs on spinal nociceptive transmission were performed in a total of 23 rat pups that had been administered an intraplantar injection of carrageenan 20 h earlier. Just before spinal cord extraction, it was clear that carrageenan treated animals had a significant increase in the diameter of the injected paw (Fig. 3a, paired *t*-test $P < 0.01$). At this time point hypersensitivity to punctate mechanical stimuli applied using von Frey filaments was detected in both the injected and non-injected hindpaws of treated animals (Fig. 3b; paired *t*-test $P < 0.01$). These changes in paw diameter and behavioural withdrawal reflexes in rat pups after carrageenan treatment are indicative of inflammatory-mediated sensitization and are consistent with previously reported data [13].

In accordance with these observations, spinal cords from all carrageenan-injected animals showed large numbers of spikes

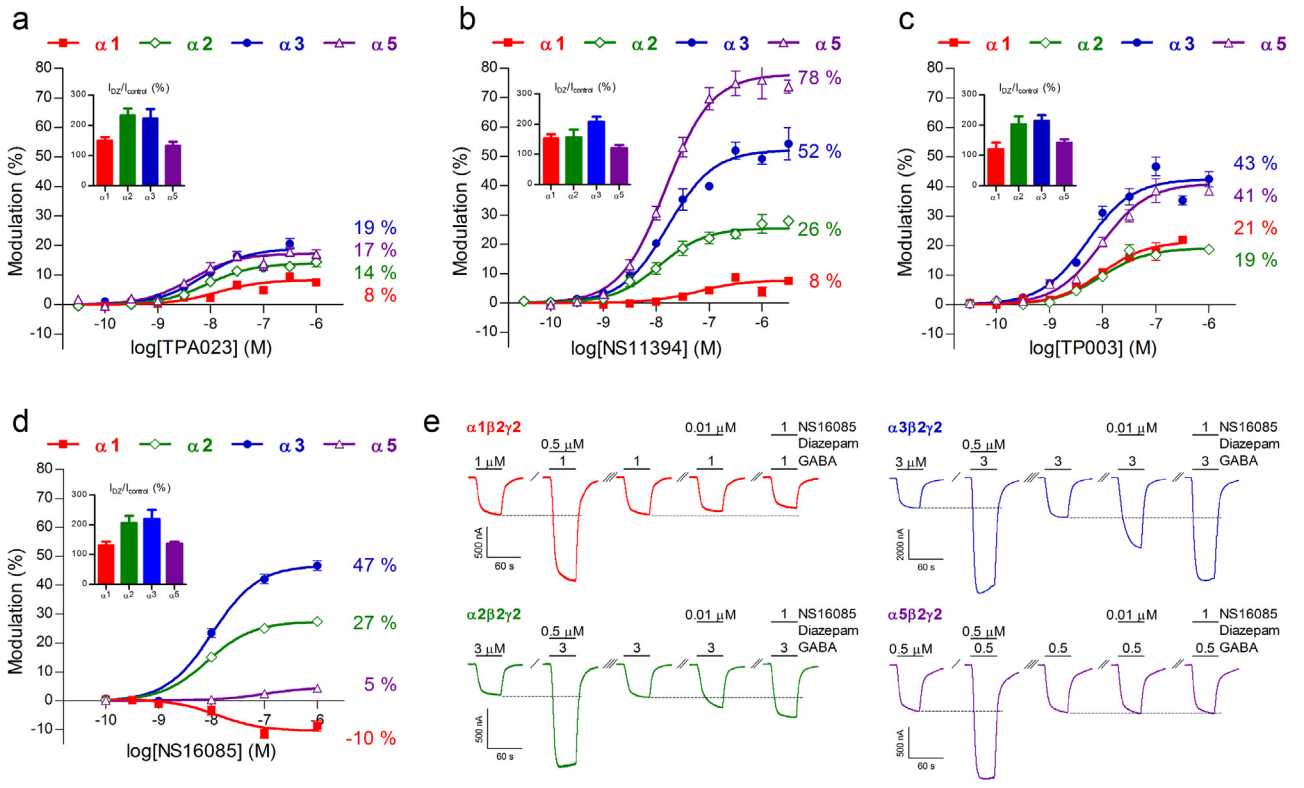


**Fig. 2.** *In vitro* efficacy profiles of $GABA_A$ receptor PAMs in two-electrode voltage-clamp experiments at human recombinant $GABA_A$ receptors expressed in *Xenopus laevis* oocytes. Oocytes were injected with cRNA mixtures for α1, α2, α3 or α5 in combination with β2 and γ2 in 1:1:2 ratios. $GABA_{control}$ concentrations (~$EC_{10}$) were 1, 3, 3 and 0.5 μM at α1, α2, α3 and α5 receptors, respectively. Baseline subtracted peak current amplitudes for $GABA_{control}$ + PAM was normalized to the amplitude of $GABA_{control}$ alone and (except for negative NS16085 actions at α1 receptors) indexed to the modulatory effect of 0.5 μM diazepam in the same oocyte. Percent modulation were plotted as a function of the PAM concentration and data points were fitted by non-linear regression to the Hill equation using GraphPad Prism 5 with fixed Hill slope of 1. (a) TPA023 (α1, α2, α3, α5 $E_{max}$ ± S.E. in % ($pEC_{50}$ ± S.E.) = 8.3 ± 0.5 (7.9 ± 0.1), 14 ± 1 (8.1 ± 0.1), 19 ± 1 (8.1 ± 0.1), 17 ± 1 (8.4 ± 0.1) respectively, $n$ = 6–16 oocytes), (b) NS11394 (α1, α2, α3, α5 $E_{max}$ ± S.E. in % ($pEC_{50}$ ± S.E.) = 7.8 ± 0.7 (7.2 ± 0.2), 26 ± 1 (7.9 ± 0.1), 52 ± 1 (7.8 ± 0.1), 78 ± 2 (7.8 ± 0.1) respectively, $n$ = 10–24 oocytes), (c) TP003 (α1, α2, α3, α5 $E_{max}$ ± S.E. in % ($pEC_{50}$ ± S.E.) = 21 ± 1 (8.0 ± 0.1), 19 ± 1 (8.0 ± 0.1), 43 ± 1 (8.3 ± 0.1), 41 ± 2 (8.0 ± 0.1) respectively, $n$ = 6–17 oocytes) and (d) NS16085 (α1, α2, α3, α5 $E_{max}$ ± S.E. in % ($pEC_{50}$ ± S.E.) = −10 ± 1 (7.9 ± 0.3), 27 ± 1 (8.1 ± 0.1), 47 ± 1 (8.0 ± 0.1), 4.7 ± 1.0 (7.0 ± 0.4) respectively, $n$ = 9–18 oocytes). Data in (b) are modified from Mirza et al., 2008 and were obtained under identical testing conditions as those used in (a), (c), (d). The numbers in each panel beside each profile quantifies the fitted maximum PAM efficacy. Modulatory effects of diazepam at each receptor subtype are shown as insets in the corresponding panels. (e) Representative $GABA_{control}$-evoked traces showing diazepam and NS16085 modulation from oocytes expressing the indicated receptors. Bars above each trace designate application periods as well as GABA and PAM concentrations. "/" between traces specifies a short wash period of 0.5 min, "//" 1–2 omitted intermittent traces whereas "///" specifies a long wash period of 20 min. Dotted lines indicates the prior $GABA_{control}$ traces used for calculation of diazepam and NS16085 modulations.

(mean value 1180 ± 185) to repetitive C-fibre stimulation of the lumbar dorsal root, in contrast to the very low values reported for untreated animals [23,28], and even some degree of spontaneous activity. Examples of original AC recordings of ventral root responses to repetitive stimulation of the dorsal root are shown in Fig. 4, where control responses clearly show a thickening of the baseline reflecting the spike wind up effect elicited by this type of stimulus. Under the present conditions, all four compounds produced concentration-dependent depressant effects on responses to repetitive stimulation of the dorsal roots at C-fibre intensity, as reflected in the progressive reduction of spikes recorded to the consecutive stimuli of the train (Figs. 4 and 5). Notably at a 1 μM concentration, which from *in vitro* oocyte electrophysiology studies leads to maximal effects at given receptor subtypes for each compound (Fig. 2), NS11394, TP003 and NS16085 clearly depressed the total spike counts to repetitive stimuli to a greater degree than TPA023 (Fig. 5 and Table 1). This aligns well with the greater efficacy of the three former compounds at α2 and α3 subunit-containing $GABA_A$ receptors compared with TPA023. Notably, the rank order of efficacy for diminishing wind-up (NS11394 > TP003 = NS16085 > TPA023) is extremely difficult to reconcile with an effect mediated *via* α5 subunit-containing $GABA_A$ receptors, given that potentiation at this subtype is far weaker for NS16085 than for NS11394 and even TP003.

Subsequently, the specificity for these compounds to interfere with mechanisms appropriate to strong repetitive stimulation were checked by examining effects on spikes evoked by single C-fibre intensity (300 μA, 200 μs) stimulation and on the monosynaptic reflex. The mean number of spike counts to the former stimulus paradigm was 45.7 ± 8.7, and all four PAMs had minor, non-significant effects on this parameter (Table 1) indicating that drugs affected the summation of stimuli underlying wind-up rather than to single responses. The monosynaptic reflex on the other hand depends on the activation of thick myelinated fibres innervating muscle spindles. The mean amplitude of this reflex to a saturating stimulus (300 μA, 200 μs) was 2.8 ± 0.48 mV ($n$ = 23). Accordingly, none of the compounds used had any effect on this variable at any of the concentrations used (Table 1) which indicates a lack of effect on non-nociceptive reflexes.

### 3.4. Efficacy of NS16085 in the rat formalin test

Next, we set about to confirm if the *ex vivo* effects observed for NS16085 on experimental wind-up, would translate into analgesic efficacy in a rat model of persistent inflammatory pain. For this purpose we injected a small volume of formalin into the rat hindpaw. This initiates spontaneous pain-like (nocifensive)

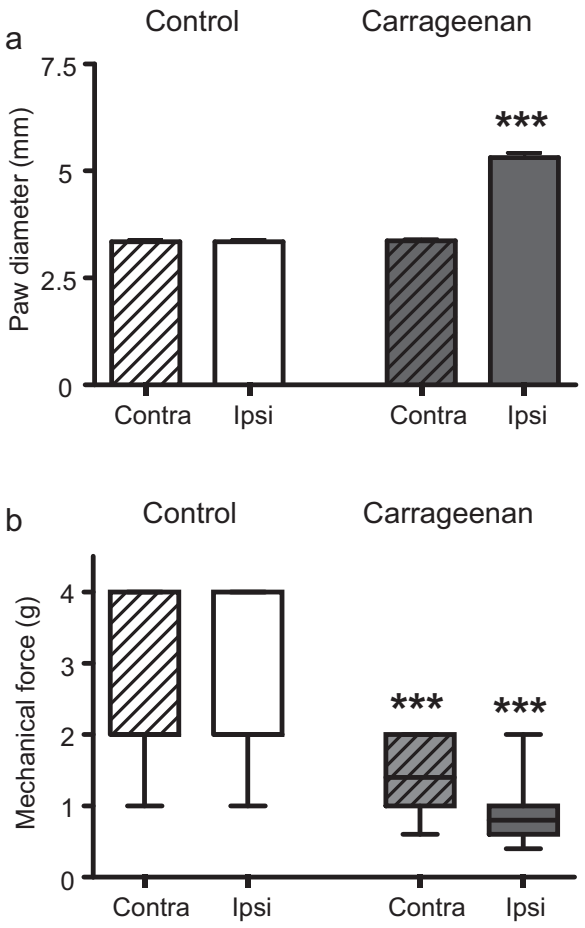


**Fig. 3.** Development of mechanical hypersensitivity and paw swelling in rats with carrageenan-induced hyperalgesia. Rat pups were given an intraplantar injection of carrageenan (3% in 50 μl saline) into the ipsilateral hindpaw. (a) Twenty hours later a significant increase in paw diameter (mm) occurred indicative of the presence of peripherally mediated neurogenic inflammatory processes. (b) This was accompanied by a decrease in the mechanical force (g) applied to the ipsilateral hindpaw by von Frey filament stimulation reflecting the presence of mechanical hyperalgesia. The reduction in mechanical force required to initiate a reflex nociceptive response on the contralateral side indicates that the hyperalgesia was in part mediated by central mechanisms. All groups $n = 24$ rats. Data are presented as mean ± S.E.M. $^{***}P < 0.001$ (Wilcoxon matched pairs test).

behaviours consisting of flinching, licking and/or biting of the injected paw and can engage mechanisms within peripheral and central pain circuits similar to a number of those described in humans with chronic pain [29,30]. A first phase can be attributed to direct chemical stimulation of nociceptors, interphase to activation of noxious inhibitory control mechanisms and a final phase to peripheral inflammatory processes that drives sensitization of spinal pain circuits [31–33]. Fig. 6 shows that administration of NS16085 (3–30 mg/kg, p.o.) 60 min prior to formalin injection significantly attenuated flinching behaviour throughout the duration of the test ($F[3,31] = 3.240$, $P = 0.037$, $F[3,33] = 5.646$, $P = 0.004$ and $F[3,30] = 19.749$, $P < 0.001$ for first phase, interphase and second phase respectively). Notably, the most potent effect was obtained during second phase with the minimal effective dose of 3 mg/kg producing a modest 27% reduction in flinching compared with vehicle ($1255 \pm 70$ total flinches, $P < 0.01$). In accordance with the dose-dependency observed, the maximal efficacy attained during second phase was 57% for the highest dose of NS16085 tested. By comparison we have previously reported that the maximal efficacy obtained for attenuation of second phase flinching behaviour was 57% and 40% for NS11394 and TPA023 (both 30 mg/kg), respectively [8,25].

Importantly, no obvious behavioural deficits such as motor impairment were observed in rats, either immediately after injection of NS16085 or 60 min later after hindpaw formalin injection and being placed in the Perspex testing chamber. In a subsequent study, shortly after oral dosing of NS16085 (1–30 mg/kg) in naive rats, we observed a strong relationship between plasma and brain concentrations each of which were in the low micromolar range (data not shown). Both parameters correlated with brain receptor occupancy, which as a pharmacodynamic marker combined with the brain concentrations achieved indicates that the efficacy observed in the formalin test was consistent with a $GABA_A$ receptor PAM mechanism.

## 4. Discussion

In the current series of experiments we set out to examine the relative contribution of α2, α3 and α5 subunit-containing receptors to $GABA_A$ PAM mediated depression of spinal pain circuits sensitized by the inflammatory algogen carrageenan. We had planned to use the α3 subtype-selective compound TP003 to dissect out a contribution of α3-containing $GABA_A$ receptors to mechanisms contributing to this process. However, in our hands TP003, and to a lesser extent TPA023 displayed only marginal selectivity across $GABA_A$ receptor subtypes. With an ongoing drug discovery programme we were able to mitigate this issue by identifying NS16085, a $GABA_A$ receptor PAM which has an entirely unique selectivity profile within this compound class. Accordingly, our hemisected cord electrophysiology data revealed that potentiation of $GABA_A$-α2 and -α3 receptor function is clearly sufficient to depress activity-dependent spinal sensitization after inflammatory injury.

Following trauma to cutaneous tissues, tissue acidification combined with the local release of peptides, cytokines and prostanoids initiates and contributes to neurogenic inflammatory processes, which acts to lower the threshold of activation of peripheral nociceptors [34]. For our experiments we simulated this process by injecting carrageenan into the rat hindpaw. The marked increase in paw diameter accompanied by the rapid development of mechanical hyperalgesia confirmed the presence of neurogenic inflammation and engagement of peripheral proinflammatory sensitizing mechanisms [13,35]. Subsequently, recording of the dorsal root ventral root reflex from carrageenan-sensitized cords revealed some degree of spontaneous activity and large numbers of spikes not normally observed in tissue obtained from naïve rats [23,28]. Together these observations indicate that peripheral sensitizing events induced activity-dependent and possibly longer term plasticity changes indicative of central sensitization within the isolated spinal cord preparation [36]. Notably, a loss of inhibitory neurotransmission mediated by $GABA_A$-receptors within the spinal cord plays a vital role in this process [15,20,37]. Clearly, the wind-up produced by repetitive C-fibre stimulation in this preparation occurs over a more compressed timescale than processes such as central sensitization or even spinal LTP. Nevertheless, in addition to recruiting NMDA receptor sensitive plasticity events [36,38], the induction and maintenance of spinal LTP has been shown to be diminished by local superfusion of diazepam [39] further validating the *ex vivo* electrophysiological methodology we implemented here.

Using this approach we observed that NS11394 and TPA023 were both capable of inhibiting wind-up of the dorsal root evoked ventral root reflex (albeit NS11394 was considerably more efficacious), complimenting behavioural studies showing that NS11394 reverses injury-induced hyperalgesia to a greater extent than TPA023 [25,26], a finding we attributed to its greater efficacy at $GABA_A$ α2/α3 receptors (Fig. 1), [16,17]. Similarly, the high *in vitro* efficacy of L838,417 at $GABA_A$ α2/α3 receptors [40] compared

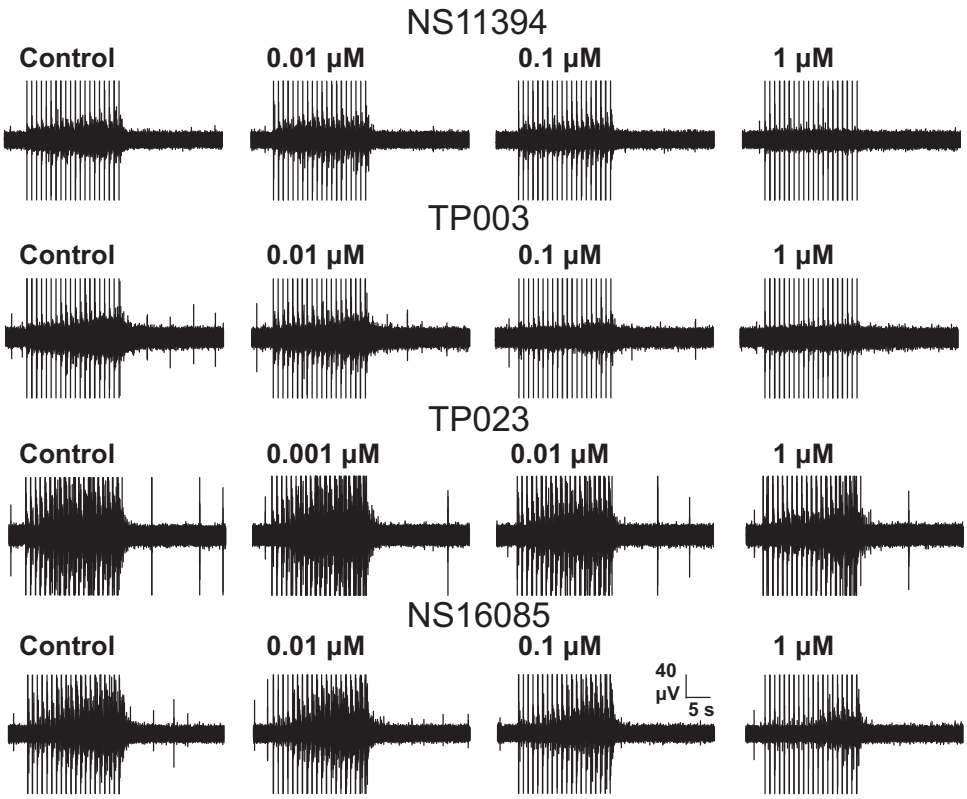


**Fig. 4.** $GABA_A$ receptor PAMs attenuate carrageenan-induced spinal sensitization. Original ventral root recordings obtained from 4 different experiments showing cumulative depressant effects of increasing concentrations of $GABA_A$ receptor PAMs on total spike counts induced by repetitive dorsal root stimulation (20 stimuli; 300 μA and 200 μs; 1 Hz). The gradual thickening of the baseline in the four individual control responses represents the accumulation of events or spikes occurring within motor neurones and reflects the presence of spinal sensitization. Long vertical lines at 1 s intervals correspond to stimulation artefacts.

to TPA023 [17] has been reported to be the important determinant explaining its superior efficacy in pathological pain models up to doses of both compounds which led to full CNS receptor occupancy [6]. Keeping in mind that other factors such as receptor affinity, pharmacokinetics and tissue free fraction concentration might also contribute, we were still left to contemplate exactly which $GABA_A$ receptors were relevant to the superior efficacy on spinal excitability obtained with NS11394.

To facilitate this process we initially tested TP003, a reportedly pure $GABA_A$ α3 selective molecule [19]. However, our oocyte electrophysiology data showed that TP003 potentiates at all receptor subtypes with efficacy at $GABA_A$-α5 receptors equivalent to that at $GABA_A$-α3 receptors. Moreover, efficacy at the latter subtype (and α2 for that matter) was essentially similar to that obtained with NS11394 [16], thereby diverging considerably from the published literature [17,19]. Clearly, differences in electrophysiology protocols between laboratories can impact efficacy and potency estimates (*e.g.* $Ltk^-$ cells *vs.* oocytes, stable cell lines *vs.* transient transfection and choice of reference compound, chlordiazepoxide *vs.* diazepam). Accordingly, caution should be applied when assigning absolute selectivity profiles to compounds unconditionally. In light of this latter consideration, and as part of the aforementioned drug discovery effort within the $GABA_A$ receptor PAM field (enabling us to utilize the exact same testing conditions as used for the other PAMs here) we identified the novel compound NS16085. The unique selectivity and efficacy profile of NS16085, exemplified by the low level of negative modulation at $GABA_A$-α1 receptors finally confirms that potentiation of $GABA_A$-α1 receptors by PAMs such as NS11394, does not contribute to the attenuation of spinal wind-up or the reversal of injury induced hyperalgesia in animal pain models, such as the formalin test used here [8,26]. This is not so surprising given that spinal diazepam can provide maximal analgesia in α1 point mutated knock-in mice [4]. More elusive though, has been the relative contribution played by α5 subunit-containing $GABA_A$ receptors to the analgesic efficacy of PAMs such as NS11394 and L838,417.

We believe that the current data now go a long way to resolving this issue based on the following comparisons. Firstly, although NS11394 and TP003 essentially potentiate α2 and α3 receptors to a similar degree *in vitro*, NS11394 has almost two fold greater efficacy at $GABA_A$-α5 receptors, and yet did not produce a greater attenuation of spinal wind-up [16]. This distinction in α5 efficacy is further amplified when comparing NS11934 and NS16085, yet with a similar outcome on the wind-up response. These findings are consistent both with the low expression of the α5 subunit in the spinal cord [15], and data from α5 point mutated knock-in mice which indicate that this subtype has a minor contribution to the hypersensitivity associated with inflammatory injury [4]. Of course this does not rule out a more significant contribution of $GABA_A$-α5 receptors to pain mediated by other types of injury [15], but the overall weight of evidence suggests a minor role for this subtype in general. From a broader drug development perspective, the lower *in vitro* efficacy of NS16085 and TP003 at $GABA_A$-α5 receptors suggests they are less likely to mediate cognitive impairment as has been reported for NS11394 [16], a safety issue which could negatively impact upon their use in chronic pain and competitiveness in the market place [41]. Furthermore, studies with $GABA_A$ receptor mutant mice have shown that α1 subunit-containing $GABA_A$ receptors are key mediators of the sedative and motor-impairing effects of benzodiazepines [40,42]. However, none of the compounds tested here affected the monosynaptic reflex to a saturating stimulus or the reflex response to single

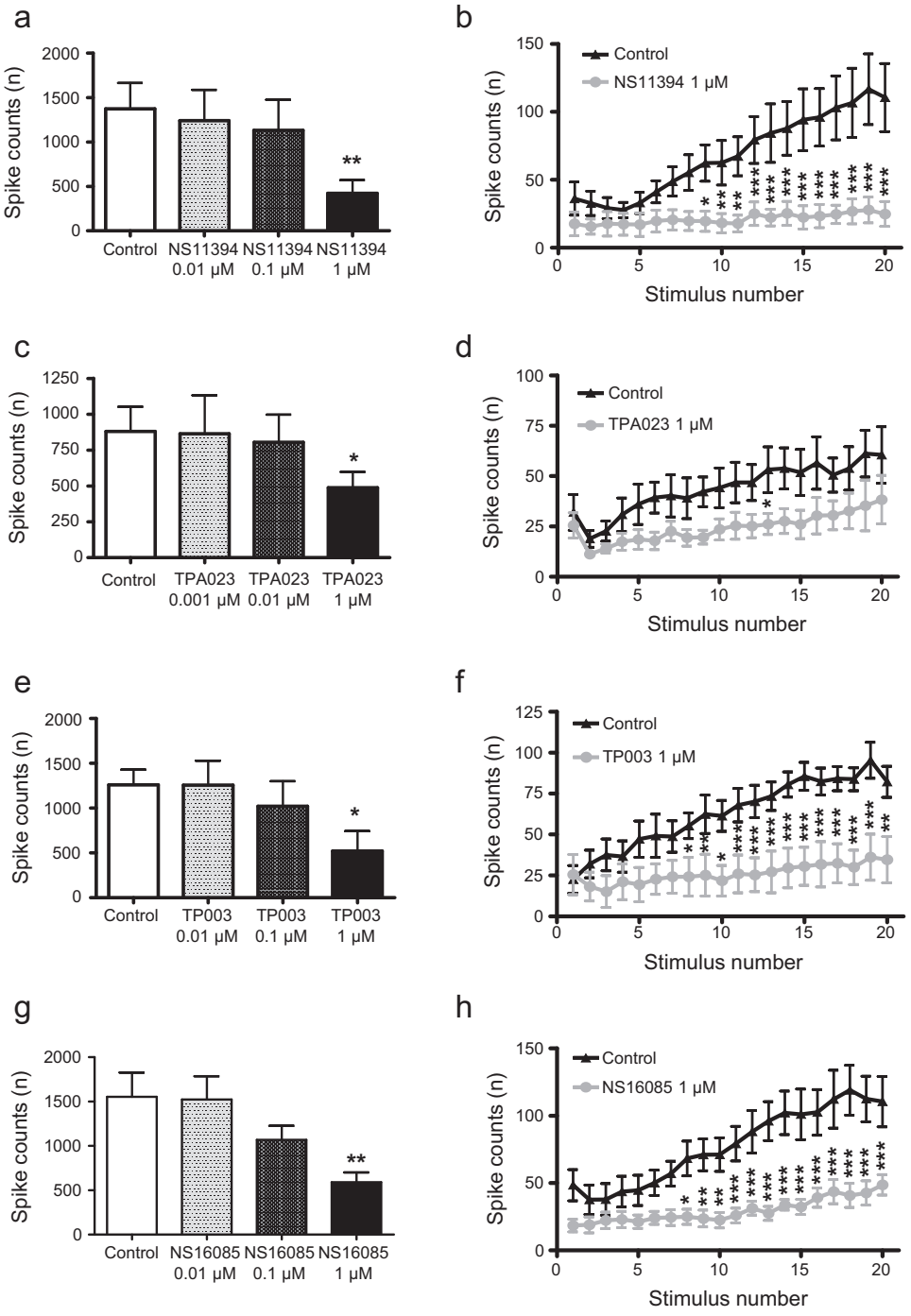


**Fig. 5.** $GABA_A$ receptor PAM mediated attenuation of carrageenan-induced spinal sensitization does not require efficacy at α5 subunit-containing receptors. Pooled data obtained from ventral root recordings showing effects of NS11394, TPA023, TP003 and NS16085 on (a, c, e, g) total spike counts induced by repetitive dorsal root stimulation (20 stimuli; 300 μA and 200 μs; 1 Hz) and (b, d, f, h) spike counts to each stimulus of the train. All compounds had a depressant effect on wind-up when applied at 1 μM albeit TPA023 did not suppress summation (d). Note the similar reduction in spike counts mediated by NS11394, TP003 and NS16085 despite their markedly different efficacy profiles at $GABA_A$ α5 subunit-containing receptors. Data are presented as mean ± S.E.M. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (ANOVA followed by Bonferonni's test).

**Table 1**
Effects of $GABA_A$ receptor PAMs on spinal reflexes recorded from carrageenan-inflamed rats. Data is shown for all compounds when superfused over the spinal cord at 1 μM. Stimulus intensity was in all cases 300 μA and 200 μs. Data are presented as mean ± S.E.M.

| | NS11394 | TPA023 | TP003 | NS16085 |
|---|---|---|---|---|
| Total spike counts to trains of stimuli (% of control) | 29.1 ± 8.0* | 58.1 ± 8.4* | 38.0 ± 11.7** | 40.7 ± 6.2* |
| Spike counts in response to C-fibre intensity single stimulus (% of control) | 84.1 ± 17.9 | 128.8 ± 37.9 | 96.9 ± 30.3 | 74.8 ± 12.0 |
| Monosynaptic reflex (% of control) | 118.7 ± 19.5 | 118.1 ± 9.4 | 104.3 ± 5.3 | 94.8 ± 5.9 |

* $P < 0.05$, Student's *t*-test.
** $P < 0.01$, Student's *t*-test.

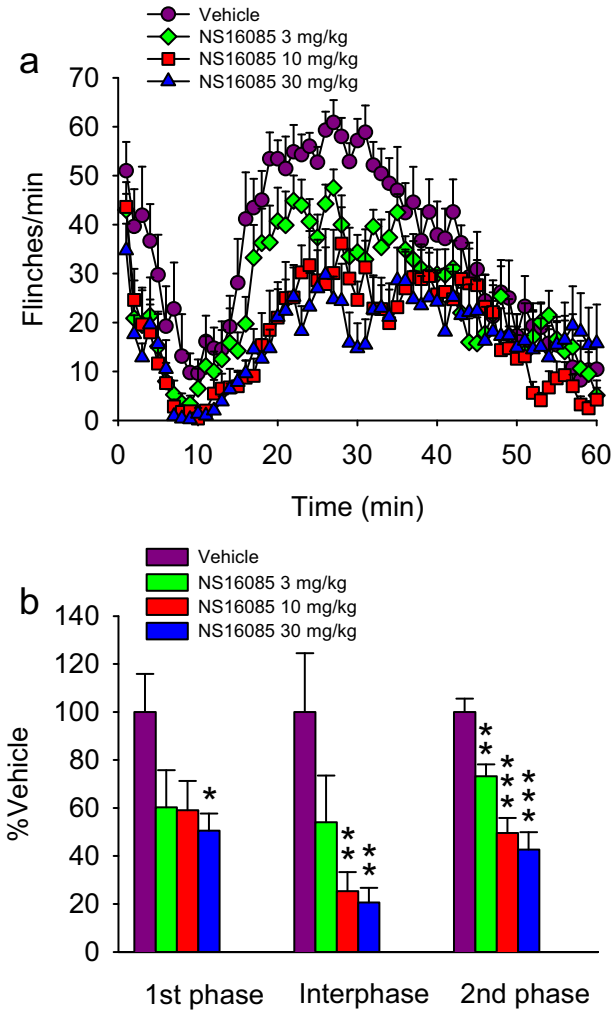


**Fig. 6.** NS16085 attenuates nocifensive behaviours in the rat formalin test of persistent pain. Rats were administered either NS16085 (3–30 mg/kg, p.o.) or vehicle 60 min prior to hindpaw formalin (5% in saline, 50 μl, s.c.) injection. Subsequently, (a) the time course of nocifensive flinching behaviours and (b) % Vehicle values for the 3 phases of the test were calculated. All groups $n = 8$ 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 Bonferonni's test).

stimuli at C-fibre intensity, consistent with behavioural observations that compounds such as NS11394, and TPA023 possessing low intrinsic efficacy at α1 subunit-containing $GABA_A$ receptors do not alter the normal response to sensory and nociceptive inputs in non-sensitizing conditions [8,16,25,26]. More importantly, as an inverse agonist at α1-containing $GABA_A$ receptors, motor dysfunction would not be expected to confound the interpretation of NS16085 analgesia obtained here in the rat formalin test.

Assuming a translational relationship between experimental wind-up in animals and temporal summation in humans, our data indicate that the former approach might be used as a pharmacodynamic biomarker to identify suitable α2/α3 subtype selective PAMs and help define dose selection for proof of concept studies in human volunteers and eventually pain patients. One word of caution regarding the latter approach is that to date all α2/α3 subtype selective PAMs synthesized are *functionally* or *efficacy* selective for these two subtypes over the α1 subtype. However, emerging phase I data from human volunteers using endpoints to assess sedative and ataxic proclivity of subtype-selective compounds (*e.g.* TPA023, TPA023B, MK-0343 and SL651498) indicates that no activity at α1 receptors is permissive if these CNS side-effects are to be obviated [18,43–45]. Indeed, we would argue that a nominal level of negative modulation at α1 receptors might be advisable, as long as it does not increase liability to seizures in longer-term toxicology and safety studies. NS16085, and like compounds, were specifically 'engineered' to incorporate nominal levels of negative modulation at α1 with the risk of sedation and ataxia in man in mind. By contrast, as far as we are aware no affinity/potency selective α2/α3 selective PAMs have been identified to date, and this is clearly an approach that should be considered in future drug discovery programmes.

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