THE ANTINOCICEPTIVE EFFECTS OF NICOTINIC RECEPTORS
ALPHA 7 POSITIVE ALLOSTERIC MODULATORS IN MURINE ACUTE
AND TONIC PAIN MODELS

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ABBREVIATIONS: nAChR(s), nicotinic acetylcholine receptor(s); s.c., subcutaneous injection; i.p., intraperitoneal; i.t., intrathecal; PAM, positive allosteric modulator; MLA, Methyllycaconitine citrate; DHβE, dihydro-β-erythroidine; ERK, extracellular signal-regulated kinase.
Abstract

The α7 nicotinic acetylcholine receptor (nAChR) subtype is abundantly expressed in the central nervous system (CNS) and in the periphery. Recent evidence suggests that α7 nAChR subtypes, which can be activated by an endogenous cholinergic tone, comprising acetylcholine (ACh) and the α7 nAChR agonist choline, play an important role in sub-chronic pain and inflammation. This study’s objective was to test whether α7 nAChR positive allosteric modulators (PAM) produce antinociception in mouse acute and persistent in vivo pain models. Testing type I (NS1738) and II (PNU-120596) α7 nAChR PAMs in acute and persistent pain, we found while neither reduced acute thermal pain, only PNU-120596 dose-dependently attenuated paw-licking behavior in the formalin test. The long-acting effect of PNU-120596 in this test was in discordance with its pharmacokinetic profile in mice, which suggest the involvement of post-receptors signaling mechanisms. Our results with selective (MEK) inhibitor U0126 argues for an important role of extracellular signal-regulated kinase (ERK1/2) pathways activation in PNU-120596’s antinociceptive effects. The α7 antagonist MLA, via intrathecal administration, reversed PNU-120596’s effects, confirming PNU-120596’s action in part through central α7 nAChRs. Importantly, tolerance to PNU-120596 was not developed after sub-chronic treatment of the drug. Surprisingly, PNU-120596’s antinociceptive effects were blocked by NS-1738. Our results indicate that type II α7 nAChR PAM PNU-120596, but not type I α7 nAChR PAM NS1738, shows significant antinociception effects in persistent pain models in mice.
Introduction

Nicotinic acetylcholine receptors (nAChRs) are pentameric structures composed of five different subunits that form a central ion-conducting pore, allowing permeability of cations, such as sodium, potassium, and calcium (Millar and Gotti, 2009). These receptors are composed of either homomeric α or heteromeric α/β subunit combinations. Currently, 12 neuronal nicotinic subunits have been identified (α2–α10; β2–β4) (Paterson and Nordberg, 2000; Gotti et al., 2006). One subtype abundantly expressed in the central nervous system and in the periphery, is the homomeric α7 subunit (Girod et al., 1999). nAChR α7 subtypes are characterized by their high calcium permeability and their rapid desensitization during agonist stimulation (Feuerbach et al., 2009) compared to other nAChR subtypes. In recent years, the α7 nAChR agonists were proposed as possible targets for cognitive enhancement, antinociception and anti-inflammatory properties (Damaj et al., 2000; Wang et al., 2005; de Jonge and Ulloa, 2007; Thomsen et al., 2010; Rowley et al., 2010). α7 nAChRs are present in supraspinal and spinal pain transmission pathways (Gillberg and Aquilonius, 1985; Wada et al., 1989; Seguela et al., 1993; Khan et al., 1994; Cordero-Erausquin et al., 2004; Cordero-Erausquin & Changeux, 2001). These receptors are also present on immune and non-immune cytokine-producing cells, including macrophages and keratinocytes (Wang et al., 2003; Pavlov and Tracey, 2004). Furthermore, α7 nAChR agonists such as choline, CDP-choline, compound B, JN403 and AR-R17779 were found to exhibit anti-inflammatory effects in various inflammation and pain models in rodents (Damaj et al., 2000; Feuerbach et al., 2009; Medhurst et al., 2008; van Maanen et al., 2009; Gurun et al., 2009; Rowley et al., 2010; Marrero et al., 2011; Munro et al., 2012). Although α7 nAChRs agonists have shown beneficial effects in inflammatory animal models in some studies, this effect was not consistently seen in others (Gao et al., 2010). Furthermore, sub-chronic treatment
with such compounds may provide suboptimal therapeutical efficacy because of sustained activation and/or desensitization of the α7 nAChRs.

Activation of α7 nAChR function can occur via direct agonist activation of the orthosteric site. Enhancement of α7 nAChR function can also occurs or via positive allosteric modulation, which can strengthen the endogenous cholinergic neurotransmission without directly stimulating the α7 nAChR. Recently, several structurally diverse and selective positive allosteric modulators (PAMs), including PNU-120596 (Hurst et al., 2005), TQS (Grønlien et al., 2007) and NS1738 (Timmermann et al., 2007) were reported. These PAMs were shown to increase the potency and/or maximal efficacy of endogenous (ACh and choline) or exogenous agonists for the α7 nAChRs. α7 nAChR PAMs have been classified as either type I, such as NS1738 or type II such as PNU-120596, on the basis of a difference in their effect on desensitization (Bertrand and Gopalakrishnan, 2007; Timmermann et al., 2007). The primary difference between these two types is in their ability to evoke a response at the receptor level. The PAMs classified as type I predominantly affects the apparent peak current with little effect on desensitization kinetics whereas Type II increases the apparent peak current and evoke a distinct weakly decaying current (Hurst et al., 2005).

Both I and II type PAMs have been shown to exhibit cognitive enhancement in vivo in rodents. For example, PNU-120596 reversed amphetamine-induced gating deficits in rats and NS1738 improved performance in the rat social recognition (Hurst et al., 2005; Timmermann et al., 2007). While these observations show that α7 nAChR PAMs, belonging to both types, are effective in certain rodent cognitive models, their effects in animal models of pain and inflammation is not well characterized. Recently, Munro et al., (2012) reported that PNU-120596 reversed mechanical hyperalgesia in the carrageenan and the CFA tests in the rat.
Therefore, the present study was designed to investigate the effects of type I and II positive allosteric modulators in acute and tonic pain models in the mouse. PNU-120596 and NS1738 effects were tested after different routes of administration in acute thermal (tail-flick and hot-plate tests) and tonic (formalin test) pain models in mice. Site of actions and receptors mechanisms were also determined. Since α7 nAChR PAMs were reported to enhance extracellular signal-regulated kinase (ERK) signalling in PC12 cells (Hu et al., 2009; El Kouhen et al., 2009), we hypothesized that activation of ERK by these allosteric modulators might play an important role in their antinociceptive effects. We therefore explored this possibility by examining the effects of U0126, a specific MEK inhibitor (Duncia et al., 1998), on the antinociceptive actions of α7 nAChR PAMs.

Here we report that systemic administration of PNU-120596 possesses significant activity in tonic but not acute pain models and thus provides the first demonstration of *in vivo* antinociceptive efficacy for α7 nAChR PAMs in the mouse. Importantly, this work demonstrates also a fundamental *in vivo* difference between type I and II α7 nAChR PAMs in pain models.
Methods

Animals

Male ICR mice obtained from Harlan Laboratories (Indianapolis, IN) and male C57BL/6 mice from Jackson Laboratories (Bar Harbor, ME) were used throughout the study. Mice null for the α7 (Jackson Laboratories) subunits and their wild-type littermates were bred in an animal care facility at Virginia Commonwealth University. For all experiments, mice were backcrossed at least 8 to 10 generations. Mutant and wild types were obtained from crossing heterozygote mice. This breeding scheme controlled for any irregularities that might occur with crossing solely mutant animals. Mice were housed in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-approved animal care facility. They were housed in groups of six and had free access to food and water. The rooms were on a 12-h light/dark cycle (lights on at 7:00 a.m.). Mice were 8–10 weeks of age and weighed approximately 20–25 g at the start of all the experiments. All experiments were performed during the light cycle (between 7:00 a.m. and 7:00 p.m.) and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All studies were carried out in accordance with the National Institute of Health guide for the Care and Use of Laboratory animals.

Drugs

(-)-Nicotine hydrogen tartrate salt [(-)-1-methyl-2-(3-pyridyl) pyrrolidine (-)-bitartrate salt] was purchased from Sigma-Aldrich (St. Louis, MO). Methyllycaconitine citrate (MLA), dihydro-β-erythroidine (DHβE) were purchased from RBI (Natick, MA). Naloxone hydrochloride dehydrate, PNU 120596 [1-(5-Chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)] and PHA-543613 [N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]furo[2,3-c]pyridine-5-
carboxamide] was obtained from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). NS 1738 [N-(5-Chloro-2-hydroxyphenyl)-N'-[2-chloro-5-(trifluoromethyl)phenyl] was purchased from Tocris Biosciences (Minneapolis, MN). U0126 [1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene monoethanolate] was purchased from Cell Signaling Technology (Danvers, MA). All drugs except for PNU 120596, NS 1738 and U0126 were dissolved in physiological saline (0.9% sodium chloride) and injected subcutaneously at a total volume of 1ml/100 g body weight unless noted otherwise. PNU 120596, NS1738 and U0126 were dissolved in a mixture of 1:1:18 [1 volume ethanol/1 volume Emulphor-620 (Rhone-Poulenc, Inc., Princeton, NJ) and 18 volumes distilled water] and administered intraperitoneal (i.p.). All doses are expressed as the free base of the drug.

**Sub-chronic PNU-120596 Administration Protocol**

Mice were administered PNU-120596 (4 mg/kg, i.p.) once a day for six days and then were challenged with PNU-120596 (4mg/kg, i.p.) on day 7 and tested in analgesic assay. Another group was exposed to vehicle for six days and then challenged with PNU-120596 on the seventh day. A vehicle-control group, in which mice were exposed to seven days of vehicle, was also included.

**Intrathecal injections**

Intrathecal (i.t.) injections were performed free-hand between the L5 and L6 lumbar space in unanesthetized male mice according to the method of Hylden and Wilcox (1980). The injection was performed using a 30-gauge needle attached to a glass microsyringe. The injection volume in all cases was 5 μl. The accurate placement of the needle was evidenced by a quick “flick” of the mouse's tail. Thus, the accurate placement of all injections could be assured by watching the tail motion of the mouse.
Antinociceptive tests

Tail-Flick Test.

The antinociceptive effect of drugs was assessed by the tail-flick method of D’Amour and Smith (1941), as modified by Dewey et al. (1970). A control response (2–4 s latency) was determined for each mouse before treatment, and test latency was determined after drug administration. To minimize tissue damage, a maximum latency of 10 s was imposed. Antinociceptive response was calculated as percent maximum possible effect (%MPE), where %MPE = [(test value−control value)/(cut-off (10 s) − control value)] × 100. Groups of 6 to 8 animals were used for each dose and for each treatment. For the tail-flick test, mice were given a 5 min pretreatment with either vehicle or nicotine (2.5mg/kg s.c.) or a 15 min pretreatment of PHA-543613 (8mg/kg s.c.), PNU-120596 (4 and 8 mg/kg i.p.), or NS1738 (10 and 30 mg/kg i.p.).

Hot-Plate Test.

Mice were placed into a 10-cm wide glass cylinder on a hot-plate (Thermojust Apparatus, Columbus, OH) as a measure of supraspinal antinociception. The hot plate is a rectangular heated surface surrounded by plexiglass and maintained at 55°C. The device is connected to a manually operated timer that records the amount of time the mouse spends on the heated surface before showing signs of nociception (e.g. jumping, paw licks). Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 8 to 12 s was assessed with a saline injection. To avoid tissue damage, the hot-plate will automatically disengage after 40 seconds. Antinociceptive response was calculated as percentage of maximum possible effect (% MPE), where %MPE = [(test value − control)/(cut-off time (40 s) − control) × 100]. The reaction time was scored when the animal jumped or licked its paws. Mice were
tested at different times after i.p. injection of NS1738 and PNU-120596. For the hot-plate test, mice were given a 5 min pretreatment with either vehicle or nicotine (2.5 mg/kg s.c.) or a 15 min pretreatment of PHA-543613 (8 mg/kg s.c.), PNU-120596 (4 and 8 mg/kg i.p.), or NS1738 (10 and 30 mg/kg i.p.).

**Mechanical Sensitivity Test.**

Mechanical sensitivity was determined according to the method of Chaplan et al. (1994). Mice were placed in a Plexiglas cage with mesh metal flooring and allowed to acclimate for 30 min before testing. A series of calibrated von Frey hairs (Stoelting, Wood Dale, IL) with logarithmically incremental stiffness ranging from 2.83 to 5.88 expressed dsLog10 of [10 £ force in (mg)] were applied to the paw with a modified up-down method (Dixon, 1965). In the absence of a paw withdrawal response to the initially selected hair, a thicker hair corresponding to a stronger stimulus was presented. In the event of paw withdrawal, the next weaker stimulus was chosen. Each hair was presented perpendicularly against the paw, with sufficient force to cause slight bending, and held 2–3 s. The stimulation of the same intensity was applied 5 times to the hind paw at intervals of a few seconds. The mechanical threshold was expressed as force in (g), indicating the force of the Von Frey hair to which the animal reacted (paw withdrawn, licking or shaking). The mechanical allodynia thresholds were measured before (pre-drug) and at 30 min after i.p treatment of the following drugs: PNU 120596 and NS1738.

**Formalin Test.**

The formalin test was carried out in an open Plexiglas cage, with a mirror placed under the floor to allow an unobstructed view of the paws. Mice were allowed to acclimate for 15 min in the test cage before formalin injection. Each animal was injected with 20 μl of 2.5% formalin in the intraplantar region of the right hindpaw. Mice were then observed (two at a time) 0-5 min
(Phase 1) and 20-45 min (Phase 2) post-formalin, and the amount of time spent licking the injected paw was recorded. The period between the two phases of nociceptive responding is generally considered to be a phase of weak activity. The amount of time spent licking the injected paw was recorded with a digital stopwatch. The Formalin test was carried out in an open Plexiglas cage, with a mirror placed at a 45-degree angle behind the cage to allow an unobstructed view of the paws.

PNU-120596, NS1738, or vehicle were injected i.p. at different times before the formalin injection. For the antagonists studies, MLA, DHBE or naloxone was injected s.c. 5 min before the formalin injection. Site studies were carried out by pretreating the mice with PNU-120596 (4mg/kg i.p.) 15 min before i.t. injection of MLA (10 µg/mouse).

**Locomotor activity**

Mice were placed into individual Omnitech photocell activity cages (28 x 16.5 cm) after 15 minutes after the i.p. administration of either i.p. vehicle or PNU-120596 at different doses. Interruptions of the photocell beams (two banks of eight cells each), which assess walking and rearing, were then recorded for the next 30 minutes. Data were expressed as the number of photocell interruptions.

**Motor coordination**

In order to measure motor coordination, we used Rotarod (IITC Inc. Life Science). The animals are placed on textured drums (1¼ inch diameter) to avoid slipping. When an animal drops onto the individual sensing platforms, test results are recorded. Five mice tested at a rate of 4 rpm. Naive mice were trained until they could remain on the rotarod for 3 min. Animals that failed to meet this criterion within three trials were discarded. Fifteen minutes after the injection of vehicle or drugs, mice were placed on the rotarod for 3 min. If a mouse fell from the
rotarod during this time period, it was scored as motor impaired. Percent impairment was calculated as follows: % impairment = [(180-test time/180)*100]. Mice were pretreated with either i.p. vehicle or PNU-120596 at different doses 15 min before the test.

**Time Course of PNU-120596 & NS1738 levels in Brain and Plasma**

For the determination of plasma and brain concentrations of the parent compound, naïve mice were dosed with the compounds as indicated and sacrificed at various time points post-dosing. For analytical determination of plasma concentrations, blood was collected into heparinized tubes and then centrifuged, and the separated plasma was frozen at -20°C until analysis. For the determination of brain concentrations, animals were decapitated at the various time points, and the brains were immediately removed and rapidly freed from blood vessels as much as possible. The resulting brain tissues were immediately frozen at -20°C, weighed and homogenized, and then the homogenate was stored at -20°C. For analysis, compounds were extracted from the samples via liquid-liquid extraction and were quantified by liquid chromatography/mass spectroscopy. As a result, it is able to measure concentrations of either NS-1738 or PNU-120596 down to 1 ng/ml in plasma (~ 3nM) and 10 ng/ml in brain (~ 30 nM) (Timmermann et al 2007).

**Statistical analysis**

The data obtained were analysed using GraphPad software programme and expressed as the mean ± S.E.M. Statistical analysis was done by one-way or two-way analysis of variance test ANOVA followed by post hoc, Tukey or Bonferroni tests. *P*-values less than 0.05 (*P* < 0.05) were considered significant.
Results

**Lack of antinociceptive effect of \( \alpha \)7 PAMs in acute thermal and mechanical pain in mice**

The tail-flick and hot-plate tests were used to determine the antinociceptive effects of the \( \alpha \)7 nAChR agonist and \( \alpha \)7 nAChR PAMs after i.p. administration in acute thermal pain models. A 5 min pretreatment of nicotine (2.5 mg/kg) induced significant antinociceptive effects in the tail-flick \([F(6,35) = 20.91, p < 0.0001]\) and hot-plate \([F(6,35) = 22.68, p < 0.0001]\) tests when compared to vehicle (Figure 1A & B). In contrast, neither of the \( \alpha \)7 PAMs showed significant antinociceptive effects at any doses tested in the tail-flick \([F(5,30) = 1.330, p = 0.2783]\) or hot-plate \([F(5,30) = 1.154, p = 0.3546]\) tests. Figure 1 (A & B) shows the lack of antinociceptive activity 15 min after administration of the PNU-120596 (4 and 8 mg/kg, i.p.), NS1738 (10 and 30 mg/kg, i.p.) and PHA-543613 (8 mg/kg, s.c.) in both tests. A similar lack of effect was observed at later pretreatment times (1 and 3 hr) after injection (Data not shown).

The effects of PNU-120596 (8 mg/kg, i.p.) and NS1738 (30 mg/kg, i.p.) on the mechanical sensitivity were measured using calibrated von Frey filaments 15 min after injection. As shown in Table 1, neither PNU-120596 nor NS1738 produced significant \([F_{2(2,3)} = 2.172, p = 0.2610]\) differences in mechanical sensitivity in response to hind paw stimulation using von Frey filaments.

**Time course and dose-response curve of type I \( \alpha \)7 PAM in the formalin test**

NS1738, a type I \( \alpha \)7 nAChR PAM, was evaluated for its effect in the formalin test, a mouse model of tonic pain. We first tested NS1738 in the formalin test at different times after injection. As shown in Figure 2 (A & B), NS1738 (10mg/kg, i.p.) did not produce a significant decrease \([F_{3(35)} = 2.108, p = 0.1169]\) in the duration of intraplantar formalin-induced nociceptive behavior in phase I and II at different times (15 min and 1 and 3 hr) after injection. We then
tested several NS1738 doses (1, 4, 10, 15 and 30 mg/kg, i.p.) 15 min after injection in the formalin test. As shown in Figure 2C, NS1738 produces a small but significant decrease $F_{5,37} = 7.660, p < 0.0001$ of formalin-induced nociceptive behavior at the two highest doses of 15 and 30 mg/kg (~30% decrease compared to vehicle). However, NS1738 failed to elicit a significant decrease $[F(3,33) = 1.450, p = 0.2326]$ in the nociceptive effects of phase II at any of the doses tested when compared to vehicle (Figure 2D).

**Time course and dose-response curve of type II α7 nAChR PAM in the formalin test**

Type II α7 nAChR PAM, PNU-120596, was evaluated for its effect in the formalin test. We first determined the time-course of PNU-120596 effects after injection. In contrast to NS-1738, PNU-120596 significantly decreased the spontaneous pain-related (nociceptive) behavioral response to formalin. As shown in Figure 3 (A & B), the onset of action for PNU-120596 (4 mg/kg, i.p.) was fairly rapid with maximum antinociception occurring between 15 and 60 min. However, the duration of PNU-120596-induced antinociception was long, having a significant effect up to 16 hr, but not at 24 hr. As illustrated in Figure 3B, PNU-120596’s effect in phase II gradually diminished to 40% decrease at 16 hr (significantly different from vehicle) $[F(6,51) = 22.99, p < 0.0001]$ and disappeared completely within 24 hr after injection. A similar time-course $[F(6,51) = 30.78, p < 0.0001]$ was seen in phase I with a rebound at time 8 hr (Figure 3A).

Dose-response relationship was then established for PNU-120596 in mice by measuring antinociception at the time of maximal effect of 15 min (Figure 3C & D). PNU-120596 produced a dose-responsive decrease in formalin nociceptive behavior with an ED50 (±CL) of 12.8 (6.9-23.3) and 2.78 (2.3-3.3) mg/kg in phases I and II respectively. The dose of 4 mg/kg of PNU-120596 was used for the subsequent studies.
Since NS1738 was not effective after peripheral administration (i.p.), we decided to test the drug centrally via the intrathecal route. Mice were pretreated i.t. with NS1738 at the dose of 15μg/mice and tested 5 min later in the formalin test. However, NS1738 elicited no significant effect in decreasing nociceptive behavior compared to vehicle-treated animals [Phase I: Vehicle = 78 ± 3, NS1738 = 71 ± 6; Phase II: Vehicle = 145 ± 12.5, NS1738 = 110 ± 14.6].

While NS1738 failed to elicit antinociceptive effects after peripheral and central administration, it blocked the actions of PNU-120596 in the formalin test. Indeed, pretreatment of mice with NS1738 (30mg/kg, i.p.) partially blocked the antinociceptive response [F(3,23) = 24.14, p < 0.0001] elicited by an active dose of PNU-120596 (4 mg/kg, i.p.) during phase II of the formalin test (Table 2).

The lack of effect of α7 type II nAChR PAM on locomotor activity and coordination of mice

In order to determine if the effects of PNU-120596 in the formalin test are not due to disruption of the locomotor activity during testing, we evaluated the effect of antinociceptive doses of PNU-120596 on spontaneous activity and motor coordination of mice. As seen in Tables 3 and 4, mice treated with PNU-120596 at doses of 4 and 8 mg/kg, i.p. did not show significant changes in locomotor activity (locomotor test) [F(2,11) = 0.8252, p = 0.4365] or motor coordination (rotarod test) [F(2,12) = 0.08455, p = 0.9195], 15 min after testing.

Time course and dose-response curve of a selective α7 nAChR agonist in the formalin test

We compared the effects of the type II α7 nAChR PAM, PNU-120596 in the formalin test to a full α7 nAChR agonist, PHA-543613. PHA-543613 at 6mg/kg given s.c. significantly reduced the formalin-induced nociceptive behavior during both early F_{2,15} = 4.366, p = 0.0320 and late F_{2,15} = 25.80, p < 0.0001 phases (Figure 4A & B). The onset of action was relatively fast.
with a maximum between 0 and 15 min and the effects disappeared within 60 min after the injection (Figure 4A & B). When a dose-response relationship was established, a U-shape curve emerged for both phase I and II (Figure 4C & D). PHA-543613 reduced formalin nociceptive behaviors at narrow range of doses (4 and 6 mg/kg in phase II) but the antinociception effect of the drug was lost at higher doses.

**Role of α7 and β2* nAChRs subtypes in PNU-120596-induced antinociception in the formalin test**

We examined the role of β2* and α7 nAChRs subtypes in mediating the antinociceptive effect of PNU-120596 (4 mg/kg i.p.). As predicted, the α7 nAChR antagonist MLA (10 mg/kg, s.c.) completely blocked PNU-120596’s effects in both phase I [F(5,34) = 30.77, p < 0.0001] and II [F(5,34) = 63.11, p < 0.0001] (Figure 5A & B). In contrast, DHβE (2 mg/kg, s.c.), a β2-containing selective antagonist, failed to block PNU-120596’s actions in the formalin test (Figure 5A & B). The blockade of PNU-120596’s effects in the formalin test being mediated through α7 nAChRs was confirmed using the α7 KO mice. As shown in Figure 5 (C & D), PNU-120596-induced antinociception in both phase I [treatment: F(1,9) = 8.136, p = 0.0106; gene: F(1,9) = 2.197, p = 0.1556; interaction: F(1,18) = 11.56, p = 0.0032] and II [treatment: F(1,9) = 107.3, p < 0.0001; gene: F(1,9) = 79.20, p < 0.0001; interaction: F(1,18) = 79.20, p < 0.0001] was lost in the α7 KO mice compared to their WT littermates.

Furthermore, pretreatment with naloxone (1 mg/kg, s.c.), an opioid receptor antagonist, did not abolish the antinociceptive effect of PNU-120596 given at the dose of 4 mg/kg (i.p.) in phase I [F(3,16) = 9.237, p = 0.0009] and phase II [F(3,16) = 69.05, p < 0.0001] (Figure 6A & B).

**Contribution of central α7 nAChRs to PNU-120596 antinociceptive response**
Since $\alpha_7$ nAChRs subtypes are present in both the periphery and the spinal cord, we examined the contribution of these sites to PNU-120596’s antinociceptive response in the formalin test. When the $\alpha_7$ nAChR antagonist MLA was given i.t. (10 µg/mouse) 5 min before PNU-120596 (4mg/kg, i.p.) administration, the effect of the $\alpha_7$ nAChR PAM II was completely reversed in phase II [$F(2,18) = 17.33, p < 0.0001$] (Figure 7).

The effects of i.t. administration of the MEK inhibitor U-0126 on PNU-120596’s antinociceptive effect in the formalin test.

We next investigated whether the antinociceptive effect of PNU-120596 was mediated through ERK activation in mice. Pretreatment of mice with i.t. injection of gradually increasing doses of U0126, a selective MEK inhibitor (Kominato et al., 2003; Aley et al., 2001), on its own did not affect nociceptive responses induced by formalin injection in U0126-treated mice compared to their vehicle controls at any of the doses tested (0.04, 0.2 and 1 µg/mouse, i.t.) (Figure 8). However, U0126 administered 5 min before PNU-120596 (4mg/kg, i.p.) dose-dependently blocked [$F(7,36) = 13.38, p < 0.0001$] PNU-120596-induced antinociception in phase II of the formalin test. U0126 at the dose of 1µg/mouse completely reversed PNU-120596’s actions in the formalin test.

Tolerance did not develop to PNU-120596’s effects in the formalin test after sub-chronic exposure

We investigated if tolerance develops to PNU-120596’s antinociceptive effects after sub-chronic exposure of the drug. The dosing protocol of PNU-120596 was based on the time-course study of the drug shown earlier (24 hr long). Animals were treated with either vehicle or PNU-120596 (4 mg/kg, i.p.) once a day (8:00 am) for 6 days. On day 7, mice were challenged with PNU (4 mg/kg, i.p.) and tested 15 min later. As seen in Figure 9A, tolerance did not develop
[F(2,15) = 17.89, p < 0.0001] after sub-chronic exposure to PNU-120596 in phase II of the formalin test. Furthermore, the sub-chronic treatment of PNU-120596 did not significantly change the weight gain of mice compared to the vehicle-treated group (Figure 9B).

Measurement of α7 type I and II PAMs levels in the brain and plasma

To estimate the ability of PAMs to permeate the blood-brain barrier, mice were administered 1 mg/kg of either NS 1738 or PNU-120596 i.p. Brain and plasma concentrations were measured at times 0.25, 2, 4 and 16 hr for PNU-120596 after dosing and it corresponded to 150 to 250 ng/ml in plasma versus brain, respectively at the 0.25 hr time point and less with the latter time points. After 4 hours after PNU-120596 injection, brain and plasma levels were close to 2 ng/ml (Figure 10A). Brain and plasma concentrations were measure at times 0.25, 2 and 4 hr for after 1 mg/kg i.p. of NS 1738 which yielded only 1 ng/ml in brain versus 1000 ng/ml in plasma samples (Figure 10B). To assess if pharmacokinetic factors are responsible for the lack of pharmacological effects for NS-1738, a 30 mg/kg i.p. dose was also assessed and brain to plasma ratio estimated at 15 min time point. As shown in Figure 10C, sufficient NS 1738 (close to 300 ng/ml) crosses the blood-brain ratio to further support pharmacodynamic differences between type I and II PAMs in regards to antinociceptive efficacy. Finally, when we examined the time course relationship of plasma and brain levels associated with antinociceptive efficacy, discordance between the pharmacokinetic-pharmacodynamic properties of PNU-120596 was observed. As depicted in Figure 10D, a large antinociceptive effect in phase II was observed at 4 h, despite substantial reduction in plasma concentration of PNU-120596 at this time point compared with the peak levels. Moreover, at the 16-h interval, significant effects were still observed, although the plasma and brain concentrations were below the detection limit.

Discussion
In the present study, we evaluated the effects of α7 nAChRs type I and II PAMs in animal models of acute and tonic pain. Our studies show that similar to α7 nAChR agonists, α7 nAChR PAMs are not active in acute thermal pain tests (hot-plate and tail-flick) and mechanical sensitivity tests. Type II α7 nAChR (PNU-120596) but not type I PAMs attenuated pain behavior in the early and late phases of the formalin test. Indeed, PNU-120596 had a long-lasting antinociceptive effect with a greater potency (6-fold) in the late (inflammatory) phase of the test. Importantly, no changes were seen in motor locomotion and coordination with antinociceptive doses of PNU-120596 in mice. In line with our data, Munro et al. (2012) recently showed that PNU-120596 produces anti-hyperalgesic and anti-inflammatory effects in the carrageenan or complete Freund’s adjuvant (CFA) tests in rats. However, the potency of PNU-120596 in the mouse formalin test was higher than the rat one. Furthermore, Munro et al., (2012) did not investigate the effects of type I PAMs in their models. In our mouse studies, NS1738, a type I α7 nAChR PAM, failed to exhibit antinociceptive effects in the late phase of the formalin test after systemic (i.p.) and central (i.t.) administration but showed a modest decrease in the early phase of the test. The lack of effect after NS1738 administration is not due to a poor drug distribution to the brain after systemic injection. Indeed, measuring brain concentrations of NS1738 after i.p. injection (30 mg/kg), yield drug levels (~0.80 µM) close to those reported to enhance ACh in expressed α7 nAChRs (Timmermann et al., 2007). In addition, the concentration of NS1738 in the mouse brain is similar to that seen in the rats after i.p. injection (Timmermann et al., 2007). Similarly, PNU-120596 brain levels after a dose of 1 mg/kg (~0.25 µM) fall near the EC50 for potentiating effects of PNU-120596 (EC50~ 0.2-1.5 µM) in various in vitro expressed and native α7 nAChRs preparations (Kalappa et al, 2010; Gronlien et al., 2007; Hurst et al., 2005; Barron et al., 2009; Gusev and Uteshev, 2010). Our data
therefore strongly suggest that type I and II α7 nAChR PAMs differentially modulate nociceptive behavior in the formalin test.

The difference in efficacy might be related to differences in α7 nAChRs regulation by these two drugs. Type I PAMs predominantly affect the apparent peak current with little effect on desensitization kinetics whereas type II PAMs increase the apparent peak current and evoke a distinct weakly decaying current causing dramatic slowing of receptor desensitization (Hurst et al., 2005). Indeed, PNU-120596 was shown to activate α7 nAChRs that would otherwise be desensitized (Papke et al., 2009).

In contrast to the type II α7 nAChR PAM, the selective α7 agonist PHA-543613 had a very narrow window of antinociceptive effect, as shown by its U-shaped dose-effect curve in the formalin test. While drug distribution and metabolism factors could account for PHA-543613 and PNU-120596 different dose-response profile, differences in α7 nAChRs activation and desensitization properties may play a more important role. Like other α7 nAChR agonists, PHA-543613, induces receptor desensitization after an initial phase of receptor activation.

The inflammatory tonic pain model consists of two distinct phases. The first phase (immediately after formalin injection), seems to be caused by the direct effect of formalin on sensory C-fibers. The second phase (starting later after formalin injection), known as the inflammatory phase, is associated with the development of a delayed inflammatory response and spinal dorsal horn sensitization (Abbott et al., 1995; Davidson and Carlton, 1998). We observed that PNU-120596 attenuated pain behavior in the early and late phases of the formalin test, suggesting that PNU-120596 acts both centrally and peripherally to reduce tonic pain.

The long-acting effect of PNU-120596 we observed in the mouse formalin test is line with PNU-12096’s effects reported in the rat carrageenan test (Munro et al., 2012), in which the
PAM II significantly blunted mechanical hyperalgesia for up to 4 h. This effect cannot be simply explained by the pharmacokinetic profile of the drug. Indeed as shown in Figure 10, most of the PNU-120596 is eliminated from plasma and brain of the animals 4 hr after injection of the drug. In this regard, PNU-120596’s properties are similar to those reported with α7 nAChR agonists such as ABT-107 (Bitner et al., 2010). PNU-120596 appears to offer prolonged efficacy that may be associated with activation of signaling pathways leading to lasting secondary functional changes linked to synaptic plasticity. Our results with the MEK inhibitor U-0126 suggest that one possible post-receptors signaling mechanism, is the extracellular-signal-regulated kinases 1 and 2 (ERK1/2) pathways. The ERK1/2 pathway regulates a diverse array of cellular functions, such as cell growth, differentiation and survival that may underlie the synaptic plasticity required for persistent pain processes (Alter et al., 2010). Furthermore, El Kouhen et al. (2009), observed a robust increase in ERK1/2 phosphorylation induced by α7 agonists in the presence of type II PAM in PC12 cells. It is also possible that the prolonged effects of PNU-120596 could be mediated by an α7-dependent regulation of anti-inflammatory chemokines such as TNF-α through a NF-κB pathway (Bernik et al., 2002). In line with this suggestive mechanism, Munro et al. (2012) recently showed that PNU-120596’s anti-inflammatory effects in rats are possibly mediated through a decrease in peripheral TNF-α and IL-6 levels.

PAMs are compounds that facilitate endogenous neurotransmission and/or enhance the efficacy and potency of agonists without directly stimulating the agonist binding sites. Our PNU-120596 data suggests the presence of a pro-antinociceptive endogenous tone mediated by α7 nAChRs. PNU-120596 may be acting in the formalin test through the enhancement of sub-threshold concentrations (i.e., 5-10μM) of endogenous α7 agonists, choline and/or ACh (Sarter and Parikh, 2005; Parikh and Sarter, 2006). Supporting the possibility of choline, PNU-120596
was recently reported to enhance the effects of sub-threshold, physiological concentrations of choline on native α7 nAChR in hypothalamic neurons (Gusev and Uteshev, 2010). Although this enhancement seen using *in vitro* α7 nAChRs preparations is greatly decreased when experimental temperature rose to near physiological levels (Sitzia et al., 2011; Williams et al., 2012), apparently it is not completely eliminated given that PNU-120596 must have enhanced endogenous α7 nicotinic activation to produce the antinociceptive effects in our tests. In addition, Williams et al., (2012) showed that while the effects of PNU-120596 are strongly temperature-dependent, physiological factors such as serum albumens reverse this temperature dependency. Alternatively, pharmacological effects of PNU-120596 could be mediated independently of α7 channel activation. Indeed, a growing body of literature suggests that α7 receptors have low intrinsic open probability and high propensity toward the induction of non-conducting ligand-bound states (Williams et al., 2011; 2012). These properties imply that some pharmacological effects of α7 ligands could be mediated independent of ion channel activation. This channel-independent regulation of signal transduction pathways by α7 nAChRs is well documented in immune cells regulating inflammation (for review see Marrero et al., 2011). Our results with the MEK inhibitor suggest such possibility in *in vivo* animal model.

In our studies, MLA, an α7 nAChR antagonist, but not DHBE, a β2* antagonist, significantly blocked PNU-120596’s antinociceptive effect in the early and late phases of the formalin test. Using both pharmacological (i.e. naloxone) and genetic approaches (i.e. α7 KO mice) we confirmed that PNU-120596’s effect is mediated by α7 nAChRs and not by opioid receptors. Similarly, α7 nAChRs seems to mediate the antihyperalgesic effects of PNU-120596 in the rat (Munro et al., 2012).
Furthermore, our results show that tolerance did not develop following sub-chronic exposure of the type II $\alpha_7$ nAChR PAM. Importantly, no changes were seen in body weight gain after sub-chronic administration of PNU-120596 in mice. Munro et al., (2012) reported the effects of PNU-120596 only after acute administration in the rat carrageenan test.

It seems that multiple sites are involved in PNU-120596’s antinociceptive effect. Indeed, the data with i.p. and i.t. MLA provides evidence of both central and peripheral involvement, respectively. Our results support the possibility that the spinal cord is an important site of action for PNU-120696 in the formalin test. However, a role for peripheral and local $\alpha_7$ nAChRs cannot be discounted. In the periphery, $\alpha_7$-nAChRs are expressed on T cells, macrophages and other immune cells, which are capable of producing ACh (Fujii et al., 2008; Kawashima and Fujii, 2003; Wang et al., 2003). Additionally, in the rat hindpaw, $\alpha_7$ nAChRs have been identified on skin keratinocytes and resident macrophages, but not on peripheral nerve endings (Kurzon et al., 2007).

Finally, our data in phase II of the formalin test show a blockade of PNU-120596’s effect by NS1738. Supporting our finding, Williams et al. (2011) recently reported that the type I PAM 5HI reduced PNU-120596’s in vitro effects in oocytes expressing human $\alpha_7$ nAChRs. These results suggest that type II and I may compete for a common allosteric transmembrane site of the $\alpha_7$ nAChRs. Indeed, Collins et al. (2011) recently showed that both type I PAM NS1738 and type II PAM PNU-120596 bind competitively at a shared or overlapping allosteric transmembrane site on the $\alpha_7$ nAChR.
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**Authorship Contributions.**
Participated in research design: Freitas and Damaj.

Conducted experiments: Freitas.

Contributed new reagents or analytic tools:

Performed data analysis: Freitas and Damaj.

Wrote or contributed to the writing of the manuscript: Carroll, Freitas and Damaj.
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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Antinociceptive effect of various α7 nicotinic compounds in the tail-flick and hot-plate tests after acute administration in mice.

Figure (A) Effects of nicotine (2.5 mg/kg, s.c.), PHA543613 (8mg/kg, s.c.), PNU-120596 (4 and 8 mg/kg, i.p.) and NS1738 (10 and 30mg/kg, i.p.) in the tail-flick test. Mice were pretreated s.c. with nicotine 5 min before testing. The other treatment groups received PHA-543613, PNU-120596 or NS1738 15 min before the tail-flick test. Figure (B) Effects of nicotine, PHA-543613, PNU-120596, and NS1738 in the hot-plate test. Mice were treated with the same doses and pretreatment time as in the tail-flick test. Each group represents the mean ± SE of 6-8 mice and *denotes p<0.05 vs. vehicle. Each point represents the mean ± SE of 6 mice. NS = NS1738; PNU = PNU-120596; Nic = nicotine; Veh = vehicle.

Figure 2. Effects of α7 type I PAM NS1738 in the mouse formalin test.

Time-course of the effects of NS1738 on (A) phase I and (B) phase II in the formalin test after i.p. administration. Mice treated with either vehicle or NS1738 (10 mg/kg, i.p) at different times after injection (15min, 1h and 3h) before intraplantar formalin injection in the right paw. Dose-response relationship for NS1738 was then established in mice 15 min after i.p. injection of various doses of drugs in (C) phase I and (D) phase II of the formalin test. Each point represents the mean ± S.E.M of total time spent licking for 8-10 mice per group and *denotes p<0.05 vs. vehicle. Veh = vehicle.

Figure 3. Effects of α7 type II PAM PNU-120596 in the mouse formalin test.
Time-course of the effects of PNU-120596 on (A) phase I and (B) phase II in the formalin test after i.p. administration. Mice treated with either vehicle or PNU-120596 (4 mg/kg, i.p) at different times after injection (15min, 1h and 3h) before intraplantar formalin injection in the right paw. Dose-response relationship for PNU-120596 was then established in mice 15 min after i.p. injection of various doses of drugs in (C) phase I and (D) phase II of the formalin test. Each point represents the mean ± S.E.M of total time spent licking for 8-10 mice per group and *denotes p<0.05 vs. vehicle. Veh = vehicle.

**Figure 4. Effects of α7 agonist PHA-543613 in the mouse formalin test.**

Time-course of the effects of PHA-543613 on (A) phase I and (B) phase II in the formalin test after s.c. administration. Mice treated with either vehicle or PHA-543613 (6 mg/kg, s.c.) at 15 and 60 min after injection before intraplantar formalin injection in the right paw. Dose-response relationship for PHA-543613 was then established in mice 15 min after s.c. injection of various doses of drugs in (C) phase I and (D) phase II of the formalin test. Each point represents the mean ± S.E.M of total time spent licking for 8-10 mice per group and *denotes p<0.05 vs. vehicle. Veh = vehicle

**Figure 5. Nicotinic receptors subtypes involved in PNU-120596-induced antinociception in the formalin test.**

Blockade of the antinociceptive effect of PNU-120596 in the (A) phase I and (B) phase II of formalin test by different nicotinic antagonists. Mice were pretreated with MLA (10 mg/kg, s.c.) or DHβE (2 mg/kg, s.c.) 15 min before an active dose of 4 mg/kg of PNU-120596. Fifteen min later, mice were injected with formalin (2.5% intraplantar, 20 μl) and then observed for pain
behaviors. Antinociceptive effects of PNU-120596 in the formalin test were tested in the α7 WT and KO mice. Mice received a dose of 4 mg/kg, i.p. of PNU-120596 in α7 WT and KO mice and 15 min later were tested in (C) the phase I and (D) phase II of formalin test. Data was expressed as mean ± SEM of licking time of 6-8 mice per group. *D enotes p<0.05 vs. vehicle-vehicle group. Veh = vehicle; PNU = PNU-120596.

Figure 6. Lack of blockade of PNU-120596-induced antinociception in the formalin test by naloxone.

Mice were pretreated with s.c. naloxone 15 min prior to PNU-120596 (4mg/kg, i.p.) injection. They were tested 15 min after the second injection in the formalin test. The time spent licking the injected paw was recorded in (A) the early and (B) late phase after the formalin injection. Data was expressed as mean ± SEM of licking time. Each group represents the mean ± SE of 6-8 mice and *denotes p<0.05 vs. vehicle-vehicle group.

Figure 7. Blockade of PNU-120596’s antinociceptive effect after i.t. MLA administration in the formalin test.

The effects of i.t. MLA on PNU-120596’s antinociceptive effect in the phase II of the formalin test. Mice were pretreated with either PNU-120596 (4mg/kg, i.p.) or vehicle (i.p.) 15 min prior to MLA (10μg/5μl i.t.) injection, and tested 5 min after later in the formalin test. Each bar represents the mean ± S.E.M for each group of 6-8 mice. *p < 0.05 versus vehicle-vehicle group; #p < 0.05 versus 4mg/kg PNU-120596. Veh = vehicle; PNU = PNU-120596.
Figure 8. Effects of MEK inhibitor, U0126, on PNU-120596-induced antinociception in the formalin test.

Mice were pretreated with the MEK inhibitor U0126 (0.04, 0.2 and 1 µg/mouse, i.t.) or vehicle 5 min prior to PNU-120596 (4mg/kg., i.p.) injection. Mice were tested 15 min later in the phase II of the formalin test. Each bar represents the mean ± S.E.M for each group of 6-8 mice. *p < 0.05 versus vehicle-vehicle group; #, p < 0.05 versus 4mg/kg PNU-120596. Veh = vehicle; PNU = PNU-120596.

Figure 9. Lack of tolerance to PNU-120596-induced antinociception in the formalin test after sub-chronic administration of the drug.

(A) Mice were treated with either vehicle or PNU-120596 (4 mg/kg, i.p.) once a day for 6 days. On day 7, mice were challenged with PNU (4 mg/kg, i.p.) and tested 15 min after in the phase II of the formalin test. Control group received vehicle for 6 days and were challenged on day 7 with vehicle and tested 15 min later in the formalin test. (B) Lack of significant effect on the body weight change of mice after sub-chronic injection PNU-120596. Body weight change (Body weight at the injection day – Initial Body weight before treatment) in the two treatment groups was recorded daily at the same time. Each bar represents the mean ± S.E.M for each group of 6-8 mice. *p < 0.05 versus vehicle-vehicle group. Veh = vehicle; PNU = PNU-120596.

Figure 10. Pharmacokinetics of NS-1738 and PNU-120596

(A) Mice (n=3) were dosed with 1 mg/kg i.p. of PNU-120596 at time 0, and both brain and plasma samples were collected at 0.25, 2 4 and 16 hours and analyzed by mass spectrometry for PNU-120596 content in order to estimate the brain/plasma ratio at each time point. (B) Mice
(n=3) were dosed with 1 mg/kg i.p. NS-1738 at time 0 and both brain and plasma samples were collected at times 0.25, 2 and 4 hours. (C). To parallel in vivo efficacious doses, mice (n=3) were dosed with either 1 mg/kg or 30 mg/kg i. p. NS-1738 at time 0 and both brain and plasma samples were collected at 15 min to compare levels. (D). Pharmacokinetic-Pharmacodynamic (PK-PD) relationships of PNU-120596. Time course of efficacy and relation to plasma and brain concentrations of PNU-120596. Antinociception [% Maximum Possible Effect (%MPE)] was assessed 0.25, 2, 4, 8, 16 and 24 h postdose of 4 mg/kg (left y-axis). Plasma and brain levels of PNU-120596 were determined in a satellite group of mice (plotted along the right y-axis). Although plasma and brain concentrations of PNU-120596 decreased to levels below 5 ng/ml by 2 h, significant effect in the formalin test still persists (p < 0.05 versus vehicle controls). All the data are represented as mean ± S.E.M.
Table 1. Effects of α7 nAChR PAMs of NS1738 (30 mg/kg, i.p.) and PNU-120596 (4 mg/kg, i.p.) on mechanical sensitivity in naïve mice. The two α7 nAChR PAMs were given i.p to animals and 30 min later, their withdrawal thresholds (g) were measured. Each value represents the mean ± SE of 6 mice.

<table>
<thead>
<tr>
<th></th>
<th>Contralateral</th>
<th>Ipsilateral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>5.33 ± 0.84</td>
<td>4.67 ± 0.67</td>
</tr>
<tr>
<td>NS1738</td>
<td>5.50 ± 0.96</td>
<td>5.00 ± 0.57</td>
</tr>
<tr>
<td>PNU-120596</td>
<td>4.33 ± 0.61</td>
<td>4.67 ± 0.42</td>
</tr>
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Table 2. **NS1738 blocks PNU-120596’s antinociceptive effect in the formalin test.** Mice were pretreated with NS1738 (30mg/kg, i.p.) 15 min before an active dose of PNU-120596 (4mg/kg, i.p.) and tested 15 min after the second injection in the formalin test. The total time spent licking the right hind paw was measured in the late phase. Each value represents the mean ± S.E.M for each group of 6-8 mice.

<table>
<thead>
<tr>
<th></th>
<th>Paw Licking (Phase II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>173.85 ± 8.06</td>
</tr>
<tr>
<td>NS1738</td>
<td>176.16 ± 7.10</td>
</tr>
<tr>
<td>PNU-120596</td>
<td>65 ± 5.06</td>
</tr>
<tr>
<td>NS + PNU</td>
<td>130.44 ± 11.46</td>
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</table>
Table 3. Effects of PNU-120596 on locomotor activity of mice. The effects of PNU-120596 (4 and 8 mg/kg, i.p.) on mouse locomotor activity. Animals were tested 15 min after injection with either PNU-120596 or vehicle and their locomotor activity were measured for 30 min. Each value represents the mean ± SE of 6-8 mice.

<table>
<thead>
<tr>
<th></th>
<th>Number of Photocell Interruptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1475.0 ± 395.0</td>
</tr>
<tr>
<td>PNU-120596 (4 mg/kg)</td>
<td>1510.7 ± 268.5</td>
</tr>
<tr>
<td>PNU-120596 (8 mg/kg)</td>
<td>1115.5 ± 177.8</td>
</tr>
</tbody>
</table>
Table 4. Effects of PNU-120596 on motor coordination of mice. The effects of PNU-120596 (4 and 8 mg/kg, i.p.) in the rotarod test after administration in mice. Animals were tested 15 min after injection with either PNU-120596 or vehicle and they were placed on the rotarod for 3 min. Each value represents the mean ± SE of 6-8 mice.

<table>
<thead>
<tr>
<th>Percent Impairment</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>10.2 ± 10.2</td>
</tr>
<tr>
<td>PNU-120596 (4 mg/kg)</td>
<td>17.2 ± 17.2</td>
</tr>
<tr>
<td>PNU-120596 (8 mg/kg)</td>
<td>11.0 ± 11.0</td>
</tr>
</tbody>
</table>
Figure 1

(A) Tail Flick

(B) Hot-Plate
Figure 2

(A) Phase I

(B) Phase II

(C) Phase I

(D) Phase II

- Vehicle
- NS1738 (10 mg/kg, i.p.)

Paw Licking (sec)

Time (hrs)

NS 1738 (mg/kg, i.p.)
Figure 5
Figure 6

(A) Phase I

(B) Phase II

Paw Licking (sec)

Veh + Veh, NaX + Veh, Veh + PNU, NaX + PNU

* indicates statistically significant difference.
Figure 7

Phase II

Paw Licking (sec)

- Veh + Veh
- PNU + Veh
- PNU + MLA

* * #
Figure 8

Phase II

Paw Licking (sec)

Veh + Veh
U-0126 (0.04) + Veh
U-0126 (0.2) + Veh
U-0126 (1) + Veh
Veh + PNU (4)
U-0126 (0.04) + PNU (4)
U-0126 (0.2) + PNU (4)
U-0126 (1) + PNU (4)

* * *

#
Figure 9

(A) Phase II

Paw Licking (sec)

Veh + Veh  Veh + PNU  PNU + PNU

(B) Body Weight Gain (g)

Veh-Veh  PNU - PNU

Days

D1  D2  D3  D4  D5  D6  D7