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The analgesic-like properties of the alpha7 nAChR silent agonist NS6740 is associated with non-conducting conformations of the receptor

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ABSTRACT

The α7 nicotinic acetylcholine receptor (nAChR) is a promising drug target for a number of neurological disorders including chronic pain and inflammatory diseases. Since α7 can function as a ligand-gated ion channel, drug development initially focused on ligands that were selective activators of the α7 ion channel. However, the best α7 drugs for chronic pain and inflammation indications may not be ion channel activators but rather "silent agonists", which bind to the receptor but preferentially induce non-conducting states that modulate signal transduction in non-neuronal cells. One such compound is NS6740. We show that NS6740 selectively induces prolonged desensitization of α7 nAChRs. There are two forms of α7 desensitization that can be distinguished by their sensitivity to the positive allosteric modulators (PAMs). At high concentrations, NS6740 preferentially induces PAM-insensitive desensitization, which over the course of several minutes reverts to the sensitive form. NS6740 was tested in several pain models after in vivo administration in the mouse. Although it had no effects in acute thermal pain, NS6740 induced significant dose- and time-dependent antinociceptive activity in formalin- and acetic acid-induced nociceptive behaviors as well as in the chronic constrictive nerve injury (CCI) model for neuropathic pain. The antinociceptive activity of NS6740 in these models was α7-dependent. In addition, NS6740 administration reversed pain-induced aversion, an important affective component of pain. The time and concentration dependence of the effects were consistent with NS6740 induction of PAM-insensitive non-conducting states, suggesting that signal transduction required for analgesia is accomplished by α7 receptors in that conformation.

1. Introduction

The α7 nicotinic acetylcholine receptor (nAChR) silent agonist (Chojnacka et al., 2013) NS6740 first appeared in the literature as a straw man in an analysis of α7 ligands for activity in a test for cognitive enhancement (Briggs et al., 2009). NS6740, which has very little efficacy for ion channel activation of α7 under control conditions, was tested along with the more efficacious analog NS6784 in a mouse inhibitory avoidance model, and while NS6784 improved performance, NS6740 did not. NS6740 was revealed to be a silent agonist, a ligand that binds to the site for orthosteric activation but more effectively promotes the conformational changes

Abbreviations: nAChRs, nicotinic acetylcholine receptors; CCI, chronic constriction nerve injury; PAM, positive allosteric modulator; RIC-3, resistance-to-cholinesterase 3; KO, knock-out; HET, heterozygous; WT, wild-type; MLA, methyllycaconitine.

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associated with desensitization than activation, when it was used in combination with the type II positive allosteric modulator (PAM) PNU-120596 and shown to generate currents. PNU-120596 destabilizes a form of desensitization that is unique to α7, thereby promoting protracted bursts of channel opening (Williams et al., 2011). The conclusion of Briggs et al. (2009) was that α7 channel activation was required for α7-mediated cognitive effects. However, even under the best of conditions the open probability of α7 nAChR is very low (Williams et al., 2011), and recent studies of α7-mediated signal transduction in non-neuronal cells has promoted the alternative hypothesis that, at least for some indications or stimuli, α7 signaling can be ion channel independent. This hypothesis was strongly supported by a subsequent study of NS6740 as a modulator of the inflammatory function of microglia (Thomsen and Mikkelsen, 2012) which showed that NS6740 and GTS-21, another α7 selective partial agonist with low ion channel efficacy, were more effective at suppressing LPS-stimulated secretion of TNF-α in rat cultured microglia than were the more efficacious agonists, SSR180711, A-582941, and choline.

The basic pharmacological data on NS6740 are limited, even in regard to its putative selectivity for α7. We have tested NS6740 on α3β4 and α4β2 as well as α7 nAChR to confirm its selectivity for α7 compared to these heteromeric nAChR subtypes. We have also investigated the time and concentration dependence of the effects of NS6740 on human α7 nAChR with and without the modulatory effects of PNU-120596. We found that NS6740 shows a remarkable concentration dependence in its ability to synergize with PNU-120596 and produce ion channel activation, and that above a certain concentration NS6740 induces non-conducting states of the receptor that are insensitive to the activation-promoting effects of the PAM.

Just as NS6740 was shown to be effective at modulating the inflammatory responses of microglia cells in vitro, we report that it is effective in well-established mouse models of chronic pain, including peripheral neuropathy (chronic constriction nerve injury, CCI) and tonic inflammatory pain (the formalin test). The behavioral and pharmacological profile of NS6740 in these models is consistent with the induction of non-conducting conformational states of the receptor. Results obtained from these studies will further understanding of the α7 nAChR subtype in pain regulation and be helpful for the targeting of novel α7 silent agonists as therapeutic agents for the treatment of chronic pain.

2. Materials and methods

2.1. In vitro methods

2.1.1. Heterologous expression of nAChRs in Xenopus laevis oocytes

Human nAChR clones were obtained from Dr. J. Lindstrom (University of Pennsylvania, Philadelphia, PA). The human resistance-to-cholinesterase 3 (RIC-3) clone, obtained from Dr. M. Treinin (Hebrew University, Jerusalem, Israel), was co-injected with α7 to improve the level and speed of α7 receptor expression without affecting the pharmacological properties of the receptors (Halevi et al., 2003). Subsequent to linearization and purification of the plasmid cDNAs, cRNAs were prepared using the mMessage mMachine in vitro RNA transfection kit (Ambion, Austin, TX).

Oocytes were surgically removed from mature Xenopus laevis frogs (Nasco, Ft. Atkinson, WI) and injected with appropriate nAChR subunit cRNAs as described previously (Papke and Stokes, 2010). Frogs were maintained in the Animal Care Service facility of the University of Florida, and all procedures were approved by the University of Florida Institutional Animal Care and Use Committee. In brief, the frog was first anesthetized for 15–20 min in 1.5 L frog tank water containing 1 g of 3-aminobenzoate methanesulfonate buffered with sodium bicarbonate. The harvested oocytes were treated with 1.25 mg/ml collagenase (Worthington Biochemicals, Freehold, NJ) for 2 h at room temperature in a calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 15 mM HEPES, and 12 mg/l tetracycline, pH 7.6) to remove the follicular layer. Stage V oocytes were subsequently isolated and injected with 50 nl of 5–20 ng nAChR subunit cRNA. Recordings were carried out 1–7 days after injection.

2.1.2. Two-electrode voltage clamp electrophysiology

Experiments were conducted using OpusXpress 6000A (Molecular Devices, Union City, CA). The OpusXpress recording system has previously been described in detail (Papke and Stokes, 2010). In brief, OpusXpress provides steady bath perfusion to each chamber from an 8-channel peristaltic pump. Solutions for drug delivery were loaded in 96-well plates (1 ml deep wells, one or two plates per experiment as required). In order to deliver drugs simultaneously to the eight oocytes in separate chambers, an eight-channel Gilson fluid handler drew up solution from a designated set of wells and then moved over to the chambers and positioned the tips over recesses in the fluid inflow path. During the data acquisition the software interrupted the peristaltic pump in synchrony with the delivery from the pipette tips of the fluid handler.

Both the voltage and current electrodes were filled with 3 M KCl. Oocytes were voltage-clamped at −60 mV. The oocytes were bath-perfused with Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, and 1 μM atropine, pH 7.2) at 2 ml/min for α7 receptors and at 4 ml/min for other subtypes. To evaluate the effects of experimental compounds compared to ACh-evoked responses of various nAChR subtypes expressed in oocytes, baseline conditions were defined by two initial applications of ACh made before co-applications of experimental compounds with the control ACh. The agonist solutions were applied from a 96-well plate via disposable tips, and the test compounds were applied alone, co-applied with ACh, or co-applied with PNU-120596. For the concentration-response study, drug applications alternated between ACh controls and experimental compounds. Unless otherwise indicated, drug applications were 12 s in duration followed by a 181 s washout period for α7 receptors and 6 s with a 241 s washout for other subtypes. A typical recording for each oocyte constituted two initial control applications of ACh, an experimental compound application, and then a follow-up control application of ACh to determine the desensitization or rundown of the receptors. The control ACh concentrations were 60 μM for α7, 100 μM for α3β4, and 30 μM for α4β2. The responses of α4β2 and α3β4-expressing cells were measured as peak current amplitudes, and the α7 data were calculated as net charge, as previously described (Papke and Papke, 2002).

Data were collected at 50 Hz, filtered at 20 Hz, analyzed by Clampfit 9.2 (Molecular Devices) and Excel 2003 (Microsoft, Redmond, WA), and normalized to the averaged peak current or net-charge response of the two initial ACh controls (Papke and Papke, 2002). Data were expressed as means ± S.E.M. from at least four oocytes for each experiment and plotted by Kaleidagraph 3.0.2 (Abelbeck Software, Reading, PA).

2.2. In vivo methods

2.2.1. Animals

Male ICR mice were purchased from Harlan Laboratories (Indianapolis, IN). Mice null for the α7 subunits (knock-out or KO), heterozygous (HET) and their wild-type (WT) littermates (The Jackson Laboratory) were bred in an animal care facility at Virginia Commonwealth University. For all experiments, mutants and wild-type controls were obtained from crossing heterozygote mice. This breeding scheme allows us to rigorously control for any anomalies that may occur with crossing solely mutant animals. Male animals were 8–10 weeks of age at the start of the experiments and were group-housed in a 21 °C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care-approved animal care facility with ad libitum access to food and water. Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All studies were carried out in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

2.2.2. Drugs

NS6740 ((1,4-diazabicyclo[3.2.2]nonan-4-yl(5-(3-trifluoromethyl) phenyl) furan-2-yl) methanone) was prepared as previously described (Peters et al., 2004; see Supplementary material). Methyllycaconitine citrate (MLA) was purchased from RBI (Natick, MA). (−)-Nicotine hydrogen tartrate salt was purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). All drugs were dissolved in physiological saline (0.9% sodium chloride) and, with the exception of MLA, injected intraperitoneally (i.p.) at a total volume of 1 ml/100 g body weight unless noted otherwise. MLA was injected subcutaneously (s.c.) 15 min before i.p. NS6740. All doses are expressed as the free base of the drug.

2.2.3. Behavioral tests

2.2.3.1. Formalin test. The formalin test was carried out in an open Plexiglas cage, with a mirror placed at a 45° angle behind the cage to allow an unobstructed view of the paws. Mice were allowed to acclimate for 15 min in the test cage prior to injection. Each animal was injected with 20 μl of (2.5%) formalin to the right hindpaw intraplantarly. Each mouse was then immediately placed in a Plexiglas box. Up to two mice at one time were observed from 0 to 5 min (phase 1) and 20–45 min (phase 2) post-formalin injection. 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.

NS6740 (0.1, 1, 3 and 9 mg/kg) or vehicle were injected i.p. 15 min before formalin injection. For the antagonist study, MLA was injected s.c. 10 min before the NS6740 (3 mg/kg; i.p.) or vehicle injection. In another separate experiment set, NS6740 (3 mg/kg; i.p.) effects in the formalin test were measured in α7 WT, HET and KO mice. The % decrease of paw licking was calculated as = [(average baseline of vehicle-drug response)/average baseline of vehicle]*100.

In a separate experiment, to determine whether NS6740 inhibits formalin-induced paw edema, we measured changes in paw diameter 1 h after formalin injection. NS6740 (9 mg/kg; i.p.) or its vehicle was administered 15 min before formalin injection. The thickness of the formalin treated and control paws were measured both before and after injections at the time points indicated above, using digital caliper (Traceable Calipers, Friendswood, TX). Data were recorded to the nearest ±0.01 mm and expressed as change in paw thickness (ΔPD = difference in the ipsilateral paw diameter before and after injection paw thickness).

2.2.3.2. Chronic constrictive nerve injury (CCI)-induced neuropathic pain model. Mice were anesthetized with pentobarbital (45 mg/kg, i.p.). An incision was made just below the hipbone, parallel to the sciatic nerve. The right common sciatic nerve was exposed at the level proximal to the sciatic trifurcation, and a nerve segment 3–5 mm long was separated from surrounding connective tissue. Two loose ligatures with 6-0 silk suture were made around the nerve with a 1.0–1.5 mm interval between each of them. Muscles were closed with suture thread and the wound with wound clips. This procedure resulted in CCI of the ligated nerve. Any suture that remained after two weeks was removed from the healed surgical wound. Mechanical allodynia (see von Frey test) was measured before and after drug injections.

2.2.3.3. Von Frey test (evaluation of mechanical allodynia). Mechanical allodynia thresholds were 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 filaments (Stoelting, Wood Dale, IL) with logarithmically incremental stiffness ranging from 2.83 to 5.88 expressed as dsLog 10 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 filament, a thicker filament 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 Log10 of [10 £ force in (mg)], indicating the force of the Von Frey hair to which the animal reacted (paw withdrawn, licking, or shaking).

NS6740 (1, 3, and 9 mg/kg) or vehicle were injected i.p. at 2 weeks after CCI surgery and tested for mechanical allodynia for possible efficacy. Mechanical stimuli thresholds were determined for each animal 5, 10, 15, 30, 60, 90, and 120 min after injection of NS6740.

In another separate experiment set, to determine whether MLA inhibits the effects of NS6740, MLA (10 mg/kg, s.c.) or its vehicle was injected 15 min before NS6740 (9 mg/kg; i.p.) or its vehicle injection and animals were then tested for mechanical allodynia. Mechanical thresholds were determined for each animal 5, 15, 30, and 60 min after last injection.

2.2.3.4. Acute thermal pain tests. The antinociceptive effect of NS6740 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 the percentage maximum possible effect (%MPE), where % MPE = {[(test value − control value)/(cut-off (10 s) - control value)] × 100}. For the tail-flick test, mice were treated with either vehicle or NS6740 (9 mg/kg, i.p.) and tested 15, 30, 45, and 60 min after injection.

The antinociceptive effect of NS6740 was also assessed by hot-plate method. Mice were placed into a 10-cm wide glass cylinder on a hot-plate (Thermojust Apparatus, Columbus, OH) maintained at 55 °C. The device was connected to a manually operated timer that recorded the amount of time the mouse spent 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. Mice with baseline latencies of less than 8 s or more than 12 s were excluded from the study. To avoid tissue damage, the hot-plate would automatically disengage after 40 s. Antinociceptive response was calculated as a percentage of maximum possible effect (% MPE), where %MPE = {[test value − control]/[cut-off time (40 s) − control] × 100}. The reaction time was recorded when the animal jumped or licked its paws. Experiments were carried out by injecting the mice with either vehicle or NS6740 (9 mg/kg, i.p.) and animals were tested 15, 30, 45, and 60 min after injection.

2.2.3.5. Acetic acid-induced writhing test. For the measurement of acetic acid-induced nociceptive behavior, each mouse was placed in a Plexiglas box and allowed to acclimate for 20 min. Then the mouse was given an i.p. injection of acetic acid (1%) or saline and then returned to the box. Counting the number of typical writhing behaviors started immediately after acetic acid administration, and the number of stretches (a stretch was operationally defined as a contraction of the abdomen followed by an extension of the hind limbs) was recorded in 10 min bins for a total of 60 min. Experiments were carried out by injecting the mice with either vehicle or NS6740 (1 and 3 mg/kg, i.p.) and 15 min later they received acetic acid (1%) and tested as described above.

2.2.3.6. Acetic acid-induced conditioned place aversion (CPA). To evaluate the negative affective component of pain, the CPA test was performed. In brief, separate groups of male ICR mice (n = 6–11 per group) were handled for 3 days prior to initiation of CPA testing. The CPA apparatus consisted of a 3-chambered box with a white compartment, a black compartment, and a center grey compartment. The black and white compartments also had different floor textures to help the mice further differentiate between the two environments. On day 1, mice were placed in the grey center compartment for a 5 min habituation period, followed by a 15 min test period of freely exploring the all compartments to determine baseline responses. A baseline score was recorded and used to randomly pair each mouse with either the black or white compartment. Drug-paired sides were randomized so that an even number of mice received drug on the black and white side. On day 2 (conditioning session), conditioning was performed as follows: the mice were given an i.p. injection of saline (10 ml/kg) as a control non-noxious stimulus or 1% acetic acid (10 ml/kg) as a noxious stimulus and then immediately confined in the drug-paired compartment for 40 min. In addition, mice were pretreated with saline as vehicle (i.p.) or NS6740 (1 and 3 mg/kg i.p.) 15 min prior to acetic acid or saline injection. On the test day (day 3) mice were allowed to freely explore the all compartments, and the day 1 procedure was repeated. Data were expressed as time spent on the drug-paired side post-conditioning minus time spent on the drug-paired side pre-conditioning. A positive number indicated a preference for the drug-paired side, whereas a negative number indicated an aversion to the drug-paired side. A number at or near zero indicated no preference for either side.

2.2.3.7. Motor coordination. In order to measure motor coordination, we used the rotarod test (IITC Inc. Life Science, Woodland Hills, CA). The animals are placed on textured drums (1¼ inch diameter) to avoid slipping. When an animal falls onto the individual sensing platforms, test results are recorded. Five mice were tested at a time using a rate of 4 rpm. Naive mice were trained until they remained on the rotarod for 3 min or 180 s. Animals that failed to meet this criterion within three trials were discarded. Fifteen min 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 NS6740 (6 and 9 mg/kg, i.p.) 15 min before the test.

2.2.4. Statistical analysis

Pain behavioral data are presented as mean ± S.E.M. Statistical analysis was done using the t-test or two-way analysis of variance test, followed by the post-hoc Bonferroni's test. All differences were considered significant at $p < 0.05$. ED_{50} (effective dose 50%) values with 95% confidence limits (CL) were calculated by unweighted least-squares linear regression as described by Tallarida and Murray (1987).

3. Results

3.1. In vitro results

We tested the effects of NS6740 at several concentrations on heteromeric α3β4 and α4β2 and homomeric α7 nAChR expressed in *Xenopus* oocytes. Responses to applications of NS6740 along with control ACh-evoked responses obtained from the same cells obtained before and after NS6740 applications are shown in Fig. 1. Our limit of detection for receptor-mediated activity is approximately 0.05% of the ACh controls, since the application of Ringer's solution alone can cause a small stimulus artifact (not shown). The heteromeric receptors did not show significant response to the NS6740 application, and these receptors remained responsive to subsequent control applications of ACh. Cells expressing α7 had barely detectable responses to the higher concentrations of NS6740, but there was a striking reduction in the response to a control application of ACh after the 12 s application of 30 μM NS6740 (Fig. 1A), suggesting that the agent was able to induce a form of desensitization that was not readily reversible.

As previously reported (Briggs et al., 2009), co-application of the α7 PAM PNU-120596 with NS6740 was able to produce activation of α7 nAChR, and, as shown in 1B, this enhancement of channel activation was not observed with co-applications of NS6740 and PNU-120596 to the heteromeric receptor subtypes tested. We have previously reported that PNU-120596 co-applications with ACh can produce potentiation that does not fully reverse after a single

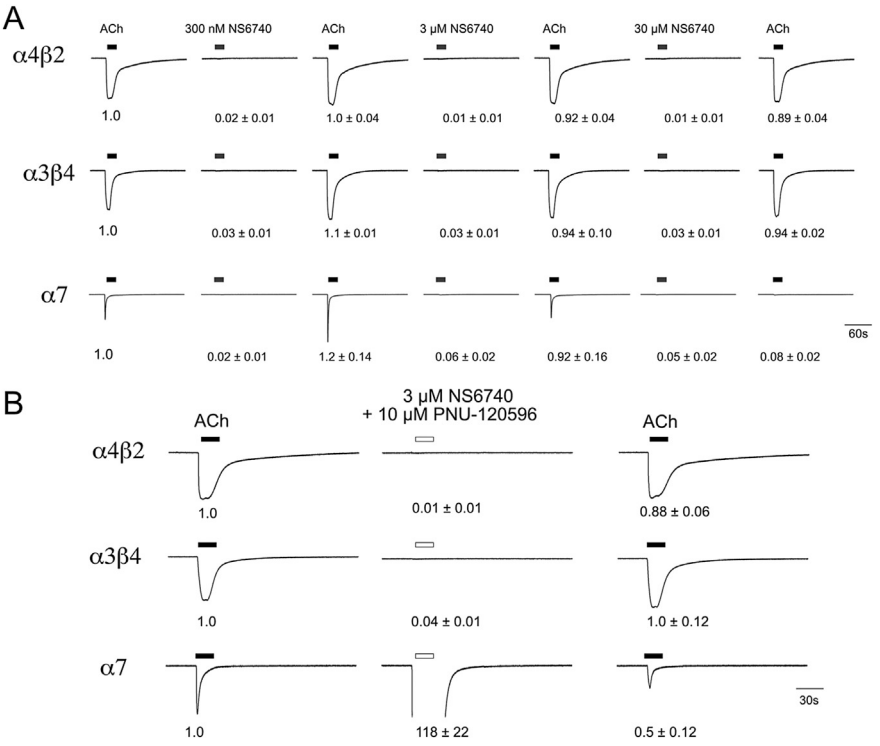

Fig. 1. A) Representative traces from single cells expressing α4β2, α3β4, or α7 nAChR. B) Representative traces from single cells expressing α4β2, α3β4, or α7 nAChR to applications of ACh or 3 μM NS6740 plus 10 μM PNU-120596. The data are scaled so that the peak amplitude of the initial ACh control is the same for each series of traces, and each trace is 210 s long. The numbers under the traces are the average peak-current amplitudes (±S.E.M.) of at least 4 cells under each condition, all calculated relative to the initial control. The ACh control concentrations were 30 μM for α4β2, 100 μM for α3β4, and 60 μM for α7. Drug applications were 12 s in duration followed by a 181 s washout period for α7 receptors and 6 s with a 241 s washout for other subtypes (see Methods).

washout period (Williams et al., 2011). However, as shown in Fig. 1B, following activation by 3 μM NS6740 plus 10 μM PNU-120596, not only there is no residual potentiation, but there's a 50% inhibition of the subsequent ACh controls.

We tested NS6740 co-applied with 10 μM PNU-120596 across a range of concentrations (Fig. 2A) and measured both the response to the co-application and the response to ACh obtained after a 4 min wash. We observed large responses to the co-applications over a limited concentration range, from 10 nM to 3 μM NS6740, with a sharp decrease in the responses when 10 μM NS6740 was co-applied with PNU-120596. As expected, ACh-evoked responses following the co-application of PNU-120596 and low concentrations of NS6740 (<1 μM) were somewhat larger than the initial ACh controls, but after co-applications with NS6740 at ≥ 3 μM, there was a concentration-dependent decrease in the subsequent ACh controls. The IC_{50} (from the potentiated levels at lower concentrations) for this inhibition was 2.7 ± 0.45 μM.

As partial agonists with very low efficacy, silent agonists like NS6740 can appear to behave as competitive antagonists in co-application experiments. As shown in Fig. 2B, co-applications of NS6740 inhibited the net-charge responses evoked by 60 μM ACh, with an IC_{50} of 4.0 ± 0.7 μM. However, as shown in Fig. 1A, after the application of NS6740, receptors remained in a desensitized state following the washout. The IC_{50} for the inhibition of ACh control responses subsequent to NS6740 applied by itself was 1.2 ± 0.15 μM, comparable to the effect observed when NS6740 was co-applied with PNU-120596 (Fig. 2A).

We have previously reported (Williams et al., 2011) that α7 nAChR dynamically convert between activatible and desensitized states as functions of time and the level of ligand binding (for both the agonist and the PAM). At intermediate levels of ACh and PAM binding, receptors may be relatively stable in PAM-sensitive nonconducting (D_s) states, while at higher levels of drug concentration and concomitant binding site occupancy they are likely to be in PAM-insensitive (D_i) states. NS6740 appears to be particularly effective at inducing and stabilizing the D_i states at levels of binding site occupancy occurring at concentrations of 3 μM or greater. To test this hypothesis, we evaluated the effects of preincubations with NS6740 alone for 30, 60, and 300 s followed by NS6740 and PNU-120596 co-applications and compared those responses to the effects of simple NS6740 and PNU-120596 co-applications. As shown in Fig. 2C, preincubations with 3 μM NS6740 for any of the time periods tested was sufficient to induce levels of D_i that were sufficient to fully suppress the response to the subsequent co-application of NS6740 and PNU-120596.

Our observations suggest that NS6740 may be a useful agent to control and track a population of receptors as they interconvert among the activatible state and the two forms of desensitization that can be distinguished by their PAM sensitivity. As shown in Fig. 3, a single application of 30 μM NS6740 was able to suppress ACh-evoked responses for a prolonged period of time. An application of PNU-120596 immediately afterward showed that some receptors were in the D_s state, but within a few minutes all of the receptors converted to D_i. After 12 min some receptors appeared to convert back to D_s from D_i, and this shift progressed, with maximal PNU-120596 responsiveness observed after 20 min.

3.2. In vivo results

NS6740 dose-dependently reduced formalin nociceptive behaviors in both phase I and II. Fig. 4A and B shows the dose-response for the antinociceptive effects of NS6740 in phase I and phase II [$F_{(4,25)} = 60.71$, $P < 0.001$] alone in mice. NS6740 was almost 30-fold more potent in phase II than phase I of the formalin

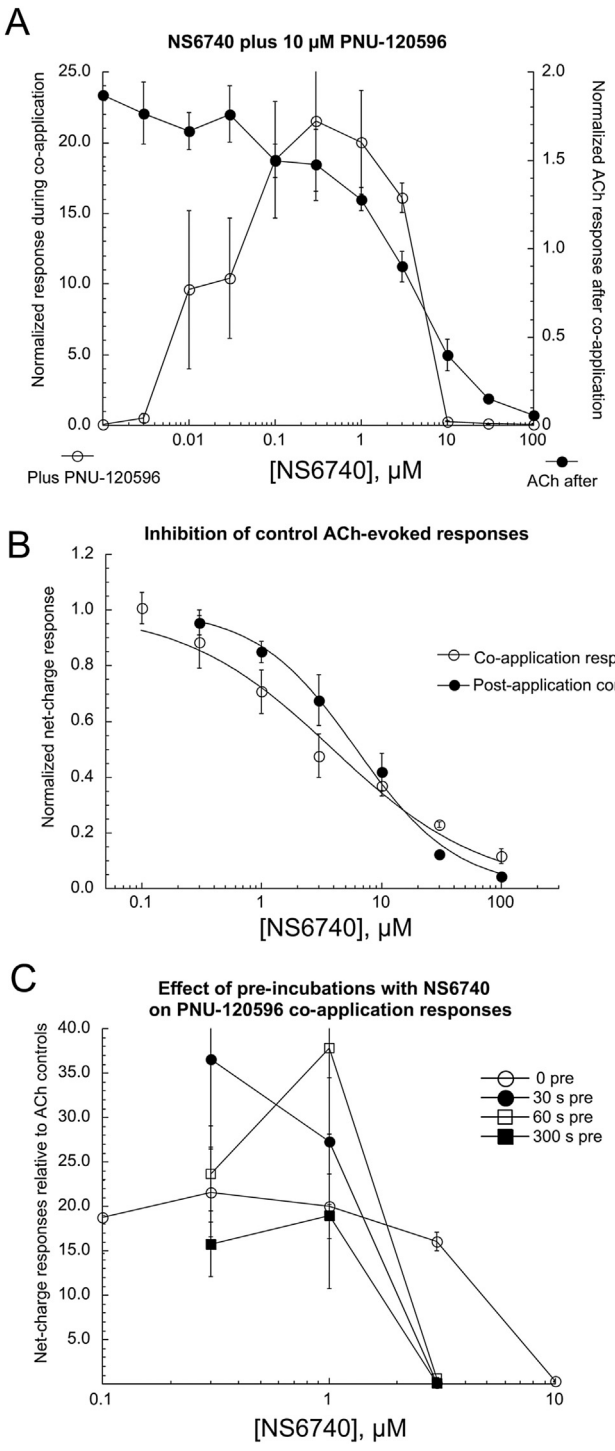

Fig. 2. Concentration-response data for the net-charge responses evoked by NS6740 co-applied with 10 μM PNU-120596 (A) and for the inhibition of ACh-evoked net-charge responses by co-applications with NS6740 (B). The effects of NS6740 preincubations for the indicated durations on the response to a subsequent co-application of NS6740 and 10 μM PNU-120596 (without washout) (C). The data were normalized to the average net-charge value of two control applications of 60 μM ACh applied before the co-application of NS6740 and PNU-120596. **(A)** The left-hand scale (open circles) refers to the co-application responses, and the right-hand scale (filled circles) refers to the ACh control responses obtained after a 4-min wash. **(B)** The open circles show the co-application responses, and the filled circles show the ACh control responses obtained after a 4-min wash. **(C)** The data were normalized to the average net-charge value of two control applications of 60 μM ACh applied before the incubations and co-application of NS6740 (3 μM) and PNU-120596. The zero preincubation data are the same as the co-application data in Fig. 2. Each point is the average (±S.E.M.) of at least 4 cells.

test, with ED_{50} (±CL) values of 1 (0.6–2.0) and 28 (2.7–299) mg/kg, respectively (Fig. 4B). NS6740's effects were mediated by α7 nAChRs. Indeed, pretreatment with the α7 antagonist MLA (10 mg/kg; s.c.) totally blocked the anti-nociceptive effect of NS6740 [$F_{(1,9)} = 22.47$, $p < 0.01$] in both phases of the formalin test (Fig. 4C). Furthermore, the antinociceptive effects of NS6740 (3 mg/kg; i.p.) in the formalin test were evaluated in α7 KO, HET, and WT mice. In phase I of the test, the effects of NS6740 were reduced in HET and KO mice compared to WT mice ($t = 0.9252$, $p > 0.05$ and $t = 2.413$, $p < 0.05$; respectively). Surprisingly, while the antinociceptive effect of NS6740 in phase II vanished in KO mice, it was preserved in HET mice compare with their vehicle controls ($t = 0.6537$, $p > 0.05$ and $t = 6.554$, $p < 0.001$; respectively; Fig. 4D). In addition, paw edema in formalin-injected mice was significantly attenuated in NS6740 (9 mg/kg, i.p.) treated mice ($t = 3.777$, $p < 0.05$ Fig. 4E).

The anti-allodynic effects of NS6740 (1, 3, and 9 mg/kg, i.p.) were explored in the CCI-induced neuropathic pain model. NS6740 induced a significant anti-allodynic effect in CCI-mice in a dose, time, and dose-time dependent manner [$F_{(3,18)} = 10.35$, $p < 0.001$; $F_{(7,126)} = 63.24$, $p < 0.001$ and $F_{(21,126)} = 3.395$, $p < 0.001$, respectively; Fig. 5A]. On the other hand, NS6740 did not show any anti-nociceptive effect in SHAM-mice [dose $F_{(3,18)} = 0.05429$, $p > 0.05$; time $F_{(7,126)} = 0.07277$, $p > 0.05$; and dose-time $F_{(21,126)} = 0.7868$, $p > 0.05$ Fig. 5B]. The anti-allodynic effect of NS6740 in the CCI test was mediated by α7 nAChRs. Indeed, pretreatment of MLA (10 mg/kg; s.c.) totally blocked the anti-allodynic effect of NS6740 (9 mg/kg, i.p.) [$F_{(1,10)} = 19.43$, $p < 0.001$; Fig. 5C].

Contrary to chronic pain models, NS6740 failed to show significant antinociceptive activity in acute thermal nociceptive tests such as tail-flick and hot-plate tests. As shown in Table 1, the highest dose of NS6740 in this study (9 mg/kg; i.p.) did not show significant antinociceptive activity in the tail flick test and hot plate test in any time point tested after injection [$F_{(1,10)} = 1.487$, $P = 0.2507$ and $F_{(1,10)} = 1.886$, $P = 0.1996$; respectively].

NS6740 reduced acetic acid-induced nociceptive behaviors in the writhing test (Fig. 6A) [$F_{(2,15)} = 9.281$, $P < 0.001$] at both doses of 1 and 3 mg/kg in mice. In the CPA test, acetic acid induced place aversion ($t = 3.207$, $P < 0.001$) in mice (Fig. 6B). However, pretreatment with NS6740 (1 or 3 mg/kg, i.p.) attenuated acetic acid-induced aversion in a dose-related manner [$F_{(4,38)} = 4.789$, $P < 0.001$]. At 3 mg/kg, NS6740 totally blocked the aversion ($t = 3.164$, $P < 0.001$) in the CPA test (Fig. 6B).

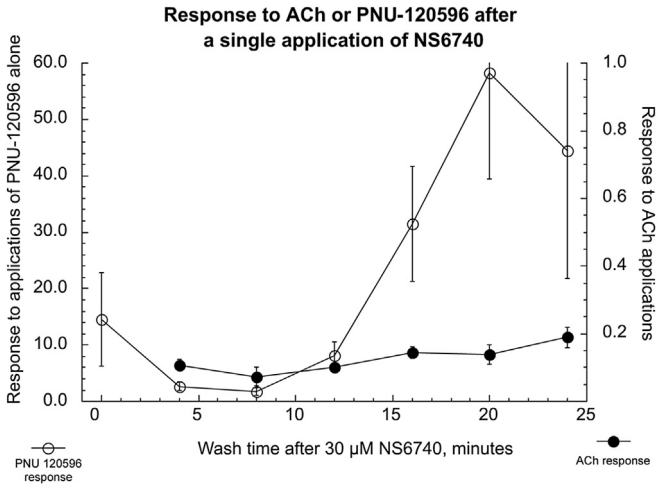

Fig. 3. The loss and recovery of PNU-120596 sensitivity following a single application of 30 μM NS6740. Cells were probed with subsequent applications of 60 μM ACh (open circles, right hand scale) or 10 μM PNU-120596. Each point is the average (±S.E.M.) of at least 4 cells.

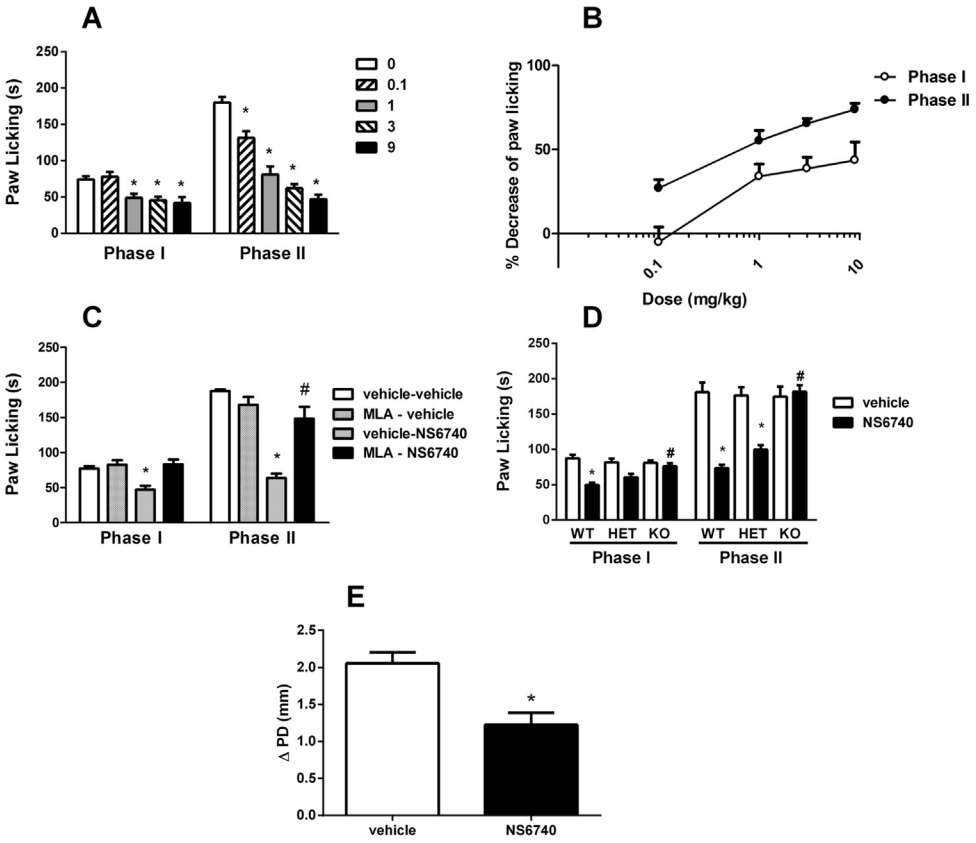

Fig. 4. The antinociceptive effects of NS6740 in the formalin test. (A) The effect after i.p. administration of various doses of NS6740 (0.1, 1, 3, and 9 mg/kg) on formalin-induced pain behavior in the mouse. Mice were treated with i.p. NS6740 15 min prior to formalin (2.5%, 20 μl) injection into the plantar region of the right hind paw. The cumulative pain response of time of licking was measured during the period of 0–5 min (first phase) and 20–40 min (second phase). **(B)** The antinociceptive effects of NS6740 in phases I and II of the formalin test presented as % decrease of paw licking behavior to compare the activity of vehicle group. **(C)** Blockade of the antinociceptive effect of NS6740 in the formalin test by the α7 antagonist MLA. MLA (10 mg/kg, s.c.) was given 15 min before an active dose of 3 mg/kg of NS6740 or vehicle. Fifteen min later, mice were injected with formalin and then observed for pain behaviors. **(D)** Antinociceptive effects of NS6740 in the α7 WT, HET, and KO mice. Mice received a dose of 3 mg/kg of NS6740 and 15 min later were tested in the formalin test. **(E)** Anti-edema effect of NS6740 (9 mg/kg) in the formalin test, as measured by the difference in the ipsilateral paw diameter before and after injection (ΔPD), in α5 WT and KO mice 1 h after intraplantar injection of 2.5% formalin. Data are given as the mean ± S.E.M. of 6–10 animals for each group. $^{*}p < 0.05$, significantly different from its vehicle group; $^{\#}p < 0.05$, significantly different from NS6740 treated group.

To determine whether the effects of NS6740 in the formalin and CCI tests are not due to disruption of locomotor activity and coordination during testing, we evaluated the effect of antinociceptive doses of NS6740 on motor coordination of mice. As seen in Table 2, mice treated the highest doses of NS6740 in this study (6 and 9 mg/kg; i.p.) did not show significant changes in motor coordination [$F_{(2,15)} = 0.6691$, $P = 0.5268$] in any time-point after testing.

4. Discussion

The identification of α7 nAChRs as a potential therapeutic target for several diseases, including pain and inflammation (de Jonge and Ulloa, 2007; Medhurst et al., 2008; Munro et al., 2012), has stimulated the development of many α7 nAChR-selective drugs. The present study investigated the in vitro and in vivo pharmacological properties of NS6740, an α7 nAChR silent agonist. Our results show for the first time that NS6740 was effective in mouse models of tonic and chronic pain via α7 nAChR mechanisms. In addition, our in vitro data suggest that the analgesic-like activity of NS6740 was not mediated by α7 nAChR ion channel activation.

NS6740 is an interesting ligand, having multiple modes of activity that are dependent on its concentration and duration of application. By itself, NS6740 does not significantly activate α7 ion channels, yet it alters the receptor's conformational state, as evidenced by the potentiated currents observed in PAM co-application experiments. When co-applied with a type II PAM like PNU 120596, NS6740 only evokes ion currents in the nM to low μM range; however, at concentrations greater than 30 μM, even a short application of NS6740 will desensitize receptors without allowing recovery from desensitization for several minutes. The time- and concentration-dependent effects of NS6740 further support the idea that there are multiple forms of receptor desensitization (Williams et al., 2011), and that molecules like NS6740 are unique tools that can be used to identify functionally significant receptor conformations. Furthermore, our data demonstrate that NS6740 is selective for α7 receptors. We provide electrophysiological evidence that NS6740 does not affect common heteromeric nAChRs, while the effects of NS6740 in pain models are blocked by the α7-selective ligand MLA and are not observed in α7 knockout mice.

The analgesic activity of NS6740 was assessed through various acute and chronic mouse pain models including chemical, mechanical, and thermal nociception assays, as well as chronic inflammatory and neuropathic pain models. Our results show that NS6740 is not active in acute thermal pain (hot-plate and tail-flick) and mechanical sensitivity tests after systemic administration. However, NS6740 attenuated pain behavior in the early and late phases of the formalin test, an inflammatory tonic pain model. The test 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

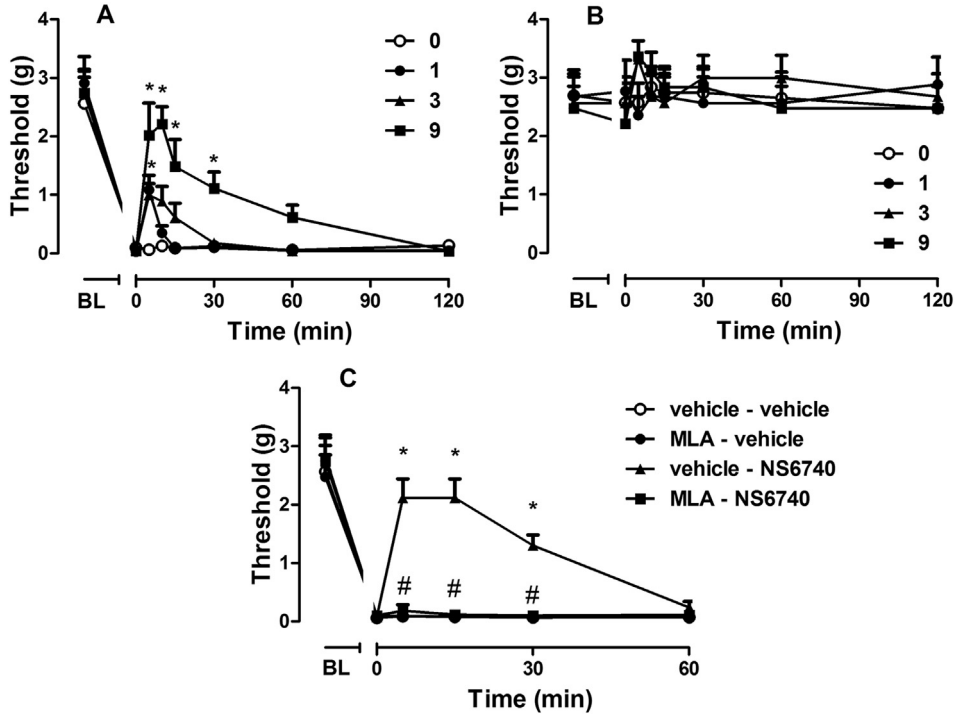

Fig. 5. The antiallodynic effects of NS6740 in chronic constrictive injury (CCI) neuropathic pain model. Two weeks after chronic constriction injury, mice were pretreated with either vehicle or NS6740 and then tested 0.5, 1, 1.5, and 2 h later, in the mechanical allodynia test. **(A)** NS6740 (1, 3, and 9 mg/kg, i.p.) significantly attenuated CCI-induced mechanical allodynia in a dose-related manner. **(B)** Mechanical sensitivity in sham mice did not differ between different doses of NS6740 (1, 3, and 9 mg/kg, i.p.) and vehicle treatments. **(C)** Blockade of the antiallodynic effect of NS6740 in the CCI test by the α7 antagonist MLA. MLA (10 mg/kg, s.c.) was given 15 min before an active dose of 9 mg/kg of NS6740 or vehicle. Data are expressed as the mean ± S.E.M. of 5–7 animals for each group. $^{*}p < 0.05$ significantly different from vehicle group; $^{\#}p < 0.05$ significantly different from NS6740-treated group.

Table 1
Effects of NS6740 in A) the tail-flick (TF), and B) hot plate (HP) tests. The antinociceptive effects of NS6740 (9 mg/kg i.p.), expressed as % MPE, were measured at multiple time points (in min) after injection. Data were presented as latency (sec) of mean ± S.E.M. of 6 animals for each group.

Treatment	Time after injection (min)							
	15		30		45		60	
	TF	HP	TF	HP	TF	HP	TF	HP
Vehicle	5 ± 2	7 ± 4	4 ± 3	3 ± 2	2 ± 1	3 ± 2	5 ± 2	2 ± 1
NS6740	2 ± 1	4 ± 2	1 ± 1	4 ± 3	1 ± 1	14 ± 3	0 ± 0	4 ± 2

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 NS6740 was more potent and more efficacious in blocking phase II than phase I nociceptive behavior, suggesting that NS6740 acts both centrally and peripherally to reduce tonic pain. In addition, formalin-induced inflammation as seen in the degree of paw edema was attenuated by NS6740. The antinociceptive activity of NS6740 on formalin-induced inflammation may result at least in part from its anti-inflammatory effect. Furthermore, using both pharmacological (i.e. the α7 nAChR antagonist MLA) and genetic approaches (i.e. α7 KO mice), we confirmed that NS6740's effect in the early and late phases of the formalin test is mediated by α7 nAChRs. In addition to the effects in the formalin test, NS6740 was also effective in the chronic neuropathic pain (CCI) model. NS6740 dose-dependently reduced mechanical allodynia via α7 nAChRs. Furthermore, it had no antinociceptive effect in sham-operated mice, indicating that it was antinociceptive under pain conditions only. Finally, NS6740 blocked, in a dose-related manner, the noxious visceral stimulus induced by i.p. administration of acetic acid in the mouse.

Since the experience of pain has both sensory and emotional-affective dimensions, we evaluated the effects of NS6740 on aversion, an important affective component of pain, using a conditioned

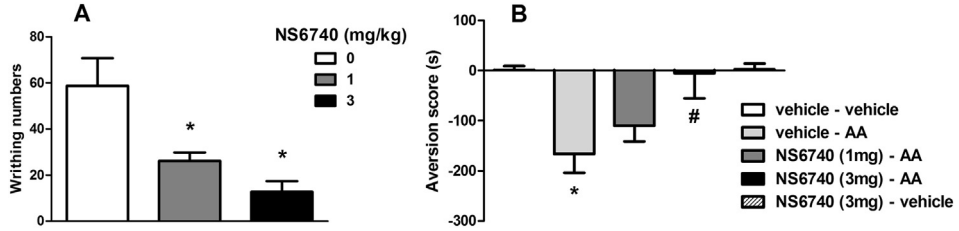

Fig. 6. Effects of NS6740 on acetic acid-induced stretching (A) and conditioned place aversion (B). A) Intraperitoneal injection of NS6740 (1 or 3 mg/kg) attenuated acetic acid-induced writhing behavior. **B)** NS6740 (1 or 3 mg/kg; i.p.) attenuated acetic acid-induced conditioned place aversion. Data are given as the mean ± S.E.M. of 6–11 animals for each group. $^{*}p < 0.05$, compared to the vehicle-injected mice; $^{\#}p < 0.05$, compared to the acetic acid-injected mice.

Table 2
Effects of NS6740 on motor coordination of mice. The effects of NS6740 in the rotarod test after administration in mice were evaluated 15 min after injection with either NS6740 or vehicle. Mice were placed on the rotarod for 3 min. Data were presented as mean ± S.E.M. % impairment of 6 animals for each group.

Treatment	Dose (mg/kg)	% Impairment
Vehicle	0	0 ± 0
NS6740	6	5 ± 5
NS6740	9	2 ± 1

place aversion (CPA) test (LaBuda and Fuchs, 2000; Johansen et al., 2001). In the present study, acetic acid i.p. injection induced an avoidance response to a pain-paired compartment compared to a saline injection, indicating that the acetic acid produced aversion. NS6740 significantly inhibited the display of CPA induced by acetic acid. The NS6740 inhibition of CPA was not the result of a reward effect since NS6740 alone does not induce conditioned place preference.

The in vivo efficacy of NS6740 in mouse models of chronic pain and inflammation is in contrast with its inactivity in a α7-sensitive cognitive improvement mouse model (Briggs et al., 2009). What are some potential explanations for the differential effects of this nicotinic ligand in cognitive vs. pain disorders? One explanation lies in the nature of the α7 nAChR itself. In contrast to neuronal cells, which are involved in cognitive and learning signaling, non-neuronal tissues, such as immune cells that are involved in inflammatory responses, have cells expressing α7 nAChRs that are not conductive in response to the endogenous ligand acetylcholine (Skok, 2009; our unpublished findings). These cells may have fundamentally different responses to α7 nAChR ligation, in terms of both receptor conformational dynamics and the intracellular signaling pathways that may by coupled to the α7 nAChR. The conformational states induced by NS6740 could have disparate signaling effects in different cell types, such that the cells involved in the inflammatory response, nociception, and pain, may produce different physiological responses than the neuronal cells involved in cognition. Finally, it is possible that poor penetrability into the brain may explain the lack of cognitive activity of NS6740 in the mouse. However, the fact that NS6740 blocked the effect of the effective full α7 agonist A-582941 in the same tests, suggest that NS6740 got into the brain and was competitive with A-582941.

The physiological effects of nicotinic ligands are manifestations of the conformational states that are induced and the context in which these interactions occur. Receptor activation and desensitization are both functionally important states, which primarily depend on ligand type, concentration and duration of activity. These studies show the preferential stabilization of different conformational states at specific ligand concentrations and time points, which could provide useful insight for therapeutic approaches with agents like NS6740. Efficacious agonists promoting ion currents through conductive receptor conformations typically cause positive enhancements in models of cognitive disease or deficit, although it is still not clear what components of these enhancements are attributed to conducting versus non-conducting states, as even activated receptors will eventually predominantly become desensitized. On the other hand, ligands that better stabilize non-conducting or desensitized conformational states show greater activity in models that involve peripheral mechanisms, such as pain and inflammation. These observations suggest there is a dichotomy for the therapeutic targeting of α7 receptors in cognitive vs. inflammatory disease, which may depend on the ligand's intrinsic ability to promote ion flux. NS6740 has differential behavior with respect to cognitive and pain mechanisms; though not effective in cognitive models (Briggs et al., 2009), it is active in these studies and in another report of peripheral inflammation (Thomsen and Mikkelsen, 2012). NS6740 selectively promotes α7 receptor desensitization over activation, suggesting that non-conducting ligand-bound conformational states are therapeutically favorable for peripheral pain and inflammatory disorders.

In conclusion, this study suggests that NS6740 and other α7 silent agonists represent novel therapeutic agents for the selective pharmacological targeting of α7 receptors in the treatment of chronic inflammatory and neuropathic pain. Further studies are needed to better understand the molecular framework of α7-mediated signaling events and how these processes may differ among cell types and organ systems.

Disclosures

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2014.12.002.

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