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## NS6740, an $\alpha 7$ nicotinic acetylcholine receptor silent agonist, disrupts hippocampal synaptic plasticity

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### Abstract

Long-term potentiation (LTP) in the dentate gyrus was previously shown to be enhanced by nicotine, an effect dependent on both homomeric  $\alpha 7$  and heteromeric  $\alpha 2\beta 2$  nicotinic acetylcholine receptors (nAChR). In our experiments, bath-applied nicotine produced no significant enhancement of LTP. The  $\alpha 7$  nAChR silent agonist NS6740, a weak activator of  $\alpha 7$  nAChR ion channels but an effective modulator of the cholinergic anti-inflammatory pathway, decreased LTP and, additionally, produced a substantial reduction in the baseline synaptic function prior to the high frequency stimulation used to induce LTP. The effects of NS6740 on the various ligand-gated ion channels associated with the generation and modulation of dentate LTP were evaluated with receptors expressed in *Xenopus* oocytes. A 60 s pre-application of 5  $\mu$ M NS6740 to  $\alpha 7$  receptors blocked the response to subsequent applications of acetylcholine (ACh). In contrast, the responses of  $\alpha 2\beta 2$  nAChR to control applications of ACh were not significantly affected by NS6740. Likewise, responses of cells expressing GluR1 + GluR2 AMPA- type glutamate receptor subunits or GABA<sub>A</sub>  $\alpha 1$ ,  $\beta 2$ , and  $\gamma 2L$  subunits to control agonist applications (100  $\mu$ M kainic acid or 10  $\mu$ M GABA, respectively), were unaffected by NS6740. The effects of NS6740 on  $\alpha 7$  were inconsistent with simple antagonism since, while unresponsive to ACh, the receptors exposed to NS6740 were effectively activated by the positive allosteric modulator PNU-120596. The results support the hypothesis that NS6740 switches the mode of  $\alpha 7$  signaling in a channel-independent manner that can reduce synaptic function.

### Keywords

Hippocampus; Dentate gyrus; LTP; Nicotinic acetylcholine receptors;  $\alpha 7$  Signaling; NS6740; PNU-120596

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## 1. Introduction

The  $\alpha 7$  subtype of nicotinic acetylcholine receptor (nAChR) has long been considered a potential therapeutic target for treating cognitive disorders, and  $\alpha 7$ -selective drugs have been shown to be effective at improving baseline or compromised behavior in animal models of learning and memory [5,13,27,45,51,53,55]. Drugs targeting  $\alpha 7$  have also been identified as effective at reducing inflammation and associated pain [43,47]. While  $\alpha 7$  channel activation appears to be important for cognitive effects [8], it does not seem to be important for the modulation of inflammation [4,26,49,54]. In fact, NS6740, which is a compound with good activity in models of inflammation and pain, is an extremely weak partial agonist of  $\alpha 7$  and is otherwise a strong de-sensitizer of  $\alpha 7$  channel activity [32]. NS6740 is, however, able to activate large  $\alpha 7$  ion channel currents in the presence of a positive allosteric modulator (PAM), identifying it as a “silent agonist”. The concept that there are two distinct types of  $\alpha 7$ -mediated signaling is supported by reports that NS6740 is not only ineffective in cognitive tests, it is able to block the effects of cognition-enhancing  $\alpha 7$  channel activators, for example, the effects of the  $\alpha 7$  agonist BMS-902483 on improving novel object recognition [44]. On its own, BMS-902483 has been also shown to increase hippocampal long-term potentiation (LTP), an effect hypothesized to be related to its positive cognitive effects.

LTP is arguably the most widely accepted neurophysiological correlate of learning and memory, involving long-lasting change in synaptic efficacy, which can be experimentally induced by high-frequency stimulation (HFS) of presynaptic axons. Mechanisms that have been shown to be important for LTP have also been demonstrated essential for spatial learning [28,41], fear conditioning [48], and passive avoidance learning [60]. LTP in the dentate gyrus of the hippocampus has been reported to be enhanced by acute application of nicotine [59]. This effect was reportedly blocked by methyllycaconitine (MLA) and absent in  $\alpha 7$ nAChR knockout mice. However, other nAChR, including heteromeric  $\alpha 2\beta 2$  receptors, have also been implicated in the induction and modulation of hippocampal LTP [30,31].

Here we report the effects of NS6740 on synaptic function and plasticity in the dentate gyrus of the rat hippocampus. Using heterologously expressed receptors, we investigated the effects of NS6740 on several ion channel receptors associated with synaptic function in the hippocampus, including important subtypes of AMPA-sensitive glutamate receptors,  $GABA_A$  receptors, and  $\alpha 2\beta 2$  subtypes of nAChR.

## 2. Methods and materials

### 2.1. Chemicals

NS6740 was supplied by Dr. Ganesh Thakur (Northeastern Univ., Boston MA). PNU-120596 was supplied by Professor Nicole Horenstein (University of Florida, Gainesville FL). All other chemicals and reagents were purchased from Sigma (St. Louis MO).

## 2.2. Animals

Procedures involving animal subjects have been reviewed and approved by the Institutional Animal Care and Use Committee and were in accordance with guidelines established by the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. Male Sprague-Dawley (SD) rats (p22-p31) were obtained from colony at Harlan Sprague Dawley Inc. through the University of Florida Animal Care and Service facility. Animals were maintained on a 12:12 h light schedule, and provided ad lib access to water.

## 2.3. LTP experiments

LTP experiments were carried out on transverse slices of Sprague-Dawley (SD) male rats (p22-p31) as previously described [59]. Rats were anesthetized with isoflurane (Halocarbon Laboratories, River Edge, NJ) and swiftly decapitated. The brains were rapidly removed, and slices were cut at a thickness of 350  $\mu\text{m}$  using a vibratome (Pelco, Redding, CA) and placed in a beaker with oxygenated high  $\text{Mg}^{2+}$ /low  $\text{Ca}^{2+}$  ice-cold artificial cerebral spinal fluid (ACSF) containing (in mM) 124 NaCl, 2.5 KCl, 1.2  $\text{NaH}_2\text{PO}_4$ , 2.5  $\text{MgSO}_4$ , 1  $\text{CaCl}_2$ , 10 D-glucose, and 25.9  $\text{NaHCO}_3$ , at room temperature for at least 2 h. The slices were then transferred to a recording chamber for submerged slices and continuously superfused at a rate of 2 ml/min at 30-32  $^{\circ}\text{C}$ . The normal recording ACSF contained (in mM): 126 NaCl, 3 KCl, 1.2  $\text{NaH}_2\text{PO}_4$ , 25.9  $\text{NaHCO}_3$ , 1.5  $\text{MgSO}_4$ , 2.4  $\text{CaCl}_2$ , 11 D-glucose, and 0.1 picrotoxin to block  $\text{GABA}_A$ -mediated activity. Pre-synaptic stimulation was applied to the medial perforant pathway of the dentate gyrus using a concentric bipolar electrode, and field excitatory post-synaptic potentials (fEPSPs) were recorded at a frequency of 0.033 Hz from the middle one-third of the molecular layer of the dentate gyrus with a borosilicate glass electrode. The inner blade of the dentate gyrus was used in all studies. To ensure pathway specificity, paired-pulse stimuli were given (40 ms apart), and paired-pulse depression was used as the criterion to confirm correct placement in the medial perforant pathway. For all experiments, the amplitude of the test fEPSP was adjusted to 35% of the maximum. LTP was induced by HFS consisting of eight trains (each of eight stimuli at 200 Hz, intertrain interval 2 s) with the stimulation current increased during the HFS to elicit the maximum fEPSP amplitude. Nicotinic drugs were bath-applied for 10 min prior to HFS.

Recordings were performed using a MultiClamp 700A amplifier (Molecular Devices, Union City CA), digitized using a Digidata 1322A (Molecular Devices), and analyzed using Clampfit 10.3 (Molecular Devices). Values are the means  $\pm$  SEM for n slices. All data were normalized to the averaged fEPSP slope for 5 min prior to HFS. Recordings were only performed in slices with the maximum fEPSP amplitude larger than 1 mV. An LTP (+) slice is defined if the fEPSP slope was  $> 120\%$  of the baseline at 60 min after HFS. All data were normalized to the averaged fEPSP slope between 30 min and 10 min prior to HFS. Values are the means  $\pm$  SEM for n slices. Two-tailed unpaired student's *t*-tests were done using Excel (Microsoft).  $P < 0.05$  is considered statistically significant. Comparisons were made based on the fEPSP ratios between the average of the last 5 response before the HFS and either the first 5 responses after the HFS (early LTP) or the last 5 responses of the recordings (55-60 min after the HFS, late LTP).

## 2.4. Heterologous expression of ligand-gated channels in *Xenopus laevis* oocytes

The human  $\alpha 7$  nAChR clone was obtained from Dr. J. Lindstrom (University of Pennsylvania, Philadelphia PA), and the human  $\alpha 2$  and  $\beta 2$  clones and concatamer were a gift from Dr. Ed Johnson (Astra Zeneca, Boston MA). Rat GluR1 and GluR2 clones were provided by Michael Hollmann and Steve Heinemann (Salk Institute, La Jolla CA), and rat GABA<sub>A</sub>  $\alpha 1$ ,  $\beta 2$ , and  $\gamma 1L$  subunit clones were provided by Dr. David Weiss (Univ. Texas, San Antonio TX). The human resistance-to- cholinesterase 3 (RIC-3) clone was provided by Millet Treinin (Hebrew University, Jerusalem, Israel) and co-injected with  $\alpha 7$  to improve the level and speed of  $\alpha 7$  receptor expression without affecting the pharmacological properties of the receptors [19]. Subsequent to linearization and purification of the plasmid cDNAs, cRNAs were prepared using the mMessage mMachine in vitro RNA transcription 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 [37]. 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 1.51 frog tank water containing 1 g of 3-aminobenzoate methanesulfonate buffered with sodium bicarbonate. The harvested oocytes were treated with 1.4 mg/ml collagenase (Worthington Bio-chemicals, Freehold, NJ) for 3 h at room temperature in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM NaHCO<sub>3</sub>, 0.82 mM MgSO<sub>4</sub>, 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. Oocytes were maintained in Barth's solution with calcium and recordings were carried out 2-11 days after injection.

Experiments were conducted using OpusXpress 6000A (Molecular Devices, Union City, CA) [37]. 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<sub>2</sub>, 10 mM HEPES, and 1  $\mu$ M atropine, pH 7.2) at 2 ml/min for  $\alpha 7$  nAChR and at 4 ml/min for the other receptors tested. To evaluate the effects of NS6740 on the various receptor sub-types expressed in oocytes, control responses were defined as the average of two initial applications of a reference agonist made before NS6740 applications. The solutions were applied from a 96-well plate via disposable tips. 400  $\mu$ l drug applications were 12 s in duration followed by a 181 s washout period for  $\alpha 7$  nAChR and 6 s in duration followed by a 241 s washout period for the heteromeric receptors. The reference agonist for the nAChR was acetylcholine (ACh). The ACh concentration was 60  $\mu$ M for the  $\alpha 7$  receptors. Note that  $\alpha 2\beta 2$  receptors can configure into two different stoichiometries, with two  $\alpha$  subunits and three  $\beta$  subunits or the reverse. The use of an  $\alpha - \beta$  concatamer expressed with either  $\beta$  or  $\alpha$  subunit monomers allows for the controlled expression of either  $\alpha 2(2)\beta 2(3)$  or  $\alpha 2(3)\beta 2(2)$  configurations, referred to as high sensitivity (HS) or low sensitivity (LS) forms, respectively [39]. The  $\alpha 2\beta 2$  experiment was run on four cells of each configuration. The ACh control was 10  $\mu$ M for the HS-expressing cells ( $n = 4$ ) and 100  $\mu$ M for the LS-expressing cells ( $n = 4$ ). Since the normalized responses were equivalent, the data for the two  $\alpha 2\beta 2$  types were pooled. The reference agonist for the cells

expressing GluR1 and GluR2 was 100  $\mu$ M kanic acid, and 10  $\mu$ M GABA was used for the cells expressing the  $\alpha$ 1,  $\beta$ 2, and  $\gamma$ 1L subunits. The oocyte responses were calculated as both peak current amplitudes and net charge, as previously described [36], and the average of the two initial controls were used for normalization purposes. Net charge responses were used for  $\alpha$ 7 [36] and peak currents for the other receptors studied.

Data were collected at 50 Hz, filtered at 20 Hz ( $\alpha$ 7) or 5 Hz (heteromeric), and analyzed by Clampfit 9.2 or 10.0 (Molecular Devices) and Excel (Microsoft, Redmond, WA). Data were expressed as means  $\pm$  SEM from at least seven oocytes for each experiment and plotted by Kaleidagraph 4.5.2 (Abelbeck Software, Reading, PA). Multi-cell averages were calculated for comparisons of complex responses. Averages of the normalized data were calculated for each of the 10,322 points in each of the 206.44 s traces (acquired at 50 Hz), as well as the standard errors for those averages.

### 3. Results

#### 3.1. Nicotine effects on LTP

We hypothesized that the nicotinic modulation of LTP in the dentate gyrus would be most effectively facilitated by  $\alpha$ 7 nAChR-selective ligands that are efficacious for ion channel activation. We first established the control LTP data in standard ACSF containing 100  $\mu$ M picrotoxin to suppress the GABA<sub>A</sub>-mediated inhibition in the dentate gyrus. The HFS-induced control LTP attained a peak amplitude of  $180 \pm 4\%$  over baseline immediately following the stimulation and declined to stable potentiation of  $143 \pm 6\%$  at 60 min following HFS ( $n = 6$ , Fig. 1A & C). The effect of nicotine on LTP was then investigated by bath perfusion of 5 or 10  $\mu$ M nicotine prior to HFS for 10 min. Bath application of nicotine had no effect on the baseline synaptic response. Although neither 5  $\mu$ M nor 10  $\mu$ M nicotine significantly increased LTP over the control values, visual inspection of the data suggest slight increases (Fig. 1A and B). Early potentiation was  $184 \pm 4\%$  and  $201 \pm 6\%$  in the presence of 5 and 10  $\mu$ M nicotine, respectively, and late potentiation was  $167 \pm 13\%$  and  $153 \pm 8\%$  in the presence of 5 and 10  $\mu$ M nicotine, respectively.

Previous studies reported the presence of high expression levels of  $\alpha$ 7 nAChRs in the rat dentate gyrus [16]. In the present study, to explore the role of  $\alpha$ 7 nAChR on the effects of nicotine on LTP in the dentate gyrus we used the  $\alpha$ 7 nAChR competitive antagonist, MLA co-applied with 10  $\mu$ M nicotine (Fig. 2A). MLA at 100  $\mu$ M, a concentration known to be selective for  $\alpha$ 7, has previously been reported to eliminate the nicotine enhancement of LTP [59]. In our experiments bath co-application of nicotine and MLA had no effect on the baseline fEPSP. Although a visual inspection of the data (Fig. 2A) suggests a small effect of MLA compared to the results with 10  $\mu$ M nicotine alone, the results were not statistically significant, probably due to the fact that we could not reproduce the previously reported robust effects of nicotine [59]. With the addition of 100 nM MLA to 10  $\mu$ M nicotine there was  $186 \pm 7\%$  early potentiation and  $145 \pm 7\%$  late potentiation.

The involvement of  $\alpha$ 7 nAChR in the dentate gyrus LTP was further investigated using the  $\alpha$ 7 silent agonist NS6740 [32] (Fig. 2B). Unexpectedly, bath application of 5  $\mu$ M NS6740 for 10 min prior to HFS demonstrated a significant ( $p < 0.001$ ) inhibitory effect on the base-

line fEPSP, reducing response amplitude by  $34 \pm 6\%$  during the 10 min incubation period (see insert Fig. 2B). The addition of  $5 \mu\text{M}$  NS6740 also significantly reduced early potentiation compared to controls ( $P < 0.05$ ) ( $148 \pm 8\%$ ) and this decayed to  $124 \pm 6\%$  after 60 min.

### 3.2. NS6740 effects on specific ligand-gated ion channels expressed in *Xenopus* oocytes

NS6740 is an  $\alpha 7$ -selective partial agonist that is a poor channel activator but particularly effective at producing long-lived desensitization [8,32,38] that can be partially reversed with PAMs. In order to confirm that the effects of NS6740 on reducing hippocampal excitatory transmission and LTP were due to  $\alpha 7$  nAChR, we tested its effects on  $\alpha 7$  and other channel receptors involved in hippocampal transmission expressed in *Xenopus* oocytes. As shown in Fig. 3A, a 60 s pre-application of  $5 \mu\text{M}$  NS6740 suppressed  $\alpha 7$  response to a following ACh application. The receptors remained desensitized and showed minimal response to two follow-up ACh applications at four-minute intervals. We have previously shown that  $\alpha 4\beta 2$  nAChR are insensitive to NS6740 activation and desensitization [32]; however,  $\alpha 2\beta 2$  nAChR have also been implicated in the nicotinic modulation of hippocampal LTP [30,31], so oocytes expressing high-or low-sensitivity  $\alpha 2\beta 2$  receptors [39] (see Methods) were tested. We also tested oocytes expressing AMPA-type glutamate receptors (GluR1 co-expressed with GluR2) [20], and GABA<sub>A</sub> receptors [10]. Using a protocol like that described for the  $\alpha 7$ -expressing cells shown in Fig. 3A, we determined that NS6740 had no significant effects on the agonist-evoked responses of any of these other receptor subtypes.

MLA, the selective  $\alpha 7$  antagonist, decreased the effects of nicotine on LTP but did not affect baseline synaptic function, at least not in the presence of nicotine. Therefore, we used the same protocol that demonstrated persistent desensitization of  $\alpha 7$  by NS6740 to determine the magnitude and duration of the effects of 100 nM MLA on  $\alpha 7$  expressed in oocytes. While a 60 s pre-application of MLA blocked the response to an initial ACh application, there was a progressive recovery of ACh responses to subsequent applications (Fig. 4A), such that the net-charge response [36] to the third ACh application shown was back up to  $75 \pm 5\%$  of the original controls. As shown in Fig. 4B, when the receptors were recovering from the MLA inhibition, they were insensitive to the allosteric modulator PNU-120596. This was in great contrast to receptors desensitized by a 60 s exposure to  $5 \mu\text{M}$  NS6740 (Fig. 4C). Under conditions when ACh responses were only  $12.6 \pm 3\%$  initial controls (Fig. 3A), an application of  $10 \mu\text{M}$  PNU-120596 alone evoked responses that were  $710 \pm 86\%$  the size of initial ACh controls. Note that the data in Fig. 4 are not “representative traces” but actual calculated multi-cell averages ( $\pm$  SEM).

In the slice experiments (Fig. 1B), 100 nM MLA was present throughout the entire nicotine treatment and should have effectively antagonized  $\alpha 7$  function without inducing desensitization. In contrast, while NS6740 would have reduced  $\alpha 7$  channel activation, it would have induced other, potentially important, conformational states [32,38].

## 4. Discussion

Our experimental design was based the methods described by Welsby et al. [59], who previously reported that 5  $\mu$ M nicotine produced a robust enhancement of dentate LTP, with an early potentiation of 250% compared to a control of 200%, and increase at 60 min after HFS of 182% compared to 143% in the control slices. Although we saw significant LTP under all conditions ( $P < 0.05$ ), we observed lower levels of LTP under control conditions and no significant effects of 5 or 10  $\mu$ M nicotine compared to controls. It should be noted that nicotine is a very non-selective agonist and at concentrations greater than 1  $\mu$ M would predominantly desensitize all heteromeric nAChR [40]. Like-wise, at such concentrations nicotine would produce only very transient activation of  $\alpha 7$  nAChR and virtually no steady-state currents [35,40].

Although our data failed to reproduce the robust enhancement of LTP in the rat dentate with nicotine that was previously reported, our results with NS6740 suggest a more fundamental role for  $\alpha 7$  receptor signaling in modulation of synaptic tone in the LTP pathway. The discovery of the cholinergic anti-inflammatory pathway (CAP) created an entirely new perspective on how  $\alpha 7$  nAChR can function and be targeted for therapeutic effects [58]. ACh released by strong electrical stimulation of the vagus nerve was shown to stimulate an anti-inflammatory response mediated by  $\alpha 7$  nAChR on macrophages and other cells of the immune system [6,47,57]. Interestingly, GTS-21, a selective but relatively weak partial agonist for  $\alpha 7$  nAChR [7] has been shown in many studies to be an effective activator of CAP [9,11,22,25,46,52,62,63]. In contrast to being a weak activator of  $\alpha 7$  ion channels, GTS-21 effectively induces prolonged desensitization of receptors that is reversible by the type II PAM PNU-120596 [35]. NS6740, like GTS-21, induces prolonged desensitization that is reversible by PNU-120596, while stimulating so little channel activation that it has been characterized as a “silent agonist” [12,32,33]. Both GTS-21 and NS6740 were shown to effectively suppress lipopolysaccharide (LPS) stimulated secretion of Tumor Necrosis Factor-alpha (TNF- $\alpha$ ) by microglia cells, an in vitro model of CAP [52]. The effects of PNU-120596 in the oocyte experiments indicate that NS6740 may affect other receptor functions such as CAP (Cholinergic Anti-inflammatory Pathway), consistent with the activity of this agent as modulatory of neuropathic pain [32].

Prior to work of Thomsen and Mikkelsen that demonstrated the CAP activity of NS6740, the drug first appeared in the scientific literature as a putatively inactive  $\alpha 7$  agonist that was able to block the cognitive effects of an efficacious  $\alpha 7$  agonist [8]. NS6740 was also recently reported to similarly antagonize the effects of BMS-902483 in a novel object recognition test [44]. In the same work, BMS-902483 was shown to enhance hippocampal LTP, an activity presumably related to cognitive enhancement. In the present study, we show that NS6740 does not behave as a simple antagonist of  $\alpha 7$  but, rather, alters the fundamental behavior of  $\alpha 7$  nAChR in the hippocampus without having effects on other types of ion channel receptors. NS6740 then appears as a key to altering the modality of  $\alpha 7$ -mediated signaling. The activity of GTS-21, NS6740, other  $\alpha 7$  silent agonists, and allosteric modulators [1,15,17,32,34,56] in models of inflammatory disease and neuropathic pain appear to be independent of ion channel activation and, rather, depend on activation and modulation of intracellular signal transduction pathways [4,14,26,49,54]. In the case of the  $\alpha 7$ -mediated

control of CAP, the cellular mediators of activity are themselves not even competent for generating nAChR channel currents, and so we must consider the complete receptor protein [50], multiple conformational states, and the complete receptor interactome [29,42] as mediators of the activity.

As we show, NS6740 does not behave like a simple receptor an-agonist but, rather, is putting receptors into conformational states consistent with the modulation of intracellular signal transduction. There are a number of possible ways that  $\alpha 7$ -mediated signaling events might be able to alter basal synaptic function, for example, through changes in presynaptic calcium homeostasis or, alternatively, by post-synaptic changes in glutamate receptor trafficking. In any case, the present study challenges the naive assumption that all  $\alpha 7$  ligands work by increasing intracellular calcium through ion channel activation. For most therapeutic/in vivo models this mode of action seems hardly even feasible since, in the absence of PAMs,  $\alpha 7$  nAChR have extremely low probability of channel opening [61] and are especially ineffective at generating currents when agonist concentrations change slowly, as they would in the brain following a subcutaneous [44] or intraperitoneal injection [8]. It seems likely that the slice experiments NS6740 is down- regulating constitutive  $\alpha 7$  ion channel receptor tone reducing tonic pre- synaptic facilitation [18].

The  $\alpha 7$  nAChR has been considered an interesting potential target for numerous therapeutic indications over the last two decades, with GTS-21 one of the first candidates proposed for Alzheimer's disease [2,3,23]. Unfortunately, although many  $\alpha 7$ -selective agonists have been developed [21], none have made it successfully through clinical trials for cognitive disorders, although small-scale trials continue with GTS-21 and schizophrenia [24]. Appreciation for the potential importance of the cholinergic anti-inflammatory pathway as an avenue for treating diverse indications such as asthma, arthritis, and neuropathic pain has redirected the field to search for different kinds of drugs for these different sorts of diseases, and it may be that attention will shift toward drugs like NS6740 that were once dismissed as an "anti-therapeutic" for cognition.

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## Abbreviations:

<b>ACh</b>	acetylcholine
<b>ACSF</b>	artificial cerebral spinal fluid
<b>CAP</b>	cholinergic anti-inflammatory pathway
<b>fEPSP</b>	field excitatory post-synaptic potentials
<b>HFS</b>	High-frequency stimulation
<b>HS</b>	high sensitivity



<b>MLA</b>	methyllycaconitine
<b>LTP</b>	long-term potentiation
<b>LS</b>	low sensitivity
<b>nAChR</b>	nicotinic acetylcholine receptors
<b>PAM</b>	positive allosteric modulator
<b>SD</b>	Sprague-Dawley
<b>TNF-<math>\alpha</math></b>	tumor necrosis factor-alpha

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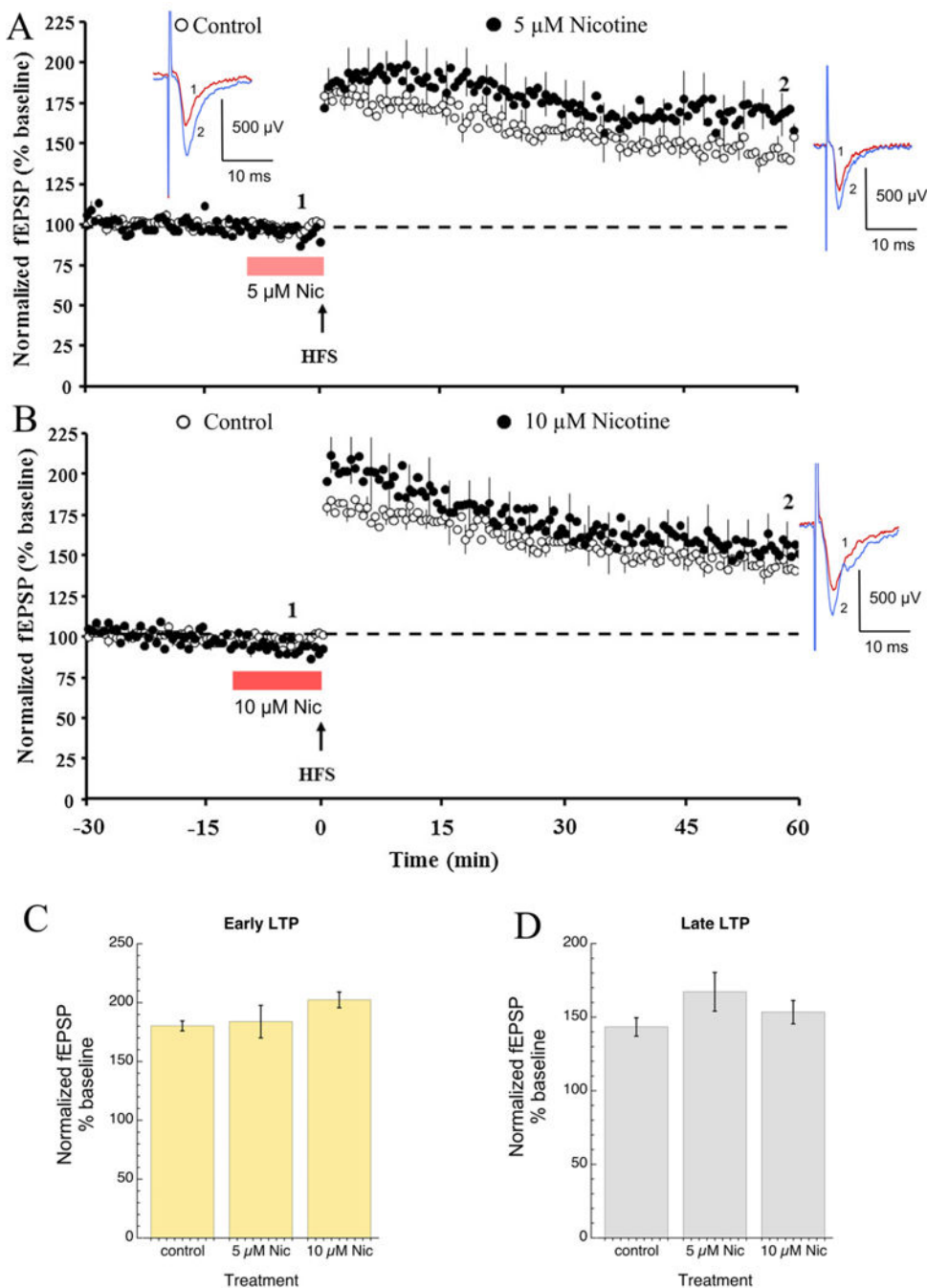
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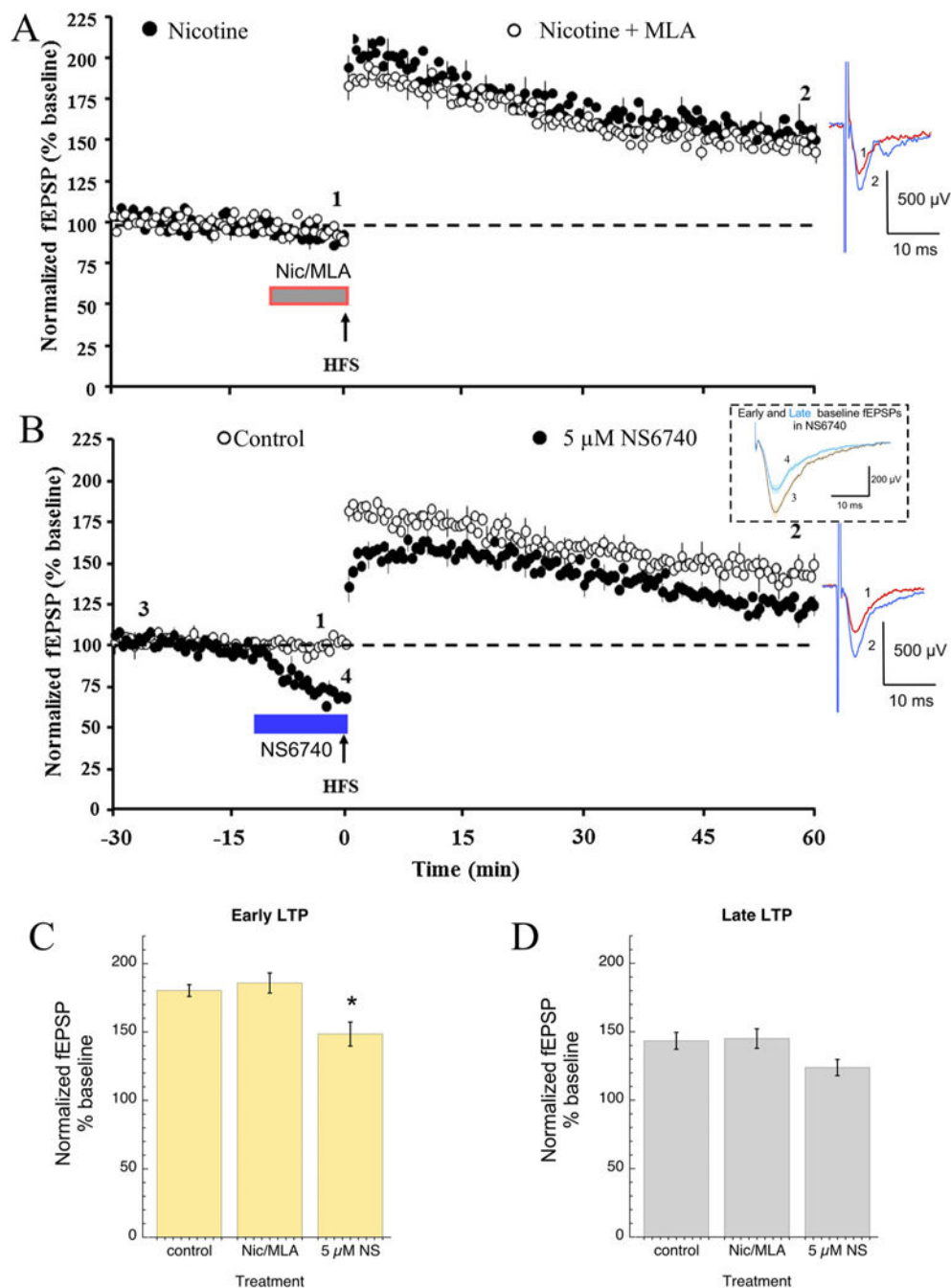
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**Fig. 1.** The nonselective nicotinic acetylcholine receptor (nAChR) agonist nicotine showed modest effects on long-term potentiation (LTP) induced by high frequency stimulation (HFS) in the dentate gyrus of hippocampal slices obtained from rats. **A)** The time course of the field EPSP (fEPSP) measurements obtained from dentate gyrus 30min before and 60min after HFS under the control condition (open circle, n = 6) and following the application of 5  $\mu$ M nicotine (filled circles, n = 7). **B)** The time course of the field EPSP (fEPSP) measurements obtained from dentate gyrus 30 min before and 60 min after HFS under the control condition

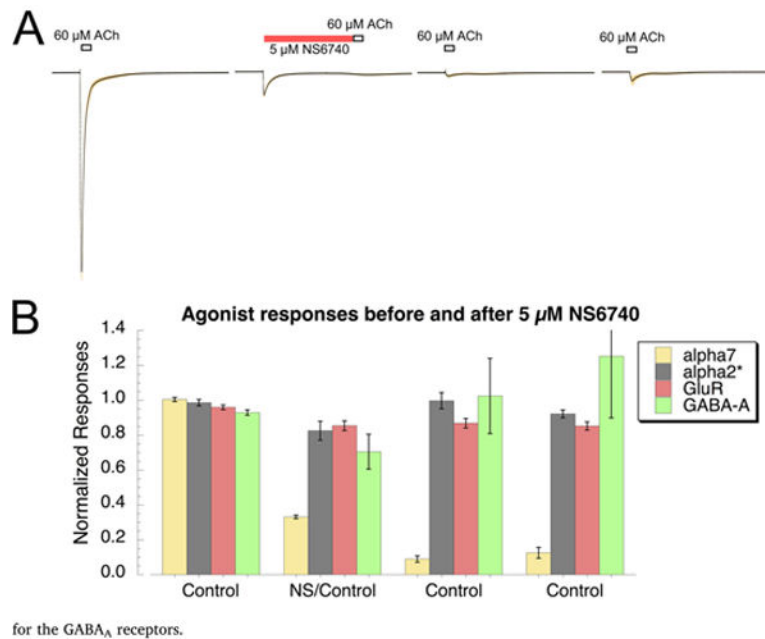
(open circle,  $n = 6$ ) and after the application of  $10 \mu\text{M}$  nicotine (filled circle,  $n = 7$ ). For both experiments, LTP was induced by HFS consisting of eight trains (each of eight stimuli at  $200 \text{ Hz}$ , inter-train interval  $2 \text{ s}$ , indicated by the arrow) with the stimulation current increased during the HFS to elicit the maximum fEPSP amplitude. The periods of nicotine bath application began  $10 \text{ min}$  prior to the HFS, as indicated by the bars. Bath applications of nicotine had no effect on the baseline synaptic response. Shown are representative fEPSP traces at the time points (1 and 2) indicated in the time course for baseline (blue) and  $60 \text{ min}$  following HFS (red). Each trace is an average of 5 consecutive traces before and after HFS under either control conditions or with  $5 \mu\text{M}$  or  $10 \mu\text{M}$  nicotine as indicated. **C)** Bar charts of the average ( $\pm \text{SEM}$ ) early (first 5 responses after HFS) and late ( $55\text{-}60 \text{ min}$  after HFS) potentiation observed under the three conditions shown in panels A & B, left and right graphs, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



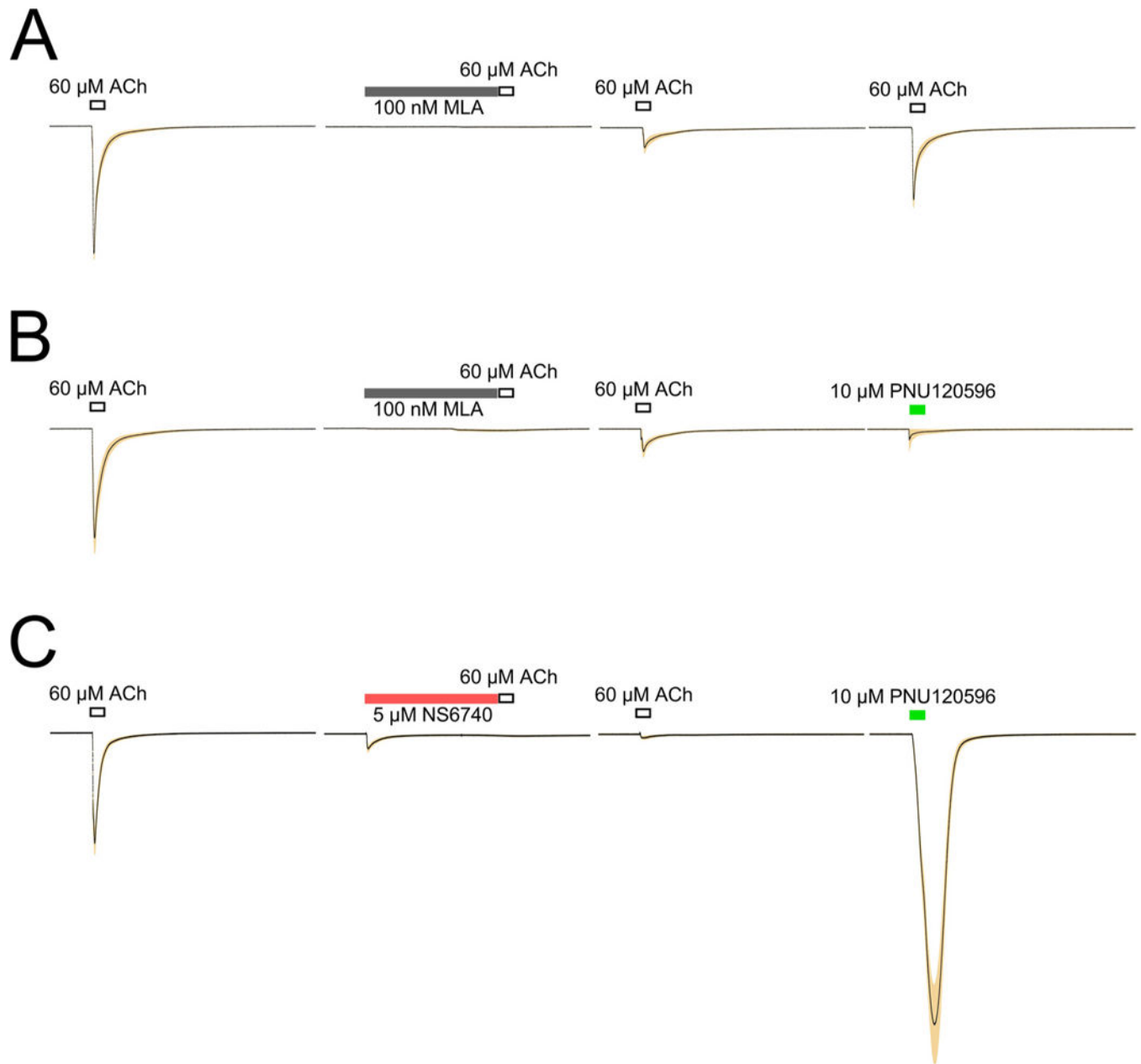
**Fig. 2.** **A)** Effects of the selective  $\alpha 7$  nAChR antagonist MLA co-applied with 10  $\mu$ M nicotine on LTP in the dentate gyrus of hippocampal slices obtained from rats. Data from 1 B with bath applied 10  $\mu$ M nicotine are shown for comparison, (open circles,  $n = 7$ ) along with data when 10  $\mu$ M nicotine was co-applied with 100nM MLA (filled circle,  $n = 7$ ). LTP was induced by HFS (indicated by the arrow). Bath co-application of nicotine and MLA had no effect on the baseline synaptic response and LTP after the HFS was slightly reduced to the level seen on control data (Fig. 1). **B)** The  $\alpha 7$  nAChR silent agonist NS6740 significantly



attenuated LTP that was induced by HFS in the dentate gyrus of hippocampal slices obtained from rats. The main panel shows the time course of the fEPSP measurements obtained from dentate gyrus 30 min before and 60 min after HFS under control condition (control data from 1A above shown for comparison, open circle,  $n = 6$ ) and in the presence of  $5 \mu\text{M}$  NS6740 (filled circle,  $n = 6$ ). The insert shows the averaged fEPSP responses of slices ( $n = 6$ ) at the beginning (time point 3, black line) and at the end of the baseline period in NS6740 (time point 4, blue line). Shaded areas are the standard errors of the mean calculated for the point-by-point averages. For both experiments, LTP was induced by HFS (indicated by the arrow). The periods of drug application began 10min prior to the HFS, as indicated by the bars. Bath application of NS6740 ( $n = 6$ ) both reduced the baseline synaptic response (time points 3 and 4) and decreased the LTP produced by HFS. Also shown are representative field EPSP (fEPSP) traces at the time points (1 and 2) indicated in the time course for baseline (blue) and 60 min following HFS (red). Each trace is an average of 5 consecutive traces before and after HFS. C) Bar charts of the average ( $\pm$  SEM) early (first 5 responses after HFS) and late (55-60min after HFS) potentiation observed under the three conditions shown in panels A & B, left and right graphs, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 3.** NS6740 selectively modulated  $\alpha 7$  nAChR ligand-gated channels. **A**) Control applications of 60 ACh were delivered to oo-cytes expressing  $\alpha 7$  ( $n = 8$ , second of two ACh controls shown). Subsequently, 5  $\mu$ M NS6740 was applied for 60 s prior to another application of ACh. This was followed by two additional control applications of ACh at four-minute intervals. The data shown are the point-by-point averages, (solid line) of the data from each cell after they were normalized to the amplitude of the initial ACh responses from each cell. The standard errors of the mean at each point are plotted as the shaded area. **B**) Responses of cells expressing  $\alpha 7$  prior to and following the application of 5  $\mu$ M NS6740 were compared to those of other receptors important for hippocampal physiology:  $\alpha 2\beta 2$  nAChR, AMPA-type glutamate receptors (cells co-expressing GluR1 and GluR2), and a representative GABA<sub>A</sub> receptor (cells co-expressing  $\alpha 1$ ,  $\beta 2$ , and  $\gamma 1L$  subunits) using the same application protocol illustrated in A. The  $\alpha 7$  responses were calculated as net charge and the others as peak currents. ACh control concentrations used were 10  $\mu$ M and 100  $\mu$ M, respectively for the high sensitivity and low sensitivity forms of the  $\alpha 2\beta 2$  receptors (see Methods), 100  $\mu$ M kainic acid was the control for the glutamate receptors, and 10  $\mu$ M GABA



**Fig. 4.**

**A)** Responses of oocytes expressing  $\alpha 7$  ( $n = 8$ ) showing the effect of 100nM MLA on  $\alpha 7$  receptor response to 60  $\mu\text{M}$  ACh application. After the acquisition of control responses to ACh (the second of two controls is shown), MLA was applied for 60s prior to a second application of ACh. This was followed by two additional control applications of ACh at four- minute intervals. **B)** Responses of oocytes expressing  $\alpha 7$  ( $n = 8$ ). After the acquisition of control responses to ACh, nM MLA was applied for 60 s prior to a second application of ACh. This was followed by an application of 10  $\mu\text{M}$  PNU-12096, a PAM of  $\alpha 7$  nAChR. **C)** Responses of oocytes expressing  $\alpha 7$  ( $n = 8$ ) showing that the effect of NS6740 on the  $\alpha 7$  receptors was distinct from simple inhibition produced by MLA, as shown by the ability of the PAM PNU-120596 to evoke large responses from the receptors that were unresponsive to

ACh. The data in each trace are the point-by-point averages (solid line) of the data from each cell after they were normalized to the amplitude of the initial ACh responses from each cell. The standard errors of the mean at each point are plotted as the shaded area.