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In vitro and in vivo characterization of a novel negative allosteric modulator of neuronal nAChRs

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Abstract

In this study, we compared the *in vitro* and *in vivo* neuronal nicotinic acetylcholine receptor (nAChR) properties of 1,2,3,3a,4,8b-hexahydro-2-benzyl-6-*N,N*-dimethylamino-1-methylindeno[1,2,-b]pyrrole (HDMP, **4**) to that of negative allosteric modulator (NAM), PCP. Patch-clamp experiments showed that HDMP exhibited an inhibitory functional activity at $\alpha 7$ nAChRs with an IC_{50} of 0.07 μM , and was 357- and 414-fold less potent at $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChRs, with IC_{50} s of 25.1 and 29.0 μM , respectively. Control patch-clamp experiments showed that PCP inhibited $\alpha 7$, $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChRs with IC_{50} s of 1.3, 29.0 and 6.4 μM , respectively. Further, HDMP did not exhibit any appreciable binding affinity to either $\alpha 7$ or $\alpha 4\beta 2$ nAChRs, suggesting its action via a non-competitive mechanism at these neuronal nAChR subtypes. The *in vivo* study showed that HDMP was a potent antagonist of nicotine-induced analgesia in the tail-flick (AD_{50} = 0.008 mg/kg), but not in the hot-plate test. All together, our *in vitro* and *in vivo* data suggest that HDMP is a novel NAM of neuronal nAChRs with potent inhibitory activity at $\alpha 7$ nAChR subtype at concentrations $\leq 1 \mu M$ that are not effective for $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChRs.

Introduction

Neuronal nAChRs are ligand-gated ion channels that consist of various combinations of $\alpha 2$ - $\alpha 10$ and $\beta 2$ - $\beta 4$ subunits and are differently distributed in various regions of the mammalian nervous system with respect to their subunit composition (Changeux and Taly, 2008). The $\alpha 4\beta 2^*$ is a major nAChR subtype of the central nervous system (Flores et al., 1992; Whiting and Lindstrom, 1987), while $\alpha 3\beta 4^*$, though detected in some brain regions, predominates in the periphery (Flores et al., 1996; Quick et al., 1999) (* indicates the possible inclusion of unspecified subunit (Lukas et al., 1999)), and $\alpha 7$ nAChRs are evenly distributed in the central and peripheral regions of the nervous system (Dickinson et al., 2008; Genzen et al., 2001; Keath et al., 2007; Lips et al., 2006; Woollorton et al., 2003).

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By definition, the nAChR receptor binding site occupied by the endogenous ligand acetylcholine (ACh) is named the orthosteric site (Jensen et al., 2005). In addition to ACh, the agonists, nicotine (**1**) and epibatidine (**2**), and antagonist, dihydro- β -erythroidine (**3**) (Fig. 1), and other natural and synthetic competitive agonists and antagonists interact with this site. Similar to many other receptor systems, nAChRs have allosteric binding sites. Unlike compounds that bind to the orthosteric binding site, compounds that bind to allosteric binding sites have no intrinsic activity. The mode of action of these allosteric modulators is to enhance or inhibit the function of nAChRs *via* a non-competitive mechanism. Compounds that increase the response to the agonist are called positive allosteric modulators (PAMs) and those that reduce the response to agonist are called negative allosteric modulators (NAMs) (Bertrand and Gopalakrishnan, 2007; Edelstein and Changeux, 1998; Jensen et al., 2005).

Non-competitive inhibition of neuronal nAChRs have been reported for a number of compounds, including bupropion (Fryer and Lukas, 1999; Slemmer et al., 2000), mecamylamine (Chavez-Noriega et al., 1997), UCI-30002 (Yoshimura et al., 2007), and phencyclidine (PCP) (Connolly et al., 1992; Fryer and Lukas, 1999).

In this study, we report the *in vitro* and *in vivo* characterization of 1,2,3,3a,4,8b-hexahydro-2-benzyl-6-*N,N*-dimethylamino-1-methylindeno[1,2-*b*]pyrrole (HDMP, **4**), a **new** compound which has structural similarity to 2,3,3a,4,5,9b-hexahydro-1-methyl-1H-pyrrolo[3,2-*h*]isoquinoline (**5**) (Glassco et al., 1993) (Fig. 1). We also compared HDMP to another NAM of neuronal nAChRs, PCP. PCP was previously reported to inhibit nicotinic currents mediated by recombinant human $\alpha 3\beta 4$ (Fryer and Lukas, 1999) and mouse splice variant of $\alpha 4\beta 2$ ($\alpha 4$ - $2\beta 2$) nAChRs (Connolly et al., 1992) with IC_{50} values close to 10 μM . The effect of PCP on $\alpha 7$ nAChRs has not been yet evaluated in functional assays. We investigated the *in vitro* profile of HDMP and PCP on various neuronal nAChR subtypes. Specifically, the antagonistic activity of both compounds was assessed at recombinant $\alpha 4\beta 2$, $\alpha 3\beta 4$ and $\alpha 7$ nAChRs. The *in vitro* studies were complemented with the investigation of *in vivo* effects of HDMP and PCP on nicotine's actions in mice. In the present study, we tested the extent to which an acute systemic administration of HDMP and PCP alters nicotine-induced hypothermia and the antinociceptive activity of nicotine in the tail-flick and the hot-plate tests.

Results

In this study we tested the *in vivo* and *in vitro* effects of HDMP on neuronal nAChRs and compared the results to those of NAM of neuronal nAChRs, PCP, as a control (Connolly et al., 1992; Fryer and Lukas, 1999).

In vitro Studies

In order to examine the concentration-dependence and potency of the inhibitory effect of HDMP with respect to subunit composition of neuronal nAChRs, we tested its effect *in vitro* on three major neuronal nAChR subtypes, $\alpha 4\beta 2$, $\alpha 7$ and $\alpha 3\beta 4$, and compared the inhibitory potency of HDMP to that of PCP. To assess *in vitro* function, the cell under recording was exposed to the EC_{50} concentration of ACh, determined previously for each nAChR subtype (20 μM for human $\alpha 4\beta 2$ (Abdrakhmanova et al., 2006), 280 μM for rat $\alpha 7$ (Moaddel et al., 2008; Xiao et al., 2009) and 100 μM for rat $\alpha 3\beta 4$ nAChRs (Abdrakhmanova et al., 2006; Zhang et al., 1999), and 0–2 min later to ACh at the same concentration in the presence of various concentrations of HDMP or PCP. When the inhibitory effect of the tested compound was reversible, two more concentrations were tested on the same cell. Co-application of ACh and HDMP following pre-exposure to the compound showed that the inhibitory effect of HDMP develops gradually in 40–60 s.

HDMP exhibited comparable inhibitory potency at $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChRs with IC_{50} s of $25.06 \pm 6.37 \mu M$ ($nH \sim 1.15$; $n=3-4$) and $28.98 \pm 3.82 \mu M$ ($nH \sim 1.06$; $n=3-7$, specifically n was 3 for 0.1 μM and 1 μM , n was 4 for 40 μM and 100 μM , and n was 7 for 10 μM), respectively (Fig. 2 & Table 1). Most interestingly, HDMP inhibited the $\alpha 7$ nAChRs with $IC_{50} = 0.07 \pm 0.01 \mu M$ ($nH \sim 0.73$; $n=3-6$), being 357- and 414-fold more potent at $\alpha 7$ than at $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChRs, respectively.

The inhibitory potency of PCP was tested under similar experimental conditions on $\alpha 4\beta 2$, $\alpha 7$ and $\alpha 3\beta 4$ nAChRs (Fig. 3 & Table 1). The peak amplitude of ACh-induced currents was decreased by PCP 23-fold more effectively at $\alpha 7$ than at $\alpha 4\beta 2$ nAChRs with IC_{50} values of $1.25 \pm 0.26 \mu M$ ($nH \sim 0.97$; $n=3-4$) versus $28.97 \pm 6.38 \mu M$ ($nH \sim 1.4$; $n=3-5$), while the inhibitory potency of PCP in $\alpha 3\beta 4$ nAChRs was comparable to that at $\alpha 7$ nAChRs with IC_{50} of $6.35 \pm 1.55 \mu M$ ($nH \sim 0.65$; $n=4$). Taken together the results show that HDMP is a potent inhibitor of neuronal nAChRs with higher selectivity at $\alpha 7$ than at $\alpha 4\beta 2$ or $\alpha 3\beta 4$ nAChR subtypes as compared to PCP (Table 1).

In order to determine whether the inhibitory effect of HDMP in neuronal nAChRs arises upon its binding to the agonist binding site or to an allosteric site its binding affinities to $\alpha 4\beta 2$ and $\alpha 7$ nAChRs was determined using [3H]epibatidine and [^{125}I]MLA binding assays, respectively. HDMP possessed no appreciable affinity to [3H]epibatidine or [^{125}I]MLA binding sites ($K_i > 10,000$ nM) suggesting that the inhibitory effect of the compound on both $\alpha 4\beta 2$ and $\alpha 7$ nAChR subtypes occurs *via* a non-competitive allosteric mechanism. Experiments shown in Figure 4 were carried out to test whether the effect of HDMP was modulated by the holding potential between -100 and $+60$ mV. These experiments revealed that the inhibitory effect of HDMP was voltage-independent in $\alpha 7$, $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChRs ($n=3$ for each nAChR subtype), and suggested that HDMP doesn't act as an ion channel blocker.

In vivo Studies

HDMP and PCP were evaluated for their ability to antagonize a 2.5 mg/kg dose of nicotine (an ED_{84} dose (effective dose 84%) (Damaj et al., 1995)) in the tail-flick procedure after s.c. injection. As shown in Table 2, both HDMP and PCP blocked in a dose-dependent manner nicotine-induced antinociception when given 15 min before nicotine with AD_{50} (half-maximal antinociception dose) values of 0.008 (0.004–0.015) and 1.1 (0.87–1.3) mg/kg, respectively. By themselves, HDMP and PCP did not significantly change tail-flick latencies at the highest dose tested (Table 2). In contrast to the tail-flick test, both HDMP and PCP failed to fully block (20% inhibition) nicotine-induced antinociception as measured in the hot-plate test at high doses (Table 2). Similarly, both compounds failed to block nicotine-induced hypothermia in mice.

Discussion

Both *in vivo* and *in vitro* studies supported the inhibitory effect of HDMP on neuronal nAChRs and confirmed the inhibitory effect of PCP. We examined the potency of the functional inhibitory effect of HDMP with respect to subunit composition of neuronal nAChRs and compared the results to that for PCP as a control. It is worth mentioning that the peak amplitude of ACh-induced currents was decreased by PCP somewhat more effectively in $\alpha 7$ than in $\alpha 4\beta 2$ or $\alpha 3\beta 4$ nAChRs with IC_{50} values of 1.25 versus 28.97 and 6.35 μM , respectively (Fig. 2 & Table 1). The IC_{50} value determined for PCP in rat $\alpha 3\beta 4$ nAChRs expressed in HEK cells in this study is similar to that reported by Fryer and Lukas (Fryer and Lukas, 1999) for human $\alpha 3\beta 4$ nAChRs expressed in SH-SY5Y cells as being close to 10 μM . The IC_{50} value for PCP determined in this study for human $\alpha 4\beta 2$ nAChRs expressed in SH-EP1 cells (28.97 μM) is comparable to that previously reported by Connolly

et al. (Connolly et al., 1992) for $\alpha 4\text{--}2\beta 2$ splice variant of rat $\alpha 4\beta 2$ nAChRs expressed in *X. oocytes* (10 μM). Furthermore, it is worth mentioning that the effective concentrations of PCP appear to be in a similar range of concentrations when compared for $\alpha 7$ nAChRs, as reported in this study (Fig. 2 and Table 1), and NMDA receptors ($\text{IC}_{50} \sim 1 \mu\text{M}$) (MacDonald et al., 1990; Morris et al., 2005).

Earlier studies demonstrated that PCP inhibits muscle nAChRs with the IC_{50} s in the range of 20–30 μM at the holding potential of -60 mV (Arias et al., 2006; Eaton et al., 2000). Further, the effect of PCP is noncompetitive, voltage-independent and accompanied by an increase of desensitization rate in muscle nAChRs. These data and the results of the current study suggest that PCP produces an inhibitory allosteric modulatory effect at both muscle and neuronal nAChRs with a similar potency in muscle, $\alpha 4\beta 2$ and $\alpha 3\beta 4$ neuronal nAChR subtypes.

Based on the IC_{50} values of 1.25 and 0.07 μM , for PCP and HDMP, respectively, HDMP is 18-fold more potent than PCP in inhibition of $\alpha 7$ nAChRs in functional assays (Fig. 2, 3 and Table 1). HDMP exhibited a potency similar to that of PCP at $\alpha 4\beta 2$ nAChRs (IC_{50} s were 25.06 μM and 28.97 μM , respectively) and was 4.6-fold less potent in inhibition of $\alpha 3\beta 4$ nAChR function than PCP (IC_{50} s were 28.98 μM and 6.35 μM , respectively). HDMP showed no inhibition of [^3H]epibatidine or [^{125}I]MLA binding and voltage-independent inhibitory action in patch-clamp experiments suggesting that the effect of HDMP on both $\alpha 4\beta 2$ and $\alpha 7$ subtypes of neuronal nAChRs occurs *via* a non-competitive allosteric mechanism. Our findings from binding assays and patch-clamp experiments suggest that HDMP is a novel NAM of neuronal nAChRs with more potent effect at $\alpha 7$ than $\alpha 4\beta 2$ or $\alpha 3\beta 4$ nAChRs at concentrations $\leq 1 \mu\text{M}$.

The comparison of the results from the tail-flick and hot-plate acute pain models is very informative on the role of various neuronal nAChR subtypes in nicotine's antinociceptive effects. Both HDMP and PCP showed an antagonist activity in the tail-flick, but not in the hot-plate test. HDMP was 125-fold more potent than PCP in the tail-flick test with $\text{AD}_{50} = 0.008 \text{ mg/kg}$ compared to 1.1 mg/kg for PCP (see Table 2). While $\alpha 4\beta 2^*$ and non- $\alpha 4\beta 2^*$ nAChR subtypes were shown to mediate nicotine-induced antinociception in mice (Marubio et al., 1999), these results suggest that both $\alpha 7$ and $\alpha 3\beta 4$ nAChRs may play an important role in this nicotinic response. The absence of an antagonist effect of HDMP and PCP in the hot-plate test, which is largely mediated by $\alpha 4\beta 2^*$ nAChRs (Marubio et al., 1999), is in agreement with the above-described functional data, showing a low potency of HDMP and PCP at $\alpha 4\beta 2$ nAChRs.

In addition, the fact that nicotine-induced hypothermia was not affected either by HDMP or PCP (see Table 2) supports the finding by Sack et al. (Sack et al., 2005) that $\alpha 4$ but not the $\alpha 7$ nAChR subunits seems to play a more important role in nicotine-elicited thermoalterations in mice. As mentioned in the introduction, few compounds are known to act as NAMs at neuronal nAChRs. However, functional examination demonstrated that none of them possessed more potent action at $\alpha 7$ than $\alpha \beta$ nAChRs. Our results with HDMP suggest that this **new** compound is an example of a NAM of neuronal nAChRs with good selectivity towards the $\alpha 7$ nAChRs. Recent studies have suggested that $\alpha 7$ nAChRs may be pivotal in the control of nicotine-induced lung cancer development and in growth signal translation-induced nicotine binding to nAChRs (Palafox et al., 2009a; Palafox et al., 2009b). Importantly, the studies showed that inhibition of $\alpha 7$ -nAChRs induces antitumor activity against non-small cell lung cancer (NSCLC) by triggering apoptosis (Palafox et al., 2009b). HDMP, which is a potent $\alpha 7$ nAChR NAM, may have a potential for treating NSCLC and is a highly useful lead compound for the development of more potent and selective $\alpha 7$ nAChR NAMs.

Methods

[¹²⁵I]IodoMLA Binding Assay was performed as described in (Navarro et al., 2002). Briefly, frozen male rat cerebral cortex (Pel-Freez Biologicals, Rogers, AK) were used in these assays. The competition binding experiments were carried out at 4 °C in 1.4 mL polypropylene tubes (Matrix Technologies Corporation, Hudson, NH) in a 96-well array. In a final volume of 0.5 mL triplicate samples contained approximately 3 mg of tissue (wet weight; added last), 70 pM [¹²⁵I]iodoMLA (s.a. 2180 Ci/mmol; RTI International, Research Triangle Park, NC) and one of 10 different concentrations of the test compounds. Total binding (buffer) and non-specific binding (300 μM (minus)-nicotine) samples were included on every plate. A 96-well cell harvester (Brandel Scientific, Gaithersburg, MD) was used to separate bound radioligand from free by rapid vacuum filtration onto GF/B filters presoaked for at least 30 min in assay buffer containing 0.15% bovine serum albumin, followed by a 3 mL ice-cold buffer wash. The amount of radioligand remaining on each filter is determined using a Packard TopCount microplate scintillation counter (70% efficiency).

[³H]Epibatidine Binding Assay

In a final volume of 0.5 mL, duplicate samples contain 3 mg wet weight male rat cerebral cortex homogenate (added last), 0.5 nM [³H]epibatidine (s.a. 47–56 Ci/mmol; Perkin-Elmer, Waltham, MA), and one of 10 different concentrations of test compound. After a 4-h incubation at room temperature, the samples are vacuum-filtered over GF/B filter papers presoaked in 0.03% PEI using a Brandel 48-well harvester followed by a buffer wash. The amount of radioactivity trapped on the filter is determined by standard liquid scintillation techniques.

Receptor Binding Data Handling

The specific binding data are fit using the non-linear regression equations in Prism (GraphPad Prism v. 3.0; GraphPad Software, San Diego, CA). The Cheng-Prusoff equation [$K_i = IC_{50}/(1 + ([L]/K_d))$] is used to calculate the K_i from the IC_{50} . The data is reported as the mean ± S.E.M. from at least three independent experiments. The K_d values for [¹²⁵I]MLA and [³H]epibatidine are 1.8 and 0.02 nM, respectively. We determined these K_d values under conditions identical to their respective assays.

Cell transfection and culture

Stably transfected HEK 293 cells expressing rat α3β4 or rat α7 and SH-EP1 cells expressing human α4β2 neuronal nAChRs, respectively, were prepared as described previously (Eaton et al., 2003; Moaddel et al., 2008; Xiao et al., 2009; Xiao et al., 1998). All three cell lines were maintained at 37°C with 5% CO₂ in the incubator. Growth medium for HEK 293 cells was minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The stably transfected cell line was raised in selective growth medium containing 0.7 mg/ml of Geneticin (Invitrogen Corp, Carlsbad, CA). Growth medium for SH-EP1 cells was Dulbecco's Modified Eagle's medium with high glucose supplemented with 10% heat inactivated horse serum, 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 8 mM L-glutamine, 1 mM sodium pyruvate, and 0.25 μg/ml amphotericin (all from Invitrogen Corp, Carlsbad, CA). This stably transfected cell line was raised in selective medium containing 0.5 mg/ml zeocin (Invitrogen) and 0.4 mg/ml hygromycin B (Roche Diagnostics Corp, Indianapolis, IN). RT-PCR analysis was used to confirm expression of nAChR subunit messages in the cells and immunoprecipitation-Western analyses using solubilized membrane samples from transfected cells clearly indicated that subunits were expressed as a protein and assembled together. Studies by others and our control experiments exclude possible activation of

muscarinic ACh receptors by ACh application in both cell lines (Abdrakhmanova et al., 2006; Lambert et al., 1989; Lukas, 1998; Xiao et al., 2009; Zhang et al., 1999). Difference in species of the nAChRs used in this study (rat versus human) is due to current unavailability to us of these nAChR subtypes of the same specie that would be functional and expressed in the cells at sufficiently high level. Rat and human nAChR subunits share 82–95 % sequence identity, and when present in neuronal nAChR receptors of the same subunit composition provide them with numerous similarities in their properties (Albuquerque et al., 2000).

Whole-cell current recording

Functional expression of nAChRs was evaluated in the whole-cell configuration of the patch-clamp technique using an Axopatch 200B amplifier (Molecular devices, Sunnyvale, CA). The patch electrodes, pulled from borosilicate glass capillaries (Sutter Instrument Company, Novato, CA), had a resistance of 2.5–3.5 M Ω when filled with internal solution containing 110 mM Tris phosphate dibasic, 28 mM Tris base, 11 mM EGTA, 2 mM MgCl₂, 0.1 mM CaCl₂ and 4 mM Mg-ATP (pH adjusted to 7.3 with Tris base) (Abdrakhmanova et al., 2008; Wu et al., 2004). In some cells ~85% of electrode resistance was compensated electronically, so that the effective series resistance in the whole-cell configuration was accepted when less than 20 M Ω . Stably transfected HEK and SH-EP1 cells were studied for 2 to 3 days after plating the cells on the 15-mm round plastic cover slips (Thermanox, Nalge Nunc, Naperville, IL). Generation of voltage-clamp protocols and acquisition of the data were carried out using pCLAMP 9.0 software (Molecular Devices). Sampling frequency was 5 kHz and current signals were filtered at 5 or 10 kHz before digitization and storage. All experiments were performed at room temperature (22–25°C).

Application of drugs and Perfusion system

Cells plated on cover slips were transferred to an experimental chamber mounted on the stage of an inverted microscope (Olympus IX50, Olympus Corporation, Tokyo, Japan) and were bathed in a solution containing 140 mM NaCl, 3 mM KCl, 2 mM MgCl₂, 25 mM D-glucose, 10 mM HEPES and 2 mM CaCl₂ (pH adjusted to 7.4 with Tris base). The experimental chamber was constantly perfused with control bathing solution (1–2 ml/min). The amplitude and time course of currents mediated by neuronal nAChRs is highly dependent on the speed of drug application. The high speed solution exchange system, HSSE-2 (ALA Scientific Instruments, Westbury, NY), is able to switch rapidly between control and four test solutions delivered through two output tubes which face each other at 90° in the same plane. Under optimal conditions, the delay in switching between solutions is ~10 ms.

The cells under recording were exposed initially to ACh at its EC₅₀ concentration. To achieve the equilibrium, this was followed by pre-exposure (1–2 min) of the cell to an antagonist at the studied concentration prior to co-application of ACh with the antagonist. Data presented herein were obtained through subtraction from the leak current.

Patch-clamp data analysis

The peak amplitude of the whole-cell currents was measured using the pCLAMP 9.0 program. The IC₅₀ and nH values were evaluated with the Origin 5.0 program (Microcal, North Hampton, MA). IC₅₀ values corresponded to the concentration of inhibiting agent causing a 50% reduction in the current evoked by a pulse of ACh at the concentration near the EC₅₀ value for each tested nAChR subtype. The ACh-evoked currents in the presence of PCP or HDMP were measured at –80 mV and normalized to the amplitude of the current elicited by ACh alone. Values were plotted against the concentrations of the inhibitor on a logarithm scale and fitted with an equation: $I = I_{\max} / (1 + (IC_{50} / [\text{antagonist}])^{nH})$, where I is the current amplitude at the antagonist concentration [antagonist], $I_{\max}$ is the maximum current,

and nH is the Hill coefficient. Results for concentration-response relationship are presented as the mean $\pm$ S.E.M for the number of cells (n) or as averaged means.

Drugs

ACh chloride, (–)-nicotine hydrogen tartrate and salts for patch-clamp experimental solutions were purchased from Sigma Aldrich (Atlanta, GA), PCP hydrochloride was a gift from NIDA. HDMP (**4**) was synthesized by a procedure analogous to that used to prepare 1,2,3,3a,4,8b-hexahydro-2-benzyl-1-methyl [1,2,-b]pyrrole (Carroll et al., 1993). HDMP had mp 55–57 °C. The calculated elemental analysis is: C, 82.31; H, 8.55; N, 7.14. The experimental values are: C, 82.17; H, 8.50; N, 9.12.

Animals

Mice were housed in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care approved animal care facility with food and water available *ad libitum*. The rooms were on a 12-h light/dark cycle (lights on at 7:00 A.M.). Mice were 8 to 10 weeks of age and weighed approximately 25–30 g at the start of the experiment. All experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Studies were conducted on male ICR mice (Harlan Laboratories, Indianapolis, IN). All studies were performed under the guidelines for the Care and Use of Laboratory Animals as promulgated by the U.S. National Institutes of Health.

Tail-flick Test

Antinociception was assessed by the tail-flick method of D'Amour and Smith. A control response (2–4 s) 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 $\%MPE = [(test - control)/(10 - control)] \times 100$. Groups of eight to twelve animals were used for each dose and for each treatment. The mice were tested 15 min after s.c. injections of PCP or HDMP for evaluating possible agonist effects. Antagonism studies were carried out by pretreating the mice either with saline, HDMP or PCP 15 min before nicotine. This 15-min time point was determined in separate experiments to be the optimum pretreatment time for both compounds (data are not shown). The animals were then tested 5 min after administration of nicotine.

Hot-plate Test

Mice were placed into a 10 cm wide glass cylinder on a hot plate (Thermojust Apparatus) maintained at 55 °C. Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 8 to 12 s. Antinociceptive response was calculated as percent maximum possible effect (%MPE), where $\%MPE = [(test - control)/(40 - control) \times 100]$. The reaction time was scored when the animal jumped or licked its paws. Groups of eight to twelve animals were injected s.c. with PCP or HDMP and tested 15 min thereafter in evaluating possible agonist effects. Antagonism studies were carried out by pretreating the mice either with saline, HDMP or PCP 15 min before nicotine. This 15-min time point was determined in separate experiments to be the optimum pretreatment time for both compounds (data are not shown). The animals were then tested 5 min after administration of nicotine.

Body Temperature

Rectal temperature was measured by a thermistor probe (inserted 24 mm) and digital thermometer (Yellow Springs Instrument Co., Yellow Springs, OH). Readings were taken

just before and at different times after the s.c. injection of either saline or PCP or HDMP. The difference in rectal temperature before and after treatment was calculated for each mouse. The ambient temperature of the laboratory varied from 21 to 24°C from day to day. Antagonism studies were carried out by pretreating the mice with either saline, HDMP or PCP 15 min before nicotine. The animals were then tested 30 min after administration of nicotine.

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Nonstandard Abbreviations

nAChR	nicotinic acetylcholine receptor
NAM	negative allosteric modulator
PCP	phencyclidine
HDMP	1,2,3,3a,4,8b-hexahydro-2-benzyl-6- <i>N,N</i> -dimethylamino-1-methylindeno[1,2- <i>b</i>]pyrrole

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Figure 1. Structures of nicotine (**1**), epibatidine (**2**), dihydro-β-erythroidine (**3**), PCP, HDMP (**4**), and **5**.

Figure 2.

Analysis of the antagonist activity of HDMP in three neuronal nAChR subtypes. A, Concentration-response relationships for HDMP constructed in $\alpha 3\beta 4$ ($\blacksquare$), $\alpha 4\beta 2$ ($\bullet$) and $\alpha 7$ ($\square$) nAChR subtypes. The peak amplitude of ACh (EC_{50})-evoked currents was taken in each cell to normalize the peak amplitude of the currents that were evoked in the presence of HDMP at different concentrations. The curves were fitted to Hill equation. Symbols and bars represent the mean $\pm$ S.E.M. B, Examples of the effect of HDMP at 0.1 μM concentration illustrate a pronounced (55%) inhibition of whole-cell currents in $\alpha 7$, but no effect in $\alpha 4\beta 2$ or $\alpha 3\beta 4$ nAChR expressing cells. Holding potential in A and B was -80 mV.

Figure 3. Comparison of PCP-induced inhibitory effect on three different neuronal nAChR subtypes. A, Concentration-response relationships for PCP constructed in $\alpha 3\beta 4$ (■), $\alpha 7$ (◆) and $\alpha 4\beta 2$ (◆) nAChRs. The peak amplitude of ACh (EC_{50})-evoked currents was taken in each cell to normalize the peak amplitude of the currents that were evoked in the presence of PCP at different concentrations. The curves were fitted to Hill equation. Symbols and bars represent the mean $\pm$ S.E.M. B, Examples of inhibitory effect of PCP at concentrations close to determined IC_{50} values in three representative cells expressing $\alpha 4\beta 2$ (top), $\alpha 3\beta 4$ (middle) and $\alpha 7$ (bottom) nAChRs. Holding potential in A and B was -80 mV.

Figure 4. Voltage-dependence of inhibitory effect of HDMP on neuronal nAChRs. Each panel shows data obtained from a single representative cell expressing $\alpha 7$ (A, triangles), $\alpha 4\beta 2$ (B, circles) or $\alpha 3\beta 4$ (C, rhombs) nAChRs. ACh(EC_{50})-induced currents were evoked at various holding potentials in the range from -100 to $+60$ mV, quantified at its maximal amplitude in the absence (closed symbols) and presence (open symbols) of HDMP at the concentration close to its IC_{50} value determined for each receptor subtype, and plotted versus the corresponding holding potential. The inserts in each panel represent superimposed traces of the ACh-induced currents in the absence and presence of HDMP.

Table 1

Potency of HDMP and PCP in recombinant $\alpha 4\beta 2$, $\alpha 7$ and $\alpha 3\beta 4$ nAChRs. The IC50 values are presented as a mean $\pm$ S.E.M., the nH values are presented as a mean

compd	$\alpha 4\beta 2$ IC ₅₀ (μ M)	$\alpha 4\beta 2$ nH	$\alpha 7$ IC ₅₀ (μ M)	$\alpha 7$ nH	$\alpha 3\beta 4$ IC ₅₀ (μ M)	$\alpha 3\beta 4$ nH
HDMP	25.06 $\pm$ 6.37	1.15	0.07 $\pm$ 0.01	0.73	28.98 $\pm$ 3.82	1.06
PCP	28.97 $\pm$ 6.38	1.4	1.25 $\pm$ 0.26	0.97	6.35 $\pm$ 1.55	0.65

Table 2

Antinociception data for HDMP and PCP. The results are presented as ED₅₀ or AD₅₀ values ($\pm$ confidence limits) in mg/kg or as a percent effect at the individual dose

compd	ED ₅₀ (mg/kg)		AD ₅₀ (mg/kg)		
	Tail-flick	Hot-Plate	Hypothermia	Tail-flick	Hot-Plate
HDMP	2% @ 10	8% @ 10	0% @ 10	0.008 (0.004–0.015)	20% @ 10
PCP	6% @ 5	5% @ 5	5% @ 5	1.1 (0.87–1.30)	20% @ 5