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The pharmacological activity of nicotine and nornicotine on nAChRs subtypes: relevance to nicotine dependence and drug discovery

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Abstract

Cigarette smoking and other forms of tobacco use deliver an array of pharmacologically active alkaloids, including nicotine and ultimately various metabolites of these substances. While nornicotine is a significant component in tobacco as well as a minor systemic metabolite of nicotine, nornicotine appears to be N-demethylated locally in the brain where it accumulates at relatively high levels after chronic nicotine administration. We have now examined the effects of nornicotine on specific combinations of neuronal nicotinic acetylcholine receptor (nAChR) subunits expressed in *Xenopus* oocytes and compared these responses to those evoked by acetylcholine and nicotine. Of the nAChR subtypes studied, we have found that α7 receptors are very responsive to nornicotine ($EC_{50} \approx$ 17 μmol/L I_{max} 50%, compared with acetylcholine (ACh)). nAChRs containing the ligand-binding domain of the α6 subunits (in the form of an α6/α3 chimera) are also strongly responsive to nornicotine ($EC_{50} \approx$ 4 μmol/L I_{max} 50%, compared with ACh). α7-type nAChRs have been suggested to be potential therapeutic targets for Alzheimer's disease, schizophrenia and possibly other pathologies. nAChRs containing α6 subunits have been suggested to have a role in nicotine-evoked dopamine release. Thus, understanding the actions of nornicotine in the brain may have significance for both emerging therapeutics and the management of nicotine dependence.

Keywords: dopamine release, nicotine metabolism, voltage clamp, *Xenopus* oocytes.

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There are many threads to the complex weave associated with tobacco addiction. Tobacco use delivers an array of pharmacologically active alkaloids including nicotine and nornicotine, and ultimately various metabolites of these alkaloids. Nornicotine is present in *Nicotiana tobaccum* in both S(–) and R(+) enantiomeric forms (Kisaki and Tamaki 1961). This contrasts with nicotine, which is only present in the optically pure S(–) form in *N. tobaccum*. While nicotine has been extensively studied, nornicotine has only recently been investigated as a potential contributor to tobacco dependence (Crooks and Dwoskin 1997a).

In addition to being a significant component of tobacco, nornicotine is a minor systemic metabolite of nicotine in various animal species, including humans, non-human primates and rodents (Bowman *et al.* 1959; McKennis *et al.* 1962; Cundy and Crooks 1984; Benowitz *et al.* 1991; Jacob and Benowitz 1993; Tsai and Gorrod 1999; Moyer *et al.* 2002). About 0.8% of nicotine is metabolized to nornicotine in the periphery (Curvall and Kazeni 1993; Jacob and Benowitz 1993). However, the biotransformation of nicotine to nornicotine also appears to occur locally in the brain, and brain concentrations of nornicotine have been shown to exceed those in the periphery (Ghosheh *et al.* 1999, 2001). Repeated administration of nicotine induces cytochrome P450 enzymes in brain (Howard *et al.* 2001, 2003), providing a potential mechanism for the increased concentrations and accumulation of nornicotine in brain following repeated nicotine administration. Pharmacokinetic studies demonstrate that the half-life of nornicotine in brain is 166 min, which is three times longer than that of nicotine (52 min) (Ghosheh *et al.* 1999), thus yielding a longer residence time for nornicotine in brain relative to nicotine.

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Abbreviations used: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; VTA, ventral tegmental area.

Accumulation of nornicotine has been reported in rat brain following repeated intermittent peripheral administration of [2′-^{14}C]nicotine using a regimen that closely models intermittent human smoking behavior (Crooks *et al.* 1995; Crooks and Dwoskin 1997b; Ghosheh *et al.* 2001). In the latter study, nornicotine concentrations were ~300 nmol/L in brain and represented ~20% of the [^{14}C]-radiolabel present in brain. Such concentrations approach those which have been shown to evoke dopamine release from nucleus accumbens and striatum (Dwoskin *et al.* 1993; Green *et al.* 2001). Furthermore, a recent *in vivo* positron emission tomography study with monkeys reported that i.v. nicotine displaced [^{18}F]fluoro-A-85380 binding to nicotinic acetylcholine receptor (nAChR) across a 6-h period (Valette *et al.* 2003), which is well beyond the residence time for [^{11}C]nicotine in brain. Importantly, i.v. (±)-nornicotine displaced [^{18}F]fluoro-A-85380 binding to nAChR for a similar time period (6 h), suggesting that the prolonged receptor occupancy following nicotine was because of its biotransformation to (±)-nornicotine and appearance in brain. Extrapolation to human cigarette smokers suggests that intermittent exposure to nicotine results in an accumulating concentration of nornicotine in brain and suggests that nornicotine could play a major role in tobacco dependence.

While both nicotine and nornicotine can activate neuronal nAChR subtypes, the specificity of these two active molecules for particular nAChR subtypes is unknown. With a dozen known neuronal nAChR subunits (α2–α10 and β2–β4), (Dani 2001) that are able to form many different pentameric assemblies, the potential for nAChR subtype diversity is immense. This diversity is further amplified by differential patterns of nAChR expression in various neuronal cell types and additionally by differential subcellular compartmentalization of receptor subtypes within a given cell (Ji *et al.* 2001). Add to this terrain of complex receptor proteins the concept that dynamic and interacting stimulation is provided by at least two potential endogenous agonists, i.e. ACh and choline, and one can begin to appreciate the background that is perturbed by the exposure to nicotine and nornicotine upon tobacco use.

The current study addresses the issue of how nicotine and nornicotine may differentially activate nAChR subtypes based on subunit composition, including α7 and commonly studied pairwise combinations of alpha and beta subunits. Additionally, the effects of co-expression with the modulatory subunits α5, α6, and β3 were investigated. The effects of nicotine and nornicotine are compared with the endogenous activator ACh. Nornicotine is confirmed as having significant biological activity that differs from that of nicotine using recombinant receptor assays. Each of the ancillary subunits creates a pharmacological fingerprint in regard to the relative efficacy and/or potency of ACh, compared with nicotine and nornicotine.

Methods

Preparation of RNA

Rat nAChR clones were obtained from Dr Jim Boulter (UCLA) and the human α5 clone was obtained from Jon Lindstrom (University of Pennsylvania). A form of the α6/α3 chimera (Dowell *et al.* 2003) was obtained from Michael McIntosh (University of Utah) and corrected for a point mutation, as previously reported (Papke *et al.* 2005). After linearization and purification of cloned cDNAs, RNA transcripts were prepared *in vitro* using the appropriate mMessage mMachine kit from Ambion Inc. (Austin, TX, USA).

Expression in *Xenopus* oocytes

Mature (> 9 cm) female *Xenopus laevis* African toads (Nasco, Ft. Atkinson, WI, USA) were used as a source of oocytes. Prior to surgery, frogs were anesthetized by placing the animal in a 1.5 g/L solution of MS222 (3-aminobenzoic acid ethyl ester; Sigma) for 30 min. Oocytes were removed from an incision made in the abdomen.

To remove the follicular cell layer, harvested oocytes were treated with 1.25 mg/mL collagenase (Worthington Biochemical Corporation, Freehold, NJ, USA) for 2 h at room temperature in calcium-free Barth's solution [88 mmol/L NaCl, 1 mmol/L KCl, 2.38 mmol/L $NaHCO_3$, 0.82 mmol/L $MgSO_4$, 15 mmol/L HEPES (pH 7.6), 0.1 mg/mL gentamicin sulfate]. Subsequently, stage 5 oocytes were isolated and injected with 50 nL (5–20 ng) each of the appropriate subunit cRNAs. Recordings were made 2–15 days after injection. Although the absolute magnitude of the evoked current responses increased over time, the normalized values of the experimental responses did not vary significantly over time.

Chemicals

S(-)-Nicotine (free base) was obtained from Aldrich (Milwaukee, WI, USA), and (±)-nornicotine (free base) was synthesized as previously described. (Swango *et al.* 1999). All other chemicals for electrophysiology were obtained from Sigma Chemical Co. (St Louis, MO, USA). Fresh ACh stock solutions were made daily in Ringer's solution and diluted.

Electrophysiology

Experiments were conducted using OpusXpress 6000A (Axon Instruments, Union City CA, USA), or manual oocyte two-electrode voltage-clamp systems as previously reported (Stokes *et al.* 2004). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Cells were automatically perfused with bath solution, and agonist solutions were delivered from a 96-well plate. Both the voltage and current electrodes were filled with 3 mol/L KCl. The agonist solutions were applied via disposable tips, which eliminated any possibility of cross-contamination. Drug applications alternated between ACh controls and experimental applications. Flow rates were set at 2 mL/min for experiments with α7 receptors and 4 mL/min for other subtypes. Cells were voltage-clamped at a holding potential of −60 mV. Data were collected at 50 Hz and filtered at 20 Hz. ACh applications were 12 s in duration followed by 181-s washout periods with α7 receptors and 8 s with 241-s wash periods for other subtypes. For manual oocyte recordings, OC–725C oocyte amplifiers (Warner Instruments, Hamden, CT, USA) were used, and data were acquired with a minidigi or digidata 1200A with pClamp9

software (Axon Instruments, Union City, CA, USA). Sampling rates were between 10 and 20 Hz and the data were filtered at 6 Hz. Cells were voltage-clamped at a holding potential of −50 mV.

Experimental protocols and data analysis

Each oocyte received two initial control applications of ACh, then an experimental drug application, and then a follow-up control application of ACh. The control ACh concentrations for α7 and α4β2 receptors were 300 μmol/L and 10 μmol/L, respectively, and 100 μmol/L for the other subunit combinations tested. Responses to each drug application were calculated relative to the preceding ACh control responses to normalize the data, compensating for the varying levels of channel expression among the oocytes. Drug responses were initially normalized to the ACh control response values and then adjusted to reflect the experimental drug responses relative to the ACh maxima. Responses for α7 receptors were calculated as net charge (Papke and Papke 2002). For subtypes other than α7, responses were calculated from the peak current amplitudes. Means and standard errors means were calculated from the normalized responses of at least four oocytes for each experimental concentration. As the application of some experimental drugs cause the subsequent ACh control responses to be reduced because of some form of residual inhibition (or prolonged desensitization), subsequent control responses were compared with the pre-application control ACh responses. When cells failed to recover to at least 75% of the previous control, they were replaced with new cells.

For concentration–response relationships, data were plotted using Kaleidagraph 3.0.2 (Abelbeck Software; Reading, PA, USA), and curves were generated from the Hill equation:

$$\text{Response} = \frac{I_{\max}(\text{agonist})^n}{(\text{agonist})^n + (EC50)^n}$$

where $I_{\max}$ denotes the maximal response for a particular agonist/subunit combination, and n represents the Hill coefficient. $I_{\max}$, n, and EC_{50} were all unconstrained for the fitting of the nicotine and nornicotine responses, and $I_{\max}$ was constrained to equal 1 for the ACh responses, as maximal ACh responses were used to define full agonist activity. Negative Hill slopes were applied for the calculation of IC_{50} values associated with residual inhibition or desensitization, when observed.

Results

Initial experiments utilized subunit combinations which are believed to represent the two predominant nAChR subtypes in brain, i.e. α7 and α4β2, as well as the α3β4 subunit combination, which is used as a model for ganglionic receptors. Responses of these subtypes to ACh, nicotine and nornicotine were compared. Figure 1 shows representative responses of oocytes expressing α7 receptors to the application of these three agonists at 300 μmol/L. This figure also represents the basic three-step experimental protocol. First, an initial ACh application was made to establish an internal control for each cell. Then, the experimental application was made, followed by another control ACh application. As shown, the amplitude of control ACh responses was comparable both before and after the application of ACh or nornicotine at 300 μmol/L, but significantly decreased after the application of 300 μmol/L nicotine. Thus, the cell exposed to 300 μmol/L nicotine would not have been used for further testing (see methods). As shown in Fig. 2, both nicotine and nornicotine were relatively potent partial agonists of rat α7 receptors with efficacies of approximately 60% and 50%, respectively, compared with ACh (Table 1).

The efficacies of nicotine and nornicotine for α4β2 receptors were relatively low, compared with ACh, although

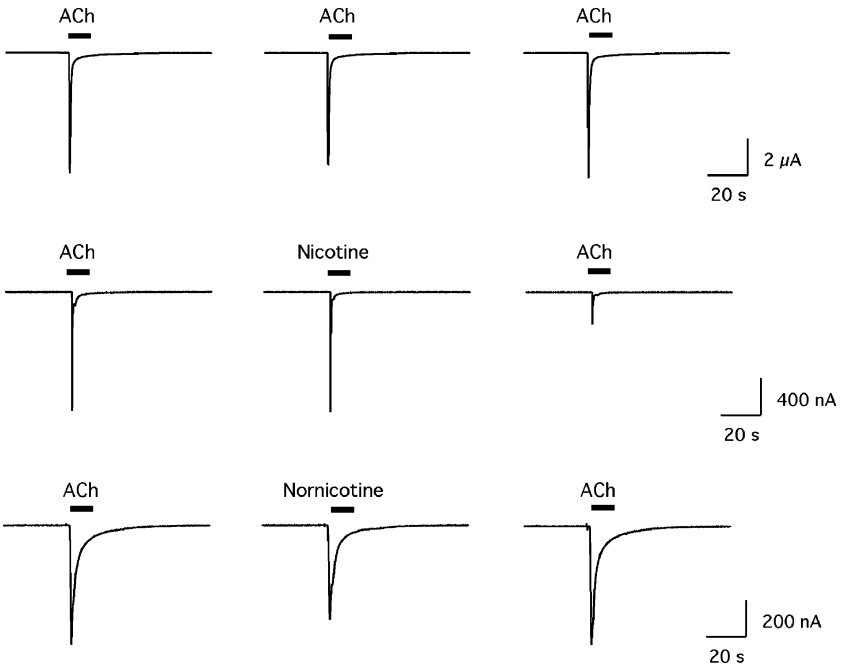

Fig. 1 Responses of oocytes expressing rat α7 receptors to the application of ACh nicotine or nornicotine. The basic experimental protocol is illustrated, showing in the upper traces three consecutive responses of an oocyte to the application of 300 μmol/L ACh. In the second set of traces, the oocyte received an initial application of 300 μmol/L ACh, followed by an application of 300 μmol/L nicotine, and then after a further wash period, another application of 300 μmol/L ACh. The second ACh response was greatly reduced compared with the first. The bottom set of traces show the responses of an oocyte to control applications of 300 μmol/L ACh and the experimental application of 300 μmol/L nornicotine.

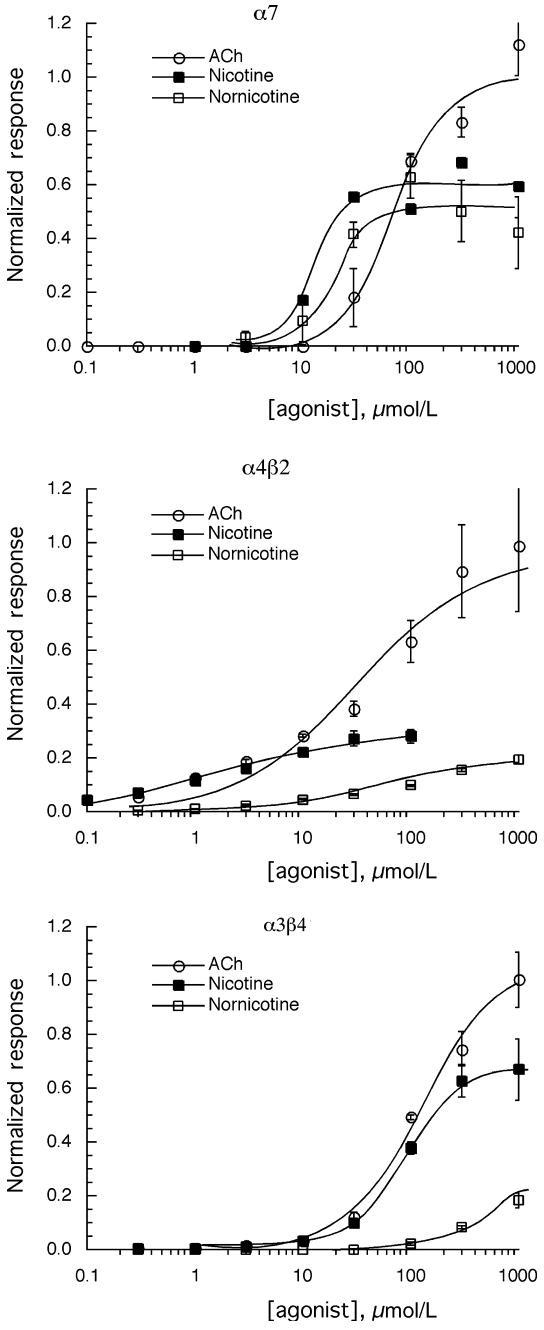

Fig. 2 Concentration response curves for oocytes expressing rat α7, α4β2 or α3β4 receptors to ACh, nicotine, or nornicotine. Responses for α7-expressing oocytes are calculated based on net charge. The α4β2 and α3β4 responses are based on peak current amplitudes, calculated relative to ACh control responses obtained from the same cells (see methods) and subsequently normalized to the ACh maximum response. Points represent the averages (±SEM) from at least four oocytes.

nicotine was rather more potent than nornicotine and ACh. It should be noted, that as previously reported (Papke *et al.* 2000), responses of α4β2 receptors evoked by nicotine are unusually long and extend into the washout time (data not shown). This was not the case for the nornicotine-evoked responses. Nicotine was relatively efficacious for α3β4 receptors, although less potent at α4β2 receptors. Nornicotine was a relatively poor agonist for α4β2 and α3β4 receptors.

Pairwise combinations of neuronal nAChR subunits are useful models for naturally occurring receptor subtypes. This is especially true for α4β2 receptors. The study of pairwise combinations has also been useful for determining subunit-dependent pharmacological effects, such as the β2 versus β4 effects on the efficacy of cytisine (Papke and Heinemann 1994). However, there is growing appreciation that recombinant receptor models need to be extended to take into account the possible effects of additional subunits in combinations more complex than the basic α/βsubunit pairing (Gerzanich *et al.* 1996; Wang *et al.* 1996; Kuryatov *et al.* 2000). Therefore, the analysis was extended to evaluate the possible effect of the α5, β3, and α6 subunits on the relative effects of nicotine and nornicotine. A series of experiments were conducted beginning with the α3β2 pairwise subunit combination. As shown in Fig. 3, the efficacy of both nicotine and nornicotine was relatively low for this receptor subtype, and nornicotine was more potent than nicotine. Then, human α5 subunits were co-expressed with α3β2, and as expected (Gerzanich *et al.* 1998), all three agonists were more potent for this receptor than for receptors containing just α3β2. The effect of α5 expression on agonist potency was greatest for nicotine, so that nicotine and nornicotine were equipotent and equi-efficacious for this α3α5β2 subunit combination.

The co-expression of β3 with α3 and β2 had relatively little effect on the concentration–response curves, except that nicotine appeared to be more potent for α3β2β3 receptors than for receptors with α3 and β2 alone (Fig. 3). To model the effects of α6 subunits, a chimera of α6 and α3 was tested, which has previously been reported to form functional α6-like receptors when co-expressed with β2 and β3 (Dowell *et al.* 2003). These receptors showed a greater response to nornicotine than did the other β subunit-containing receptors studied (Fig. 3).

As shown in Fig. 1, nicotine applications had prolonged effects on α7 receptors that could be measured as decreases in subsequent ACh-evoked control responses. Similar long-lasting effects of nicotine were observed for the other subunit combinations tested (Fig. 4), but were not observed for ACh or nornicotine (data not shown). Receptors containing the α6/3 chimera appeared to be most sensitive to this inhibitory or desensitizing effect of nicotine (Table 2).

Discussion

The current findings taken together with previous results show that the conversion of nicotine to nornicotine in the

Table 1 EC_{50} and I_{max} values generated by Hill equation curve fits

	Agonist					
	ACh		Nicotine		Nornicotine	
Receptor	EC_{50} μmol/L	I_{max}	EC_{50} μmol/L	I_{max}	EC_{50} μmol/L	I_{max}
α7	68 ± 9	1*	13.2 ± 2.6	0.60 ± 0.04	17.4 ± 4.9	0.51 ± 0.05
α4β2	37 ± 7.4	1*	2.5 ± 0.6	0.32 ± 0.02	375 ± 262	0.32 ± 0.05
α3β4	130 ± 19	1*	87 ± 4	0.69 ± 0.13	614 ± 136	0.28 ± 0.03
α3β2	72 ± 15	1*	52 ± 7	0.15 ± 0.01	6.6 ± 1.6	0.10 ± 0.01
α3β2hα5	21 ± 4	1*	6.6 ± 3.2	0.16 ± 0.02	3.5 ± 0.9	0.18 ± 0.01
α3β2β3	87 ± 22	1*	7.3 ± 3.7	0.14 ± 0.02	4.1 ± 1.1	0.11 ± 0.01
α6/3β2β3	16 ± 2.4	1*	1.7 ± 1.05	0.31 ± 0.03	3.7 ± 1.5	0.53 ± 0.04

*I_{max} expressed relative to the maximum ACh evoked responses.

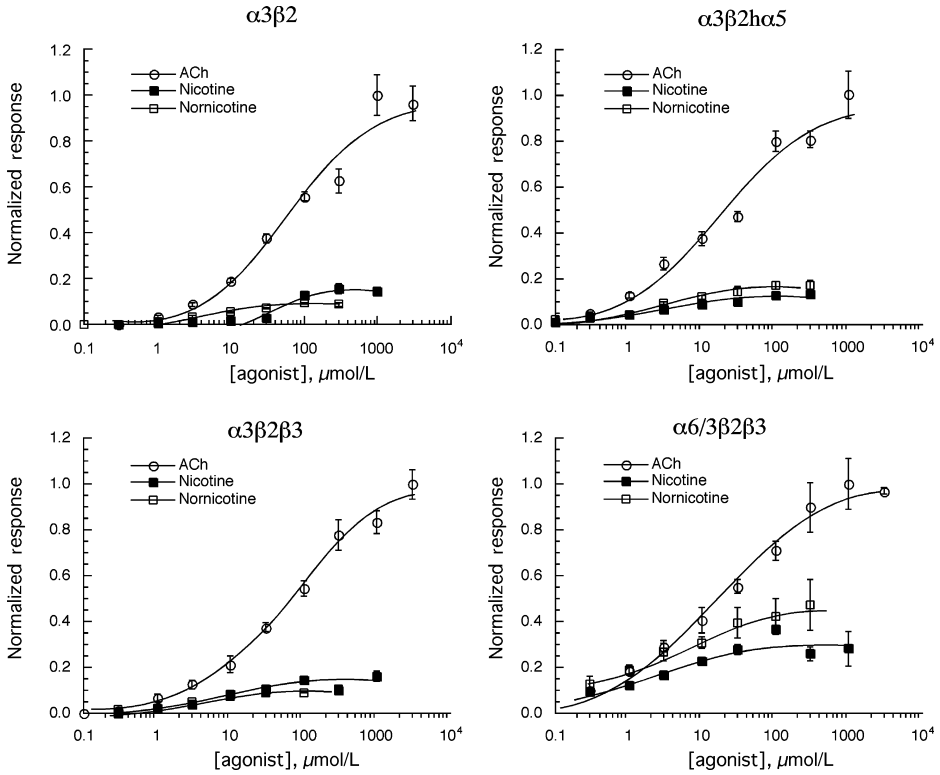

Fig. 3 Concentration response curves for oocytes expressing rat α3β2, rat α3β2 and human α5, rat α3β2β3, or rat α6/3β2β3 receptors to ACh, nicotine, or nornicotine. The responses are based on peak current amplitudes, calculated relative to ACh control responses obtained from the same cells (see methods), and subsequently normalized to the ACh maximum response. Points represent the averages (±SEM) from at least four oocytes.

brain and the presence of nornicotine as a tobacco alkaloid may result in a broadening of the array of pharmacological effects arising from tobacco use. Specifically, the current study shows that exposure to nornicotine will result in additional activation of α7-type receptors, which may be important for effects on cognition and attention. Likewise, the effects of nornicotine on α6-containing receptors may contribute to the reinforcing effects of nicotine, and therefore, is relevant to dependence on tobacco. In contrast to nicotine, nornicotine had relatively low activity on receptors other than those containing α7 or α6 subunits.

Our data obtained from heterologously expressed nAChR indicate that nornicotine can be included in the armamentarium of available compounds that can be utilized to pharmacologically characterize the subunit composition of native nAChRs. Our data also provide additional perspective on the actions of both nicotine and nornicotine in animal studies and other, more complex, *in vitro* systems. While the concentra-

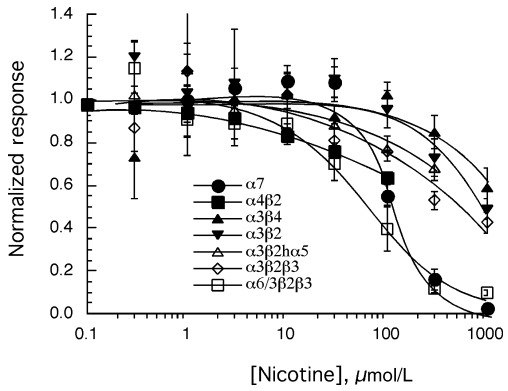

Fig. 4 The effect of nicotine on the amplitude of subsequent ACh control responses. Responses for α7-expressing oocytes are expressed as net charge; for all other subtypes, the responses are based on peak current amplitudes, calculated relative to ACh control responses obtained from the same cells (see methods). Points represent the average (±SEM) from at least four oocytes.

Table 2 Effect of nicotine application on subsequent responses to ACh

nAChR subtype	IC_{50} μmol/L ± 95% CI
α7	117 ± 16
α4β2	324 ± 26
α3β4	1680 ± 914
α3β2	887 ± 289
α3β2hα5	900 ± 21
α3β2β3	492 ± 186
α6/3β2β3	64 ± 14

tions of both nicotine and nornicotine required to fully activate the nAChR expressed in oocytes are higher than the plasma concentrations normally associated with 'smoking dosages' of nicotine, our data indicate that nornicotine is at least as potent as nicotine itself for important receptor subtypes. The fact that nornicotine has a longer half-life in the brain than nicotine (Ghosheh *et al.* 1999), combined with our data, which indicate that nornicotine is equipotent as nicotine at activating some receptor subtypes, and may also be less desensitizing than nicotine, suggests that nornicotine is important for the penumbra of effects following nicotine self-administration.

Evidence indicates that nicotine reward depends, at least in part, on activation of specific nAChRs on neurons in the mesolimbic dopamine system that project from the midbrain ventral tegmental area (VTA) to the nucleus accumbens. Dopamine antagonists decrease the reinforcing effect of nicotine assessed in either rats (Corrigall and Coen 1991) or human smokers (Dawe *et al.* 1995; Caskey *et al.* 1999). Further, lesion of the nucleus accumbens or the VTA with the neurotoxin 6-hydroxydopamine disrupts the rewarding effect of nicotine in rats (Clarke 1990; Corrigall *et al.* 1992; Louis and Clarke 1998). Moreover, local microinjection of nAChR antagonists into the VTA also decreases nicotine self-administration (Corrigall *et al.* 1994). Interestingly, nornicotine has been shown to displace [^{3}H]-nicotine binding to rat brain membranes (Reavill *et al.* 1988; Copeland *et al.* 1991; Zhang and Nordberg 1993). Nornicotine evokes a concentration-dependent and calcium-dependent release of dopamine from rat and mouse striatal slices and synaptosomes (Grady *et al.* 1992; Dwoskin *et al.* 1993). The observation that nAChR antagonists inhibit dopamine release evoked by nornicotine (100 nmol/L–100 μmol/L) (Teng *et al.* 1997), supports the involvement of a nAChR-mediated mechanism.

Different nAChR subtypes may play important roles in evoking dopamine release depending on the dopaminergic terminal field stimulated. Knockout mice have been used as an approach to elucidate the specific subunits comprising the nAChR subtypes mediating nicotine-evoked dopamine release. β2, α6 and α4 knockout studies clearly demonstrate the involvement of β2-containing nAChRs in nicotine-evoked dopamine release (Picciotto *et al.* 1998; Whiteaker *et al.* 2000; Grady *et al.* 2002; Champtiaux *et al.* 2003; Salminen *et al.* 2004). Additionally, α5 and β3 contribute to this response; whereas β4- and α7-containing nAChRs may not (Salminen *et al.* 2004). Knockout studies suggest that perhaps six different nAChR subtypes (α6β2*, α6β2(β3)*, α4β2*, α4α6β2*, α4α6β2(β3)* and α4α5β2*) mediate nicotine-evoked dopamine release and immunoprecipitation studies using antibodies directed at specific nAChR subunits provide similar results (Zoli *et al.* 2002), which are generally consistent with findings from the aforementioned knockout studies. While it remains to be determined if the same nAChR subtypes are also involved in nornicotine-evoked dopamine release, the likely involvement of α6-containing receptors in dopamine release and the sensitivity of these receptors to nornicotine strongly support a role for nornicotine in the reinforcing effects of nicotine self-administration.

In addition to its neurochemical effects, acute nornicotine produces nicotine-like effects on locomotor activity in mice and rats (Mattila 1963; Stolerman and Jarvis 1995). The locomotor stimulant effect of repeated nornicotine appears to be dependent on dopamine D_2 receptors in the mesolimbic system (Green *et al.* 2002). Other research indicates that nornicotine produces a nicotine-like discriminative stimulus effect (Rosecrans and Meltzer 1981; Goldberg *et al.* 1989; Bardo *et al.* 1997; Desai *et al.* 1999, 2003), as well as nicotine-like effects on schedule-controlled operant responding (Risner *et al.* 1985, 1988). Thus, nicotine and nornicotine have some similar pharmacological properties. However, the current research also reveals some important differences in the profile of nAChR stimulation specifically with regard to the α7-containing nAChRs. Utilization of the different profiles of nicotine and nornicotine obtained from the

recombinant nAChR studies described herein may provide a means for identifying native nAChRs involved in tobacco dependence.

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