

Characterization of Nicotinic Acetylcholine Receptors that Modulate Nicotine-evoked [³H]Norepinephrine Release from Mouse Hippocampal Synaptosomes

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Nonstandard abbreviations: CTX, conotoxin; DA, dopamine; LC, locus ceruleus;
nAChR, nicotinic acetylcholine receptors; NE, norepinephrine; WT, wildtype.

Abstract

Nicotine's modulation of hippocampal noradrenergic neurotransmission may contribute to its mnemonic properties, but the nAChR subtypes that modulate terminal release of norepinephrine are unknown. In the present study, we have used a number of subtype selective α -conotoxins in combination with nicotinic receptor subunit deficient mice to characterize nAChRs that modulate [^3H]norepinephrine release from synaptosomes. The results indicate that at least 2 populations of nAChRs contribute to this release. These are a novel $\alpha 6(\alpha 4)\beta 2\beta 3\beta 4$ subtype and an $\alpha 6(\alpha 4)\beta 2\beta 3$ subtype. These are distinct from subtypes that modulate synaptosomal norepinephrine release in the rat hippocampus where an $\alpha 6/\beta 2$ and/or $\alpha 6/\beta 4$ ligand-binding interface is *not* present. Whereas α -conotoxin MII fully inhibits nicotine-evoked [^3H]norepinephrine release in mouse, it is ineffective in blocking [^3H]norepinephrine release in rat. Block of [^3H]norepinephrine release by α -conotoxin BuIA, a toxin that kinetically distinguishes between $\beta 2$ - and $\beta 4$ -containing nAChRs, was partially reversible in mouse but irreversible in rat. This indicates that in contrast to rat, mouse nAChRs are made of both $\beta 4$ and non- $\beta 4$ -containing populations. Results from $\beta 2$ and $\beta 4$ null mutant mice confirmed this conclusion, indicating the presence of the $\beta 2$ subunit in all nAChRs, and the presence of the $\beta 4$ subunit in a subpopulation of nAChRs. Additionally, both $\alpha 4$ and $\beta 3$ subunits are essential for formation of functional nAChRs on mouse noradrenergic terminals. Cytisine, a ligand formerly believed to be $\beta 4$ -selective, was a highly efficacious agonist for $\alpha 6\beta 2$ -containing nAChRs. The sum of these results suggests a possible novel nAChR subtype that modulates noradrenergic neurotransmission within the mouse hippocampus.

Presynaptic nicotinic acetylcholine receptors (nAChRs) modulate release of many neurotransmitters within the central nervous system, including dopamine (DA), serotonin, GABA and norepinephrine (NE) (Wonnacott, 1997). The nicotinic receptors regulating DA release from striatal terminals have been extensively characterized in both rats and mice. In both species, at least two types of nAChR subtypes have been identified based on sensitivity to α -conotoxin (CTX) MII (Grady et al., 2002; Kulak et al., 1997) and α -CTX PIA (Azam and McIntosh, 2005). Knockout studies have shown that both subtypes require the β 2 subunit, as nicotine-evoked [3 H]DA release is completely abolished in β 2 null mutant mice (Grady et al., 2001; Salminen et al., 2004; Zoli et al., 2002). Knockout and immunoprecipitation studies have identified at least 4 different subtypes on the DAergic terminals: α 6 β 2 β 3, α 6 α 4 β 2 β 3, α 4 β 2 and α 4 α 5 β 2 (Champiaux et al., 2003; Zoli et al., 2002).

In contrast to the striatal DAergic system, there is limited data on nicotinic regulation of hippocampal noradrenergic neurotransmission in rats (Fu et al., 1998; Sacca et al., 1996; Sershen et al., 1997) and a lack of data in mice. Nicotine-stimulated [3 H]NE release from rat hippocampal synaptosomes is insensitive to block by α -CTX MII and partially blocked by the α 3 β 4 selective α -CTX AuIB (Kulak et al., 1997; Luo et al., 1998). The rat locus ceruleus (LC), which provides the sole noradrenergic projection to the hippocampus, expresses a variety of nAChR subunits, including α 3- α 7 and β 2- β 4 (Lena et al., 1999; Vincler and Eisenach, 2003; Winzer-Serhan and Leslie, 1997). Besides α 3 β 4, the involvement of other nAChR subtype(s) in nicotine-stimulated [3 H]NE release from rat hippocampal synaptosomes remains unknown.

Several novel nAChR subtype-selective α -conotoxins have recently been discovered, including α -CTX PIA and α -CTX BuIA (Azam et al., 2005; Dowell et al., 2003). α -CTX PIA has been used in the characterization of nAChRs that regulate [3 H]DA release from striatal synaptosomes and confirmed a role for α 6-containing subtypes (Azam and McIntosh, 2005). α -CTX BuIA can kinetically distinguish between β 2 and β 4-containing nAChRs (Azam et al., 2005). In the present study, we have used these toxins in combination with subunit null-mutant mice to characterize murine nAChRs that modulate [3 H]NE release. To our knowledge, this is the first pharmacological characterization of nAChRs on mouse hippocampal noradrenergic terminals. The results indicate that at least 2 different nAChR subtypes are involved. In addition, there are substantial species differences between mice and rats in both the pharmacology and developmental regulation of nAChR subtypes that modulate hippocampal [3 H]NE release.

MATERIALS AND METHODS

Materials. The chemicals were obtained from the following sources: (-)Nicotine hydrogen tartrate, pargyline HCl, bovine serum albumin, ascorbic acid, nisoxetine HCl (Sigma, St. Louis, MO), [3 H]NE (Norepinephrine, Levo-[Ring-2,5,6- 3 H]; 52-53 Ci/mmol) (Perkin-Elmer, Boston, MA), Ecolume scintillation cocktail (MP, Costa Mesa, CA). α -Conotoxins were synthesized as previously described (Azam et al., 2005; Cartier et al., 1996; McIntosh et al., 2004).

Tissue Preparation. Adult male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) were kept two per cage on a 12:12 hr light/dark cycle, with food and water available *ad libitum*. Male and female Sprague-Dawley rat pups (2-3 weeks old) were

kept in the cage with the dam and both sexes were used for the experiments. C57Bl/6J wildtype and null mutant mice that were bred onto C57Bl/6J background were provided by The Institute for Behavioral Genetics (University of Colorado, Boulder, CO) and used by permission from Dr. Arthur Beaudet (Baylor College of Medicine, Baylor, TX). The breeding triads (2 females, 1 male) were kept in the same cage and allowed to mate. Only the first generation pups for each genotype were used for the experiments. For each experiment, hippocampi from 2 adult male rats between 60 and 90 days old, 6 postnatal rats between 14-21 days old, or 2-3 adult male mice or 4-6 mice pups (males and females) between 14-20 days old were used. The animals were decapitated and brains quickly removed. This procedure was approved by the Institutional Animal Care and Use Committee and is consistent with Federal guidelines. Synaptosomes were prepared as described by Azam and McIntosh (2005). Briefly, the hippocampus was quickly dissected on ice and placed in ice-cold 0.32 M sucrose buffer, pH 7.4-7.5. The dissected hippocampus was homogenized by 14 gentle up and down strokes, followed by centrifugation at 1000g for 10 min at 4°C. The supernatant was centrifuged at 12,000g for 20 min at 4°C. The resulting P2 pellet was resuspended in 2 ml of Krebs-HEPES buffer (superfusion buffer) with composition (in mM): NaCl 128, KCl 2.4, KH₂PO₄ 1.2, MgSO₄ 0.6, CaCl₂ 3.2, HEPES 25, Glucose 10 and supplemented with 1 mM ascorbic acid, 0.1 mM pargyline and 0.1 mg/ml BSA. The synaptosomes were incubated for 10 min at 37°C to equilibrate with the superfusion buffer, followed by another 10 min incubation with 0.13 μM [³H]NE (specific activity 52-53 Ci/mmol) at 37°C. For the experiment determining uptake specificity, 0.6 μM nisoxetine HCl was present in the buffer throughout the pre-incubation and incubation periods. The synaptosomes were

centrifuged at 3500 rpm for 5 min in order to get rid of excess radiolabeled NE. The pellet was re-suspended in 4 ml of superfusion buffer and 1 ml was transferred into each of four conical tubes containing 3 ml of superfusion buffer and subsequently loaded into the superfusion chambers containing 13 mm diameter A/E glass fiber filters (Gelman Sciences, Ann Arbor, MI). One tube of the final synaptosomal preparation (4 ml total volume) contained enough synaptosomes for six chambers of the superfusion apparatus.

Superfusion. The superfusion system had 12 identical channels and was set up as described in Kulak et al. (1997), except the peristaltic pumps were switched to Brandel pumps. Once synaptosomes were loaded into the superfusion apparatus, they were washed for 20 min with either superfusion buffer alone or buffer plus varying concentrations of the toxins, at a rate of 0.5 ml/min. For studies where the reversibility of α -CTX BuIA or α -CTX AuIB was examined, synaptosomes were first perfused with buffer containing toxin for 20 min and subsequently perfused for an additional 10 or 20 min with toxin-free buffer. Following the wash period, 2-min fractions were collected in 6 ml polypropylene vials containing 4 ml of Ecolume scintillation cocktail. At the end of the third 2-min fraction, a 1-min pulse of nicotine, nicotine plus toxin, cytisine or cytisine plus toxin was applied, followed by a 10-min wash with superfusion buffer alone. In experiments examining the reversibility of toxins, no toxin was present when the nicotine or cytisine pulse was applied after the toxin washout period. At the end of the superfusion, filters containing the synaptosomes were taken out and placed directly in vials containing 4 ml Ecolume to determine total [3 H]NE uptake. Radioactivity collected in each fraction was quantitated by liquid scintillation spectroscopy, with a Beckman 5801 liquid scintillation counter, tritium efficiency approximately 50%.

Data Analysis. Throughout this paper, tritium release is presumed to correspond directly to amounts of radiolabeled transmitter release, as it has been shown previously that tritium released by nAChR agonists is proportional to total radiolabeled transmitter released (Rapier et al., 1988).

To account for experimental variations in tissue amount, release was calculated relative to the baseline. Baseline release was determined as the average of two fractions before (fractions # 2 and 3) and two fractions after (fractions #6 and 7) the peak release (fractions #4 and 5). Average baseline was subtracted from the evoked release and the resulting values divided by the baseline to yield the evoked release as a percent over baseline. The percent release above baseline in fractions 4 and 5 were then added together to yield total evoked release (or “area-under-the-curve”). Since the total [³H]NE uptake among chambers within each experiment was very consistent (less than 10% deviation), it was assumed that similar amount of tissue was loaded into each chamber. For all data, except those in figures 1B, percent release over baseline was normalized to average release by 100 μM nicotine (or cytosine in case of Fig. 9) alone or to release by 100 μM nicotine in wildtype mice, as indicated. The IC₅₀ for α-CTX BuIA inhibition was determined by non-linear regression analysis using Prism (Graphpad, San Diego, CA). All statistical analysis was performed with Prism. Toxin effects were analyzed by one-way ANOVA, followed by Dunnett’s *post-hoc* for comparisons with nicotine control.

Results

Uptake specificity

The total [^3H]NE uptake into hippocampal synaptosomes prepared from mouse and rat hippocampus is shown in Figure 1A. Tissue from postnatal rat and mouse and adult mouse displayed similar [^3H]NE uptake. Synaptosomes from adult rat, however, displayed significantly higher [^3H]NE uptake (* $p < 0.05$, Dunnett's *posthoc* with adult rat as control). To determine the extent of [^3H]NE uptake into non-noradrenergic terminals within the mouse hippocampus, the synaptosomes were exposed to 0.6 μM nisoxetine, a potent and specific inhibitor of the NE transporter, prior to incubation with radiolabeled neurotransmitter. Similar to rat hippocampal synaptosomes (Barik and Wonnacott, 2006), total [^3H]NE uptake into synaptosomes prepared from mouse hippocampus was reduced by $80 \pm 1.1\%$, suggesting that the majority of radiolabeled NE is taken up by noradrenergic terminals. Additionally, 100 μM nicotine stimulated only $9.3 \pm 4.2\%$ [^3H]NE release above baseline from synaptosomes exposed to 0.6 μM nisoxetine, suggesting that almost all of radiolabeled NE release from mouse hippocampal synaptosomes occurs from noradrenergic terminals.

Figure 1B shows a representative profile of nicotine-evoked [^3H]NE release in adult and postnatal rats and mice. Adult and postnatal rats exhibited similar [^3H]NE release above baseline upon stimulation by 100 μM nicotine, despite the lower total [^3H]NE uptake in postnatal rats (Fig. 1A). Postnatal mouse exhibited lower nicotine-evoked [^3H]NE release than did rat at the same age. Adult mouse exhibited a comparatively low level of nicotine-evoked [^3H]NE release, despite similar total [^3H]NE uptake to both postnatal mouse and rat (Fig. 1A and B). When calculated as the area-under-the-curve, adult and postnatal rats exhibited $199 \pm 16\%$ and $215 \pm 39\%$ [^3H]NE

release above baseline, respectively, and adult and postnatal mice release was $20 \pm 2.2\%$ and $78 \pm 4.5\%$ above baseline, respectively ($p < 0.001$, postnatal mouse release significantly different from adult mouse, Student's t-test).

The $\beta 4$ subunit is implicated in all the nAChRs that modulate [^3H]NE release from adult rat hippocampal synaptosomes

To further assess the contribution of the $\beta 4$ subunit to nicotinic modulation of [^3H]NE release from the adult rat hippocampus, we took advantage of a novel α -conotoxin, α -CTX BuIA, that kinetically distinguishes between $\beta 2^{*1}$ and $\beta 4^*$ nAChRs. Block of rat, human and mouse nAChRs by this toxin is rapidly reversed in subtypes that have an $\alpha x/\beta 2$ interface ($t_{1/2}$ for recovery from block < 1.5 min for all except $\alpha 6\beta 2\beta 3$ where $t_{1/2} \approx 10$ min); in contrast its block is very slowly reversed in nAChRs containing an $\alpha x/\beta 4$ interface ($t_{1/2} > 30$ min) (Azam et al., 2005). α -CTX BuIA dose-dependently inhibited [^3H]NE release from adult rat hippocampal synaptosomes evoked by $100 \mu\text{M}$ nicotine, with an IC_{50} of 88 nM (95% confidence interval: $57\text{-}136 \text{ nM}$) and Hill slope of 1.1 ± 0.23 . At a concentration of $1 \mu\text{M}$, α -CTX BuIA completely inhibited nicotine-evoked [^3H]NE release (Fig. 2). The reversibility of toxin inhibition was next examined. After superfusing the synaptosomes with α -CTX BuIA for 20 min, the synaptosomes were washed with toxin-free buffer. As shown in Fig. 3A, even after a 20-min wash with toxin-free buffer, nicotine-evoked [^3H]NE release did not recover from the level of initial toxin block. To ascertain that the lack of reversibility was a true toxin-dependent effect rather than an artifact of the experimental procedure, the reversibility of the block by α -CTX AuIB was also examined. α -CTX AuIB blocks $\alpha 3\beta 4$ nAChRs more potently than $\alpha 6\beta 4$ nAChRs, with IC_{50} s of $0.5 \pm 0.14 \mu\text{M}$ and $> 5 \mu\text{M}$, respectively ($5 \mu\text{M}$ toxin blocks

rat $\alpha 6\beta 4$ nAChRs expressed in *Xenopus* oocytes by $35.6 \pm 2.7\%$, $n=5$). Toxin block is rapidly reversed for both subtypes ($t_{1/2} < 1.5$ min) (Luo et al., 1998); unpublished observations). $5 \mu\text{M}$ α -CTX AuIB blocked nicotine-evoked [^3H]NE release from adult rat hippocampal synaptosomes by $\sim 50\%$. A 10-min wash with toxin-free buffer was sufficient to completely reverse the partial inhibition of nicotine-evoked [^3H]NE release by α -CTX AuIB (Fig. 3B).

Nicotinic-receptor modulation of [^3H]NE release in C57Bl/6J mice is developmentally regulated

As discussed above, in adult rat hippocampal synaptosomes, nicotine maximally evoked [^3H]NE release at 200% above baseline. In contrast, in adult C57Bl/6J mouse synaptosomes, $100 \mu\text{M}$ nicotine only stimulated [^3H]NE release at $20 \pm 2.2\%$ above baseline, with no additional release at $300 \mu\text{M}$ nicotine. However, a 1-min pulse of 25 mM K^+ stimulated [^3H]NE release at $528 \pm 10.3\%$ above baseline, indicating that the hippocampal terminals of adult mice have the exocytotic machinery required for neurotransmitter release. The lower level of nicotine evoked [^3H]NE release in adult mouse was not further investigated.

In contrast to adult mouse, 2-3 week old pups displayed significantly higher nicotine-evoked [^3H]NE release (Fig. 1B). $100 \mu\text{M}$ nicotine stimulated [^3H]NE release at $78 \pm 4.5\%$ above baseline, with no additional release observed at $300 \mu\text{M}$ nicotine. Therefore, the pharmacological characterization of nicotine-evoked [^3H]NE release in both wildtype (WT) and null mutant mice was carried out during the 2nd to 3rd postnatal week at a nicotine concentration of $100 \mu\text{M}$. Thus, the results drawn from these studies do not necessarily apply to adult mouse.

Nicotine modulation of [³H]NE release from postnatal C57Bl/6J WT mouse hippocampal synaptosomes is mediated by a mixed population of β 2 and β 4-containing nAChRs

Similar to adult rat, 1 μ M α -CTX BuIA almost completely inhibited [³H]NE release from 2-3 week old WT mouse hippocampal synaptosomes. However, in contrast to adult rat (where inhibition was pseudo-irreversible), the inhibition by α -CTX BuIA was partially reversed after toxin washout (Fig. 4A), suggesting the presence of both β 2 (without β 4) and β 4-containing nAChRs.

To further characterize the identity of the nAChR subtypes that regulate [³H]NE release from postnatal WT mouse hippocampal synaptosomes, release was examined in the presence of three subtype-selective α -conotoxins: α -CTX MII (selective for α 3 β 2, α 6 β 2* and α 6 β 4 nAChRs (Cartier et al., 1996; Champiaux et al., 2002; Vailati et al., 1999), α -CTX PIA (selective for α 6 β 2* and α 6 β 4 nAChRs (Dowell et al., 2003)) and α -CTX AuIB (selective for α 3 β 4 (Luo et al., 1998) > α 6 β 4). In contrast to adult rat, where α -CTX MII does not inhibit [³H]NE release (Kulak et al., 1997; Luo et al., 1998), 100 nM α -CTX MII almost completely blocked nicotine-evoked [³H]NE release from postnatal mouse hippocampal synaptosomes (Fig. 5A). 10 nM α -CTX PIA, a concentration that blocks α 6 β 2* nAChRs by ~ 85% and blocks α 6 β 4 nAChRs by ~ 20%, but only blocks α 3 β 2 by ~10% (Dowell et al., 2003), inhibited nicotine-evoked [³H]NE release by $80 \pm 9.4\%$ (Fig. 5A). α -CTX AuIB, at 5 μ M, blocked nicotine-evoked [³H]NE release by $14 \pm 3.8\%$ (Fig. 5A). Since the α -CTX AuIB-sensitive fraction was quantitatively similar to the α -CTX PIA-insensitive release, both toxins were co-applied

to determine if the α -CTX PIA insensitive component could be eliminated by α -CTX AuIB. Co-application of 10 nM α -CTX PIA and 5 μ M α -CTX AuIB did not produce a greater inhibition than that seen with 10 nM α -CTX PIA alone (Fig. 5A).

To ascertain whether the difference in the pharmacology of nicotinic regulation of [3 H]NE release between mice and rats is due to age differences, nicotine-evoked [3 H]NE release was examined in 2-3 week old rat pups. Similar to adult rats, 1 μ M BuIA almost completely and irreversibly inhibited nicotine-evoked [3 H]NE release from postnatal rat hippocampal synaptosomes (Fig. 4B). 100 nM α -CTX MII was ineffective in blocking nicotine-evoked [3 H]NE release from hippocampal synaptosomes of rat pups (Fig. 5B), in contrast to the complete block in postnatal mice (Fig. 5A). 5 μ M α -CTX AuIB partially but significantly blocked [3 H]NE release. Surprisingly, 10 nM α -CTX PIA potentiated [3 H]NE release (Fig. 5B). This was not further investigated.

The α 4, β 2 and β 3 subunits are critical components of nAChRs that modulate [3 H]NE release from postnatal C57Bl/6J mouse hippocampal synaptosomes

To further elucidate the role of the different subunits in nAChRs that modulate [3 H]NE release from postnatal mouse hippocampal terminals, mutant mice that lack a specific nAChR subunit were examined for nicotine-modulation of hippocampal [3 H]NE release. Nicotine-evoked [3 H]NE release was abolished in β 2 null mutant mice (β 2 $^{-/-}$) and significantly decreased in α 4 $^{-/-}$ and β 3 $^{-/-}$ mice (Fig. 6). Interestingly, nicotine-evoked [3 H]NE release in β 4 $^{-/-}$ pups was similar to the level in the WT (Fig. 6), despite the fact that the partial reversibility of α -CTX BuIA indicated the presence of the β 4 subunit in a subpopulation of nAChRs that modulate [3 H]NE release in WT pups (see above). However, α -CTX BuIA block was fully reversed in β 4 $^{-/-}$ pups (Fig. 7B),

suggesting the presence of the $\beta 2$ subunit in all nAChRs. In addition, α -CTX AuIB failed to block [^3H]NE release in $\beta 4^{-/-}$ mice (Fig. 7A), consistent with α -CTX AuIB being inactive on $\beta 2$ -containing nAChRs (Luo et al., 1998).

Nicotine-evoked [^3H]NE release was also pharmacologically examined in synaptosomes from mouse pups that lack either the $\alpha 4$ or the $\beta 3$ subunit. In $\alpha 4^{-/-}$ pups, release was decreased by almost 80% relative to the WT (Fig. 6, 8A), suggesting that the $\alpha 4$ subunit is present in a large proportion of functional nAChRs on hippocampal terminals. The residual release in these animals was completely blocked by α -CTX MII and α -CTX BuIA and blocked approximately 76% by α -CTX PIA (Fig. 8A). In the $\beta 3^{-/-}$ pups, [^3H]NE release was decreased by almost 90% relative to the WT. The residual release was not sensitive to any of the α -CTXs (Fig. 8B).

We also examined whether the developmental decline in nicotine-evoked [^3H]NE release observed in WT mice also occurs in $\beta 4^{-/-}$ mice. Similar to WT mouse, $\beta 4^{-/-}$ adult mouse displayed only $16.9 \pm 10.5\%$ release above baseline in response to $100 \mu\text{M}$ nicotine, with no additional release ($16.6 \pm 4\%$) at $300 \mu\text{M}$ nicotine.

Cytisine is an efficacious agonist at $\beta 2$ -containing nAChRs present on mouse noradrenergic terminals

Cytisine is a nAChR agonist formerly reported to be highly efficacious at $\beta 4$ -containing nAChRs (Chavez-Noriega et al., 1997; Colquhoun and Patrick, 1997; Luetje and Patrick, 1991), but only poorly efficacious at $\beta 2$ -containing nAChRs (Papke and Heinemann, 1994). In WT mouse hippocampal synaptosomes, $100 \mu\text{M}$ cytisine was $90 \pm 6\%$ as efficacious as nicotine in stimulating [^3H]NE release. α -CTX BuIA almost completely inhibited cytisine-stimulated [^3H]NE release in WT mouse, and this block

was reversed by $80.5 \pm 3.4\%$ after a 20-min wash with toxin-free buffer (Fig. 9A), suggesting involvement of mostly $\beta 2$ -containing nAChRs. The high efficacy of cytisine at $\beta 2$ -containing nAChRs was further confirmed in $\beta 4^{-/-}$ mice. In these mice, 100 μM cytisine was $82 \pm 6\%$ as efficacious as nicotine in stimulating [^3H]NE release. This release was almost completely blocked by 1 μM α -CTX BuIA and the block was fully reversed after a 20-min wash with toxin-free buffer (Fig. 9B).

Discussion

In this study, we have utilized a combination of novel subtype selective ligands and nAChR subunit knock-out mice to examine the molecular composition of nAChRs that modulate hippocampal [^3H]NE release. To our knowledge this is the first report to examine nicotine-evoked [^3H]NE release in mouse hippocampus. The data indicate species differences in both the developmental as well as pharmacological profile of nAChRs in mouse vs. rat. Although in rats, nicotine-evoked [^3H]NE release during 2nd to 3rd postnatal week is similar to adult levels (this study and Leslie et al., 2002), NE release decreases with age in mice. A first major pharmacological difference between the two species is that mouse nAChRs are potently blocked by $\alpha 6^*$ -selective antagonists whereas rat nAChRs are not. Second, in addition to a population of $\beta 2$ and $\beta 4$ -containing nAChRs, there is a separate sub-population of only $\beta 2$ -containing nAChRs on mouse noradrenergic terminals, whereas in the rat, all nAChRs appear to contain a $\beta 4$ subunit. The results from the present study are summarized in Table 1.

nAChRs on rat hippocampal noradrenergic terminals

Previous work identified a subpopulation of $\alpha 3\beta 4$ -like nAChRs in nicotine-evoked [^3H]NE release from adult rat hippocampal synaptosomes (Clarke and Reuben,

1996; Luo et al., 1998). In the present study, it was shown that the $\beta 4$ subunit is present in most, if not all, nAChRs that modulate nicotine-evoked [^3H]NE release in both adult and postnatal rats, as evidenced by the pseudo-irreversible block by α -CTX BuIA. In addition, there is an absence of nAChRs that have an $\alpha 6/\beta x$ or $\alpha 3/\beta 2$ subunit interface in both developmental time-points, as evidenced by the lack of block by α -CTX MII (Kulak et al., 1997; Luo et al., 1998). The partial block by 5 μM α -CTX AuIB indicates the presence of a population of $\alpha 3\beta 4^*$ and/or $\alpha 6\beta 4^*$ nAChRs. However, lack of block by α -CTX MII excludes the $\alpha 6\beta 4^*$ subtype. This is in contrast to LC nAChRs that modulate adult rat hippocampal NE release. Microinjection of α -CTX MII and α -CTX AuIB into the LC blocks hippocampal NE release by 67% and 44%, respectively. Co-administration of the two toxins does not produce a greater inhibition than α -CTX MII, suggesting presence of nAChRs with both the $\beta 2$ and the $\beta 4$ subunits (Fu et al., 1999). The greater inhibition by α -CTX MII suggests the possible presence of additional $\alpha 3\beta 2^*$ and/or $\alpha 6\beta 2^*$ subtype(s), without $\beta 4$, that are not sensitive to block by α -CTX AuIB. The present findings, together with those of Fu et al., 1999, indicate that systemic nicotine stimulates hippocampal NE release by targeting different subtypes of nAChRs that are present in rat LC and on rat hippocampal NE terminals.

Possible candidates for the α -CTX AuIB-resistant nAChRs on rat hippocampal noradrenergic terminals are those that contain an $\alpha 4/\beta 4$ or $\alpha 2/\beta 4$ interface. However, in light of the absence of $\alpha 2$ mRNA in both postnatal and adult LC (Lena et al., 1999; Vincler and Eisenach, 2003), the $\alpha 2\beta 4^*$ subtype can be excluded. The $\alpha 4$ subunit mRNA and protein have been detected in the rat LC (Lena et al., 1999; Vincler and

Eisenach, 2003). The $\beta 2$ subunit may also be present on the terminals, but the irreversible block by α -CTX BuIA suggests that any $\beta 2$ -containing nAChRs also contain a $\beta 4$ subunit at the other ligand-binding interface. The presence of the putative structural subunits $\alpha 5$ and $\beta 3$ in these nAChRs is also a possibility, especially since the mRNA for both subunits have been detected in the LC (Lena et al., 1999).

nAChRs on mouse hippocampal noradrenergic terminals

In the mouse hippocampus, much more detailed studies were able to be performed due to the availability of nAChR subunit deficient mice. In contrast to postnatal and adult rats, results from the null mutant mice indicate that all nAChRs that regulate nicotine-evoked [3 H]NE release contain a $\beta 2$ subunit. A large proportion of these nAChRs also contain $\beta 3$ and/or $\alpha 4$ subunits (Table 1). In WT mice, partial reversibility of block by α -CTX BuIA indicates the presence of both $\beta 4^*$ and $\beta 2^*$ (without $\beta 4$) nAChR subtypes. The $\beta 2$ subunit, however, appears to be able to compensate for the $\beta 4$ subunit in $\beta 4^{-/-}$ mice, as evidenced by the lack of a decrease in the total amount of [3 H]NE release, lack of effect of α -CTX AuIB and, most notably, the complete reversibility of α -CTX BuIA inhibition.

In WT mice, block of [3 H]NE release by the $\alpha 6\beta x/\alpha 3\beta 2$ -selective α -CTX MII and the $\alpha 6\beta x$ selective α -CTX PIA indicates that most, if not all, receptors also contain an $\alpha 6$ subunit. The lack of co-additivity of inhibition by 10 nM α -CTX PIA and 5 μ M α -CTX AuIB suggests a common site of action, possibly the $\alpha 6\beta 4^*$, rather than an $\alpha 3\beta 4^*$, subtype. However, the presence of a small population of $\alpha 6(\alpha 3)\beta 4^*$ subtype that is sensitive to block by both α -CTX AuIB and α -CTX PIA can not be ruled out. This is in

contrast to the rat, where approximately half of nicotine-evoked [^3H]NE release is modulated by $\alpha 3\beta 4^*$, but not $\alpha 6\beta 4^*$ or $\alpha 6\alpha 3\beta 4^*$ nAChRs (Luo et al., 1998).

To further investigate the pharmacology of nAChRs on mouse noradrenergic terminals, cytosine, a ligand formerly believed to only activate $\beta 4$ -containing nAChRs with high efficacy (Chavez-Noriega et al., 1997; Colquhoun and Patrick, 1997; Luetje and Patrick, 1991) was used. More recent studies, however, have shown that cytosine binds to α -CTX MII sensitive sites, although with low affinity (Whiteaker et al., 2000). Additionally, one study has demonstrated that cytosine is as efficacious as nicotine and acetylcholine in activating α -CTX MII-sensitive nAChRs that modulate [^3H]DA release from mouse striatal synaptosomes (Salminen et al., 2004). Similar to the latter study, in the present study cytosine was nearly as efficacious as nicotine in stimulating [^3H]NE release from WT hippocampal synaptosomes. In addition, cytosine was also effective in stimulating [^3H]NE release from hippocampal synaptosomes of $\beta 4^{-/-}$ mice, where all the nAChRs contain the $\alpha 6\beta 2$ interface and are α -CTX MII sensitive. These results confirm that cytosine is a highly efficacious agonist at the $\alpha 6\beta 2^*$ nAChRs present on mouse noradrenergic terminals.

Nicotine-evoked [^3H]NE release was significantly reduced in mice lacking the $\alpha 4$ subunit, suggesting that along with the $\beta 2$ subunit, the $\alpha 4$ subunit is a critical component of the majority of nAChRs that stimulate [^3H]NE release in postnatal mouse hippocampus. The residual release in $\alpha 4^{-/-}$ mice is abolished by 100 nM α -CTX MII and by $\sim 80\%$ by 10 nM α -CTX PIA. This suggests that the small residual release in $\alpha 4^{-/-}$ mice may be mediated by a population of $\alpha 6\beta 2\beta 3(\beta 4)$ receptors that is still functional in the absence of the $\alpha 4$ subunit.

Deletion of the $\beta 3$ subunit largely eliminates nicotine-evoked [^3H]NE release from mouse hippocampal synaptosomes. This result suggests that almost all the nAChRs on mouse noradrenergic terminals contain a $\beta 3$ subunit. It has been shown that the α -CTX MII sensitive component of nicotine-evoked [^3H]DA release from striatal synaptosomes is substantially reduced in $\beta 3^{-/-}$ adult mice (Cui et al., 2003; Salminen et al., 2004). In light of the finding that nicotine-evoked [^3H]NE release from hippocampus of mouse pups is completely α -CTX MII sensitive (this study), the loss of the [^3H]NE release in $\beta 3^{-/-}$ is consistent with this subunit being a critical component of native α -CTX MII sensitive nAChRs that modulate catecholamine release in the CNS. Notably, it has recently been shown that the deletion of the $\beta 3$ subunit decreases the number of $\alpha 6$ -containing nAChRs on mouse DAergic terminals (Gotti et al., 2005), indicating that $\alpha 6$ and $\beta 3$ subunits co-participate in formation of native nAChRs.

Additional implications

NE is a neurotransmitter that is important for attentiveness, working memory and learning. Compounds that enhance memory also increase the release of NE within the hippocampus (Lee et al., 1993; Lee and Ma, 1995). The cognitive-enhancing properties of nicotine may, in part, be mediated by the release of NE within the hippocampus. SIB-1553A, a nicotinic receptor ligand with selectivity for $\beta 4$ -containing nAChRs, improves attention and working memory in both rats and mice (Bontempi et al., 2003; Bontempi et al., 2001; Terry et al., 2002). SIB-1553A's beneficial effects on working memory/attention may, in part, be mediated by release of ACh (Bontempi et al., 2001; Rao et al., 2003b), and/or NE within the hippocampus (Rao et al., 2003a). The results of the present study, which indicate the presence of the $\beta 4$ subunit in nAChRs that modulate

[³H]NE release in both rat and mouse hippocampus, provides additional support for the latter.

In addition to its role as a neurotransmitter, NE acts as a neurotrophic factor in the immature CNS, regulating cell proliferation, differentiation and synaptogenesis. In the rat cerebellum, another late maturing structure, nicotine-evoked [³H]NE release is significantly higher during the 2nd to 3rd postnatal week as compared to the adult, similar to the situation observed in the present study for the mouse hippocampus (O'Leary and Leslie, 2003). The authors attributed the transient [³H]NE release to key developmental events occurring during the period of observed peak release. It is possible that similar developmental events also occur in the mouse hippocampus during the postnatal period when nicotine-evoked [³H]NE release is observed.

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Footnotes

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¹Asterisk indicates presence of other subunits

Figure legends

Figure 1: Total [³H]NE uptake and release profile in adult and postnatal rat and mouse.

(A) Total synaptosomal [³H]NE uptake, shown as counts per minute (CPM), is similar in postnatal mouse and rat and adult mouse, but significantly higher in adult rat. **p*<0.05, Dunnett's *posthoc* with adult rat as control. Values are mean ± SEM from three experiments. (B) Representative nicotine-evoked [³H]NE release profile from hippocampal synaptosomes of rat and mouse. Adult and postnatal (2-3 weeks old) rats display similar [³H]NE release above baseline in response to 100 μM nicotine. In contrast, although postnatal (2-3 weeks old) mice show nicotine-evoked [³H]NE release, adult mice do not display significant release above baseline. Values are averages from three experiments. Nic: nicotine.

Figure 2: Concentration-response curve of α-CTX BuIA inhibition of nicotine-stimulated [³H]NE release from adult rat hippocampal synaptosomes. Percent release above baseline is normalized to 100 μM nicotine control (100%). Inhibition by α-CTX BuIA is significant at concentrations ≥10 nM and complete at 1 μM. The IC₅₀ for inhibition is 88 nM (Confidence Interval: 57-136 nM) with a Hill coefficient of 1.1 ± 0.23. 100 μM nicotine was used. ****p*<0.001, Dunnett's *posthoc* with 100 μM nicotine alone as control. Values are mean ± SEM from at least three experiments.

Figure 3: Reversibility of α-CTX BuIA and α-CTX AuIB block of nicotine-evoked [³H]NE release from adult rat hippocampal synaptosomes. Percent release above baseline is normalized to 100 μM nicotine control (100%). (A) α-CTX BuIA (100 nM

and 1 μM) inhibition of nicotine-stimulated [^3H]NE is not reversed after a 10 or 20 min wash with toxin-free buffer. The “control” bars represent release in presence of the toxin without a toxin-free wash. (B) α -CTX AuIB blocks nicotine-evoked [^3H]NE release from adult rat hippocampal synaptosomes by 50%, but in contrast to α -CTX BuIA, the block is completely reversed after a 10 min wash with toxin-free buffer. *** $p < 0.001$, significantly different from 100 μM nicotine control, Dunnett’s *posthoc*. Values are mean \pm SEM from at least three experiments. Nic: nicotine.

Figure 4: α -CTX BuIA inhibition of nicotine-evoked [^3H]NE release from postnatal mouse and rat hippocampal synaptosomes. Percent release above baseline is normalized to 100 μM nicotine control (100%). (A) 1 μM α -CTX BuIA blocks [^3H]NE release from mouse hippocampal synaptosomes and its block is partially reversed after 10 and 20-min washes with toxin-free buffer. (B) 1 μM α -BuIA almost completely blocks nicotine-evoked [^3H]NE release from postnatal rat hippocampal synaptosomes, but its block is not reversed even after a 20-min wash with toxin-free buffer. *** $p < 0.001$, significantly different from 100 μM nicotine control, Dunnett’s *posthoc*. Values are mean \pm SEM from at least three experiments. Nic: nicotine.

Figure 5: Effect of selective antagonists on nicotine-evoked [^3H]NE release from WT postnatal mouse and postnatal rat hippocampal synaptosomes. Percent release above baseline is normalized to 100 μM nicotine control (100%). A) α -CTX MII completely blocks nicotine-evoked [^3H]NE release from postnatal mouse hippocampal synaptosomes, whereas α -CTX PIA blocks release by ~80%. α -CTX AuIB blocks

release by only about 15%. Co-application of α -CTX PIA and α -CTX AuIB does not produce a greater inhibition than α -CTX PIA alone. B) α -CTX MII is ineffective in blocking nicotine-evoked [3 H]NE release from postnatal rat hippocampal synaptosomes, whereas α -CTX PIA significantly potentiates release. α -CTX AuIB blocks release by ~30%. * p <0.05, *** p <0.001, significantly different from 100 μ M nicotine control, Dunnett's *post-hoc*. Values are mean \pm SEM from at least three experiments.

Figure 6: Nicotine-evoked [3 H]NE release from hippocampal synaptosomes of WT (+/+) and nAChR subunit null-mutant mice. Percent release above baseline is normalized to release by 100 μ M nicotine in WT mice (100%). β 4 $^{-/-}$ mice show the same amount of [3 H]NE release as the WT mice. However, release is abolished in hippocampal synaptosomes of β 2 $^{-/-}$ mice, whereas it is significantly reduced in α 4 $^{-/-}$ and β 3 $^{-/-}$ mice. *** p <0.001 Dunnett's *posthoc*, with WT release as control. Values are mean \pm SEM from at least three experiments.

Figure 7: Effect of selective antagonists on nicotine-evoked [3 H]NE release from postnatal β 4 $^{-/-}$ mouse hippocampal synaptosomes. Percent release above baseline is normalized to 100 μ M nicotine control (100%). A) α -CTX BuIA block of nicotine-evoked [3 H]NE release from β 4 $^{-/-}$ mouse hippocampal synaptosomes is almost completely reversed after 10- and 20-min washes with toxin-free buffer. B) Both α -CTX MII and α -CTX PIA inhibit nicotine-evoked [3 H]NE release, whereas α -CTX AuIB is ineffective in inhibiting this release. *** p <0.001, Dunnett's *post-hoc* with 100 μ M

nicotine alone as control. Values are mean \pm SEM from three experiments. Nic:
nicotine.

Figure 8: Effect of α -CTX MII, α -CTX PIA and α -CTX BuIA on nicotine-evoked [3 H]NE release from hippocampal synaptosomes of $\alpha 4^{-/-}$ and $\beta 3^{-/-}$ postnatal mice. Percent release above baseline is normalized to release by 100 μ M nicotine in WT mice (100%) (A) α -CTX MII and α -CTX BuIA completely inhibit the residual [3 H]NE release in $\alpha 4^{-/-}$ -mice. Although α -PIA inhibits the release by \sim 80%, this inhibition does not reach significance. (B) Nicotine-evoked [3 H]NE release is almost completely abolished in $\beta 3^{-/-}$ mice. The residual release is not sensitive to block by any of the toxins. Values are mean \pm SEM from three experiments. Nic: nicotine.

Figure 9: Cytisine (Cyt) stimulation of [3 H]NE release from WT and $\beta 4^{-/-}$ mouse hippocampal synaptosomes. Percent release above baseline is normalized to 100 μ M cytisine control (100%). A) α -BuIA blocks cytisine-stimulated release in WT mouse by more than 80%; however, this block, although still significant, is mostly reversed after a 20-min toxin washout period. B) α -BuIA inhibits cytisine-stimulated [3 H]NE release in $\beta 4^{-/-}$ mouse, but its block is fully reversed after a 20-min washout. * $p < 0.05$, *** $p < 0.001$, significantly different from 100 μ M cytisine control, Dunnett's *post-hoc*. Values are mean \pm SEM from three experiments.

Table 1: Composition of nAChR subtypes expressed on hippocampal noradrenergic terminals of rat and mouse. For each subtype, the percent contributing to NE release has been determined from the portion of the release that is sensitive to the particular subtype-selective α -conotoxin.

nAChR Subtype	% contributing to NE release	α -conotoxin
Adult Rat $\alpha 3\beta 4$	50%	AuIB sensitive; slow BuIA reversibility
$\alpha x\beta 4$ (not $\alpha 6$)	50%	AuIB and MII insensitive; slow BuIA reversibility.
Postnatal rat $\alpha 3\beta 4$	30%	AuIB sensitive; slow BuIA reversibility
$\alpha x\beta 4$ (not $\alpha 6$)	70%	AuIB and MII insensitive; slow BuIA reversibility.
Adult mice	nAChR-mediated release too small to perform pharmacological studies	
Postnatal mice $\alpha 6(\alpha 4)\beta 2(\beta 3)$	65%	MII and PIA sensitive; rapid BuIA reversibility
$\alpha 6(\alpha 4)\beta 2\beta 4(\beta 3)$	35%	MII and PIA sensitive; slow BuIA reversibility

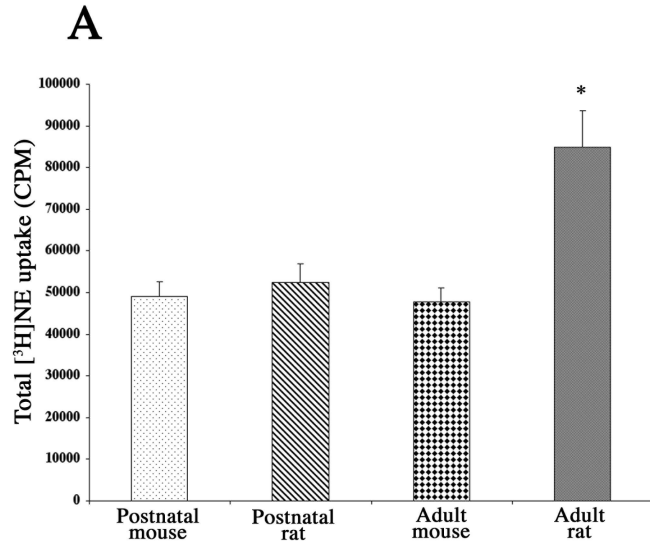


Figure 1

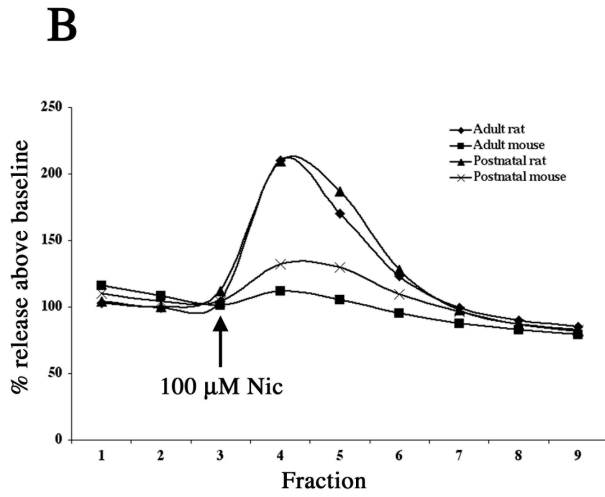
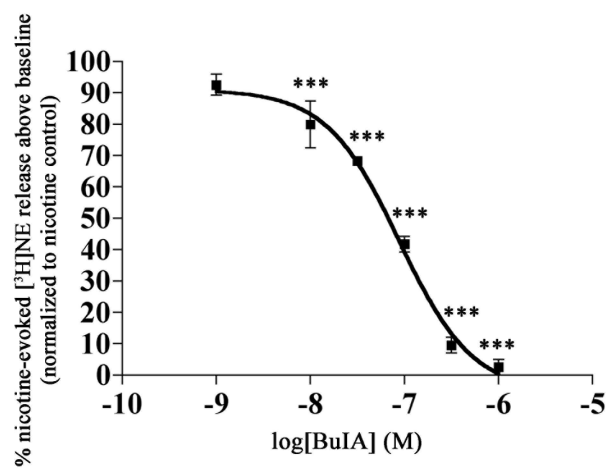


Figure 2



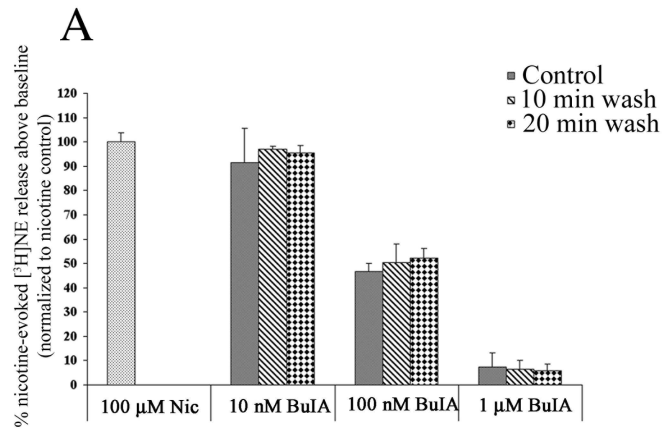


Figure 3

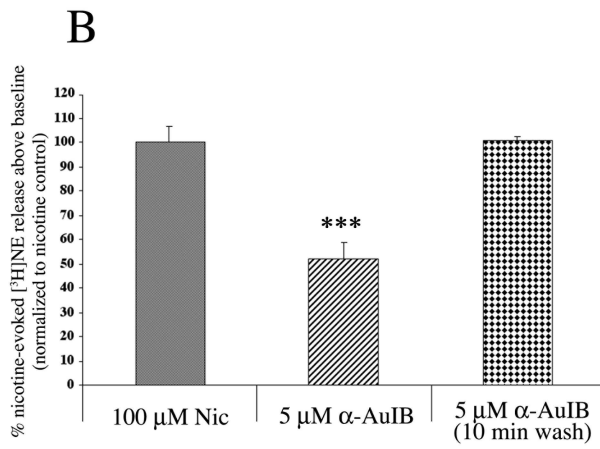


Figure 4

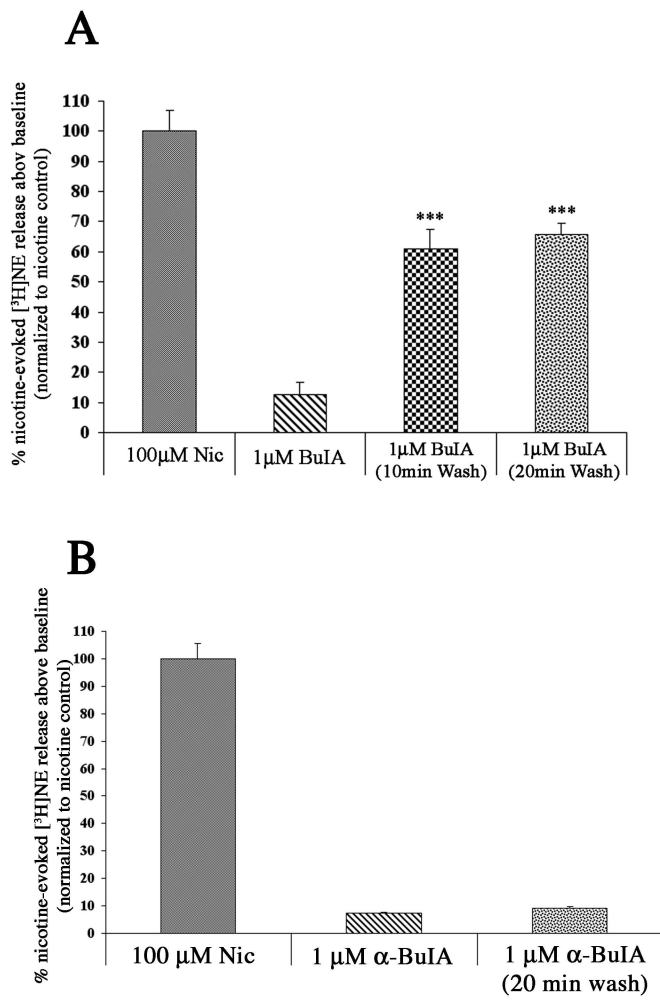


Figure 5

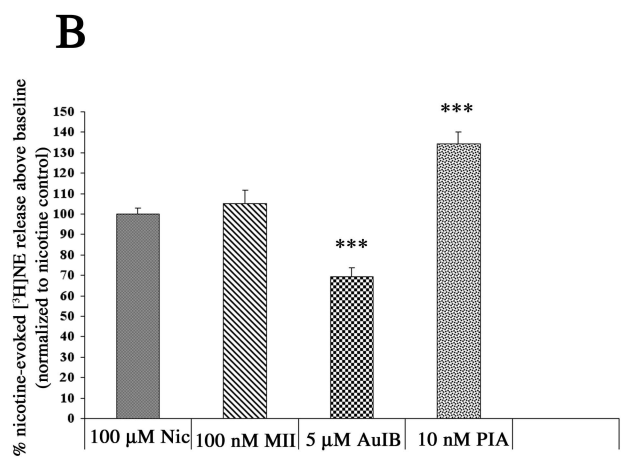
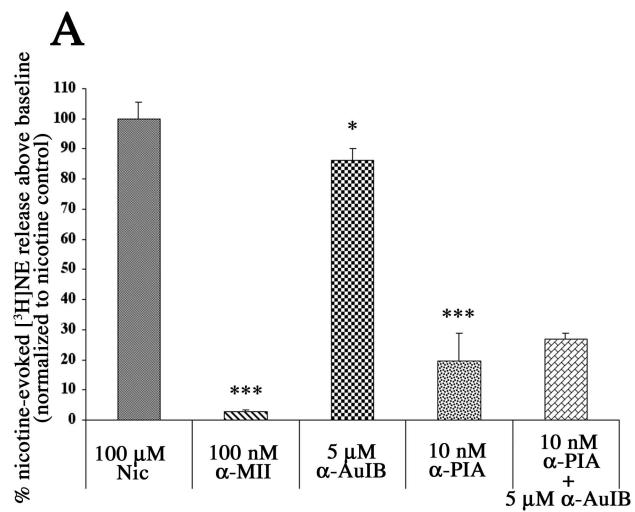


Figure 6

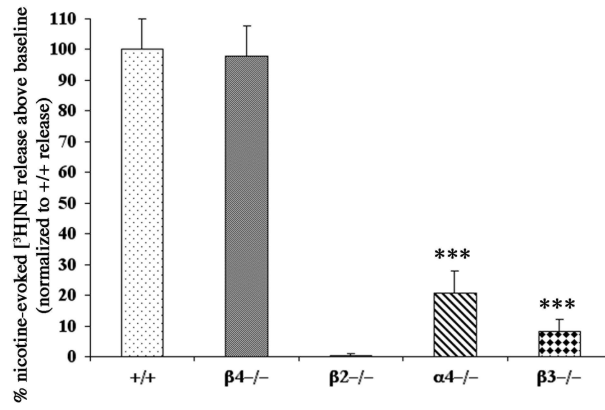


Figure 7

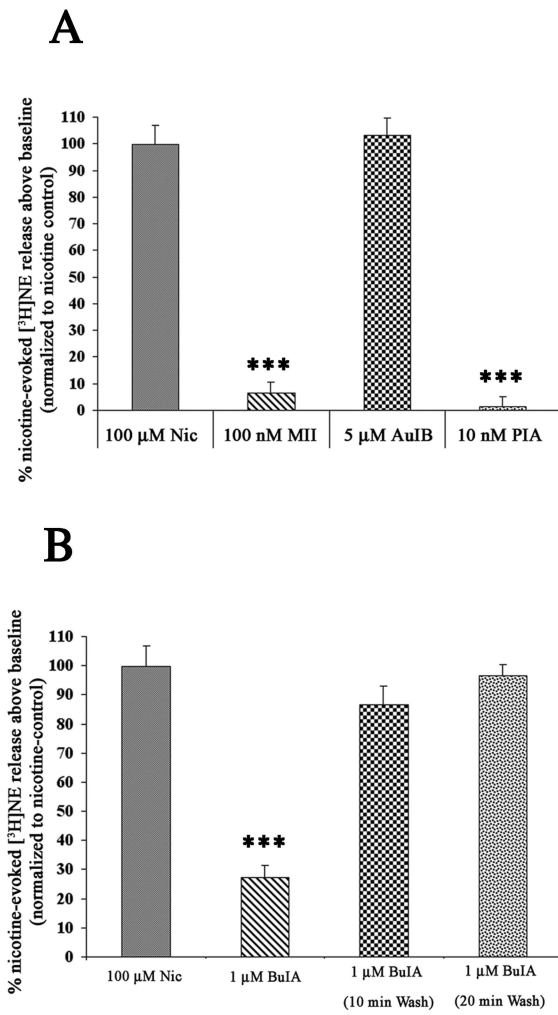


Figure 8

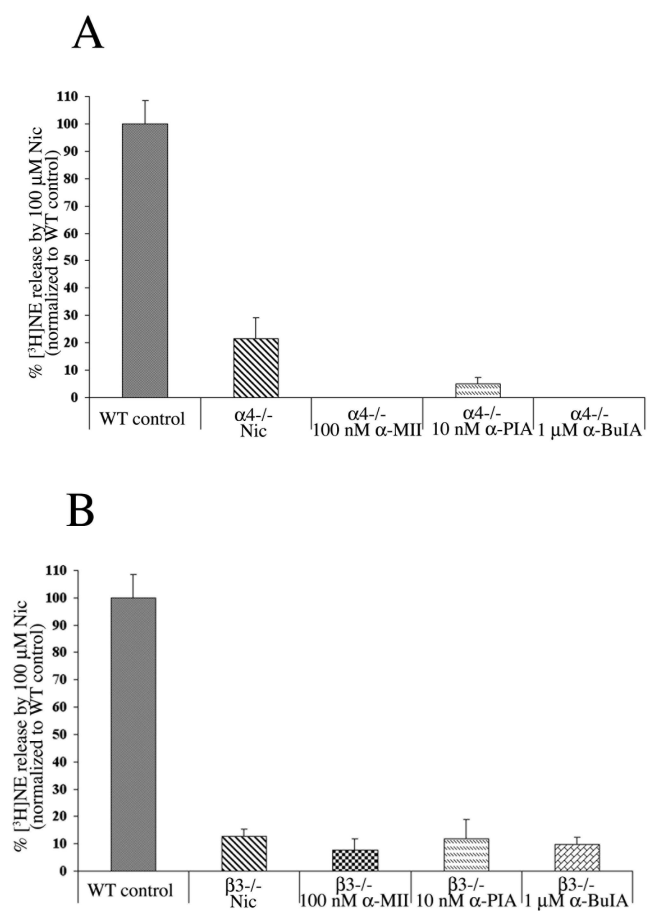


Figure 9

