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Characterization of Nicotinic Acetylcholine Receptors That Modulate Nicotine-Evoked [^{3}H]Norepinephrine Release from Mouse Hippocampal Synaptosomes

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ABSTRACT

Nicotine's modulation of hippocampal noradrenergic neurotransmission may contribute to its mnemonic properties, but the nicotinic acetylcholine receptor (nAChR) subtypes that modulate terminal release of norepinephrine are unknown. In the present study, we used a number of subtype-selective α-conotoxins in combination with nicotinic receptor subunit-deficient mice to characterize nAChRs that modulate [^{3}H]norepinephrine release from synaptosomes. The results indicate that at least two populations of nAChRs contribute to this release: a novel $\alpha6(\alpha4)\beta2\beta3\beta4$ subtype and an $\alpha6(\alpha4)\beta2\beta3$ subtype. These are distinct from subtypes that modulate synaptosomal norepinephrine release in the rat hippocampus in which an $\alpha6/\beta2$ and/or $\alpha6/\beta4$ ligand binding interface is not present. Whereas α-conotoxin MII fully inhibits nicotine-evoked [^{3}H]norepinephrine release in mouse, it is ineffective in blocking [^{3}H]norepinephrine release in rat. Block of [^{3}H]norepinephrine release by α-conotoxin BuIA, a toxin that kinetically distinguishes between $\beta2$- and $\beta4$-containing nAChRs, was partially reversible in mouse but irreversible in rat. This indicates that in contrast to rat, mouse nAChRs are made of both $\beta4$ and non-$\beta4$-containing populations. Results from $\beta2$ and $\beta4$ null mutant mice confirmed this conclusion, indicating the presence of the $\beta2$ subunit in all nAChRs and the presence of the $\beta4$ subunit in a subpopulation of nAChRs. In addition, both $\alpha4$ and $\beta3$ subunits are essential for the formation of functional nAChRs on mouse noradrenergic terminals. Cytisine, a ligand formerly believed to be $\beta4$-selective, was a highly effective agonist for $\alpha6\beta2$-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 (Kulak et al., 1997; Grady et al., 2002) and α-CTX PIA (Azam and McIntosh, 2005). Knockout studies have shown that both subtypes require the $\beta2$ subunit, because nicotine-evoked [^{3}H]DA release is completely abolished in $\beta2$-null mutant mice (Grady et al., 2001; Salminen et al., 2004). Knockout and immunoprecipitation studies have identified at least four different subtypes on the DAergic terminals: $\alpha6\beta2\beta3$, $\alpha6\alpha4\beta2\beta3$, $\alpha4\beta2$, and $\alpha4\alpha5\beta2$ (Zoli et al., 2002; Champtiaux et al., 2003).

In contrast to the striatal DAergic system, there are limited data on nicotinic regulation of hippocampal noradrenergic neurotransmission in rats (Sacaan et al., 1996; Sershen et al., 1997; Fu et al., 1998) 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 $\alpha3\beta4$-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 $\alpha3$–$\alpha7$ and $\beta2$–$\beta4$ (Winzer-Serhan and Leslie, 1997; Lena et al., 1999; Vincler and Eisenach, 2003). Besides $\alpha3\beta4$, 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

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; CTX, conotoxin; DA, dopamine; LC, locus ceruleus; NE, norepinephrine; WT, wild-type.

recently been discovered, including α-CTX PIA and α-CTX BuIA (Dowell et al., 2003; Azam et al., 2005). 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 α6-containing subtypes (Azam and McIntosh, 2005). α-CTX BuIA can kinetically distinguish between β2- and β4-containing nAChRs (Azam et al., 2005). In the present study, we 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 two 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, and nisoxetine HCl were from Sigma (St. Louis, MO); [^{3}H]NE (L-[ring-2,5,6-^{3}H]norepinephrine; 52–53 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Boston, MA); and Ecolume scintillation cocktail was from MP Biomedicals (Irvine, CA). α-Conotoxins were synthesized as described previously (Cartier et al., 1996; McIntosh et al., 2004; Azam et al., 2005).

Tissue Preparation. Adult male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) were kept two per cage on a 12:12-h 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 wild-type 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 were used by permission from Dr. Arthur Beaudet (Baylor College of Medicine, Baylor, TX). The breeding triads (two female rats, one male rat) were kept in the same cage and were 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 to 21 days old, or 2 to 3 adult male mice or 4 to 6 mice pups (male and female) between 14 and 20 days old were used. The animals were decapitated, and brains were removed quickly. 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). In brief, the hippocampus was quickly dissected on ice and placed in ice-cold 0.32 M sucrose buffer, pH 7.4 to 7.5. The dissected hippocampus was homogenized by 14 gentle up and down strokes, followed by centrifugation at 1000*g* for 10 min at 4°C. The supernatant was centrifuged at 12,000*g* for 20 min at 4°C. The resulting P2 pellet was resuspended in 2 ml of Krebs-HEPES buffer (superfusion buffer) with composition of 128 mM NaCl, 2.4 mM KCl, 1.2 mM KH_2PO_4, 0.6 mM $MgSO_4$, 3.2 mM $CaCl_2$, 25 mM HEPES, 10 mM glucose, and supplemented with 1 mM ascorbic acid, 0.1 mM pargyline, and 0.1 mg/ml bovine serum albumin. 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 [^{3}H]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 preincubation and incubation periods. The synaptosomes were centrifuged at 3500 rpm for 5 min to get rid of excess radiolabeled NE. The pellet was resuspended 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 in which 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. After 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 of 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 article, 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 percentage over baseline. The percentage release above baseline in fractions 4 and 5 were then added together to yield total evoked release (or area under the curve). Because the total [^{3}H]NE uptake among chambers within each experiment was very consistent (less than 10% deviation), it was assumed that a similar amount of tissue was loaded into each chamber. For all data, except those in Fig. 1B, the percentage release over baseline was normalized to average release by 100 μM nicotine (or cytisine in case of Fig. 9) alone or to release by 100 μM nicotine in wild-type mice, as indicated. The IC_{50} value for α-CTX BuIA inhibition was determined by nonlinear regression analysis using Prism (GraphPad Software Inc., San Diego, CA). All statistical analysis was performed with Prism. Toxin effects were analyzed by one-way analysis of variance, followed by Dunnett's post hoc test for comparisons with nicotine control.

Results

Uptake Specificity. The total [^{3}H]NE uptake into hippocampal synaptosomes prepared from mouse and rat hippocampus is shown in Fig. 1A. Tissue from postnatal rat and mouse and adult mouse displayed similar [^{3}H]NE uptake. Synaptosomes from adult rat, however, displayed significantly higher [^{3}H]NE uptake ($*p < 0.05$, Dunnett's post hoc test with adult rat as control). To determine the extent of [^{3}H]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, before incubation with radiolabeled neurotransmitter. Similar to rat hippocampal synaptosomes (Barik and Wonnacott, 2006), total [^{3}H]NE uptake into synaptosomes prepared from mouse hippocampus was reduced by 80 ± 1.1%, suggesting that the majority of radiolabeled NE is taken up by noradrenergic terminals. In addition, 100 μM nicotine stimulated only 9.3 ± 4.2% [^{3}H]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 [^{3}H]NE release in adult and postnatal rats and mice. Adult and postnatal rats exhibited similar [^{3}H]NE release above baseline upon stimulation by 100 μM nicotine, despite the lower total [^{3}H]NE uptake in postnatal rats (Fig. 1A). Postnatal mouse exhibited lower nicotine-evoked [^{3}H]NE release than did rat at the same age. Adult mouse exhibited a comparatively low level of nicotine-evoked [^{3}H]NE release, despite similar total [^{3}H]NE uptake to both postnatal mouse and rat (Fig. 1, A and B). When calculated as the area under the curve, adult and postnatal rats exhibited 199 ± 16% and 215 ± 39% [^{3}H]NE release above baseline, respectively, and adult and postnatal mice release was 20 ± 2.2% and 78 ± 4.5% above baseline, respectively ($p < 0.001$, postnatal mouse release significantly different from adult mouse, Student's t test).

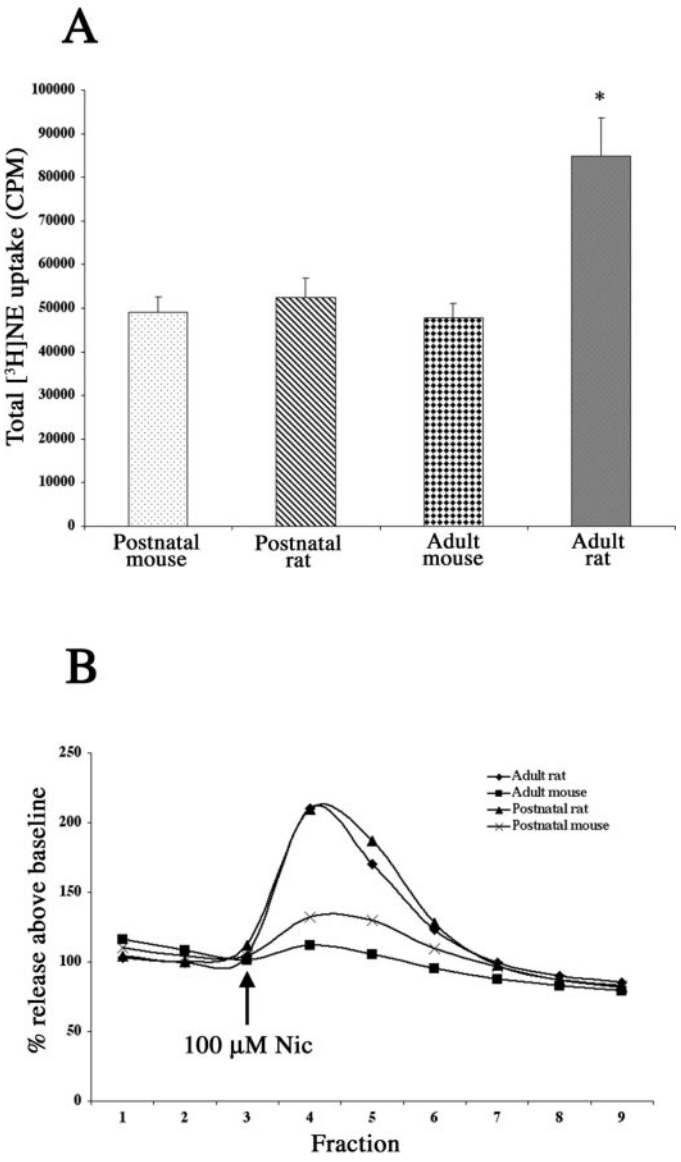

Fig. 1. Total [^{3}H]NE uptake and release profile in adult and postnatal rat and mouse. A, total synaptosomal [^{3}H]NE uptake, shown as counts per minute (CPM), is similar in postnatal mouse and rat and adult mouse but significantly higher in adult rat. *, $p < 0.05$, Dunnett's post hoc test with adult rat as control. Values are mean ± S.E.M. from three experiments. B, representative nicotine-evoked [^{3}H]NE release profile from hippocampal synaptosomes of rat and mouse. Adult and postnatal (2–3 weeks old) rats display similar [^{3}H]NE release above baseline in response to 100 μM nicotine. In contrast, although postnatal (2–3 weeks old) mice show nicotine-evoked [^{3}H]NE release, adult mice do not display significant release above baseline. Values are averages from three experiments. Nic, nicotine.

The β4 Subunit Is Implicated in All of the nAChRs that Modulate [^{3}H]NE Release from Adult Rat Hippocampal Synaptosomes. To further assess the contribution of the β4 subunit to nicotinic modulation of [^{3}H]NE release from the adult rat hippocampus, we took advantage of a novel α-conotoxin, α-CTX BuIA, that kinetically distinguishes between β2* (* indicates the presence of other subunits) and β4* nAChRs. Block of rat, human, and mouse nAChRs by this toxin is rapidly reversed in subtypes that have an αx/β2 interface ($t_{1/2}$ for recovery from block <1.5 min for all except α6β2β3, where $t_{1/2} \approx 10$ min); in contrast, its block is very slowly reversed in nAChRs containing an αx/β4 interface ($t_{1/2} > 30$ min) (Azam et al., 2005). α-CTX BuIA dose-dependently inhibited [^{3}H]NE release from adult rat hippocampal synaptosomes evoked by 100 μM nicotine, with an IC_{50} value of 88 nM (95% confidence interval, 57–136 nM) and Hill slope of 1.1 ± 0.23. At a concentration of 1 μM, α-CTX BuIA completely inhibited nicotine-evoked [^{3}H]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 [^{3}H]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 α3β4 nAChRs more potently than

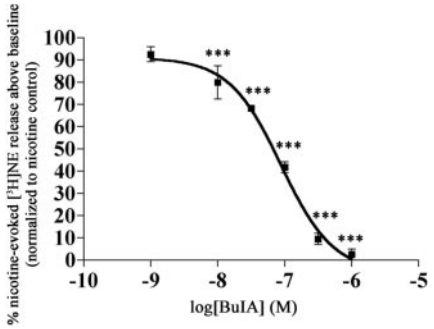

Fig. 2. Concentration-response curve of α-CTX BuIA inhibition of nicotine-stimulated [^{3}H]NE release from adult rat hippocampal synaptosomes. Percentage of release above baseline is normalized to 100 μM nicotine control (100%). Inhibition by α-CTX BuIA is significant at concentrations ≥10 nM and complete at 1 μM. The IC_{50} value for inhibition is 88 nM (confidence interval, 57–136 nM) with a Hill coefficient of 1.1 ± 0.23. ***, $p < 0.001$, Dunnett's post hoc test with 100 μM nicotine alone as control. Values are mean ± S.E.M. from at least three experiments.

$\alpha6\beta4$ nAChRs, with IC_{50} values of 0.5 ± 0.14 μM and >5 μM, respectively (5 μM toxin blocks rat $\alpha6\beta4$ nAChRs expressed in *Xenopus laevis* oocytes by 35.6 ± 2.7%, $n = 5$). Toxin block is rapidly reversed for both subtypes ($t_{1/2} < 1.5$ min) (Luo et al., 1998; L. Azam, unpublished observations). α-CTX AuIB (5 μM) blocked nicotine-evoked [^{3}H]NE release from adult rat hippocampal synaptosomes by ~50%. A 10-min wash with toxin-free buffer was sufficient to completely reverse the partial inhibition of nicotine-evoked [^{3}H]NE release by α-CTX AuIB (Fig. 3B).

Nicotinic-Receptor Modulation of [^{3}H]NE Release in C57BL/6J Mice Is Developmentally Regulated. As discussed above, in adult rat hippocampal synaptosomes, nicotine maximally evoked [^{3}H]NE release at 200% above baseline. In contrast, in adult C57BL/6J mouse synaptosomes, 100 μM nicotine only stimulated [^{3}H]NE release at 20 ± 2.2% above baseline, with no additional release at 300 μM nicotine. However, a 1-min pulse of 25 mM K^+ stimulated [^{3}H]NE release at 528 ± 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 [^{3}H]NE release in adult mouse was not further investigated.

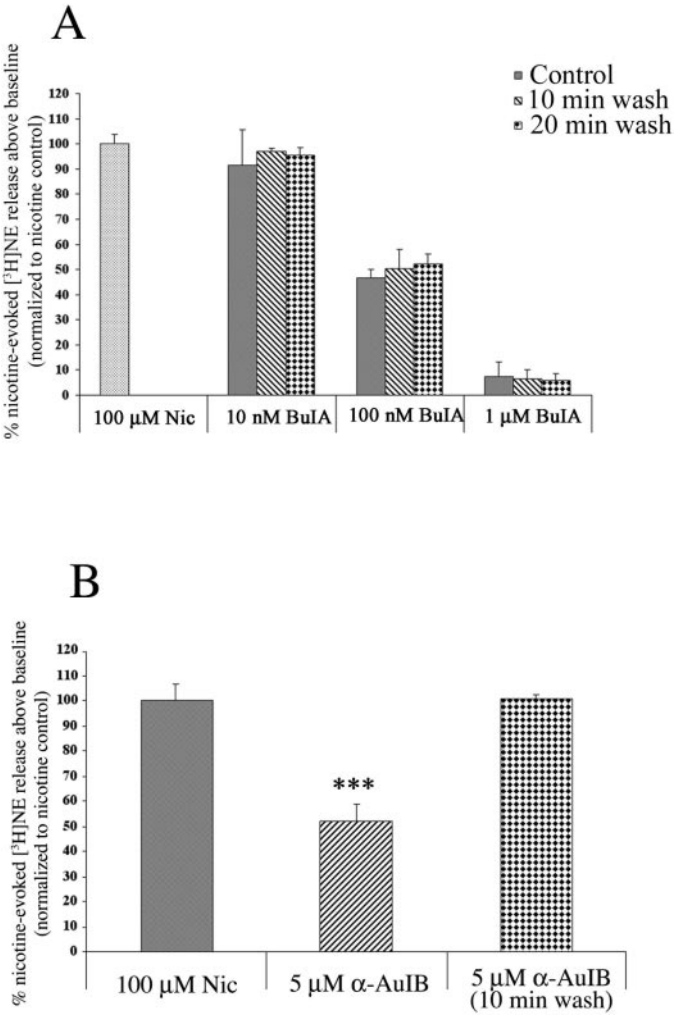

Fig. 3. Reversibility of α-CTX BuIA and α-CTX AuIB block of nicotine-evoked [^{3}H]NE release from adult rat hippocampal synaptosomes. Percentage of release above baseline is normalized to 100 μM nicotine control (100%). A, α-CTX BuIA (100 nM and 1 μM) inhibition of nicotine-stimulated [^{3}H]NE is not 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 [^{3}H]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 post hoc test. Values are mean ± S.E.M. from at least three experiments. Nic, nicotine.

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

Nicotine Modulation of [^{3}H]NE Release from Postnatal C57BL/6J WT Mouse Hippocampal Synaptosomes Is Mediated by a Mixed Population of $\beta2$- and $\beta4$-Containing nAChRs. Similar to adult rat, 1 μM α-CTX BuIA almost completely inhibited [^{3}H]NE release from 2- to 3-week-old WT mouse hippocampal synaptosomes. However, in contrast to adult rat (where inhibition was pseudoirreversible), the inhibition by α-CTX BuIA was partially reversed after toxin washout (Fig. 4A), suggesting the presence of both $\beta2$ (without $\beta4$) and $\beta4$-containing nAChRs.

To further characterize the identity of the nAChR subtypes that regulate [^{3}H]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 $\alpha3\beta2$, $\alpha6\beta2$*, and $\alpha6\beta4$ nAChRs) (Cartier et al., 1996; Vailati et al., 1999; Champtiaux et al., 2002), α-CTX PIA (selective for $\alpha6\beta2$* and $\alpha6\beta4$ nAChRs) (Dowell et al., 2003), and α-CTX AuIB [selective for $\alpha3\beta4$ (Luo et al., 1998) > $\alpha6\beta4$]. In contrast to adult rat, where α-CTX MII does not inhibit [^{3}H]NE release (Kulak et al., 1997; Luo et al., 1998), 100 nM α-CTX MII almost completely blocked nicotine-evoked [^{3}H]NE release from postnatal mouse hippocampal synaptosomes (Fig. 5A). α-CTX PIA (10 nM), a concentration that blocks $\alpha6\beta2$* nAChRs by ~85% and blocks $\alpha6\beta4$ nAChRs by ~20%, but only blocks $\alpha3\beta2$ by ~10% (Dowell et al., 2003), inhibited nicotine-evoked [^{3}H]NE release by 80 ± 9.4% (Fig. 5A). α-CTX AuIB, at 5 μM, blocked nicotine-evoked [^{3}H]NE release by 14 ± 3.8% (Fig. 5A). Because the α-CTX AuIB-sensitive fraction was quantitatively similar to the α-CTX PIA-insensitive release, both toxins were coapplied to determine whether the α-CTX PIA-insensitive component could be eliminated by α-CTX AuIB. Coapplication of 10 nM α-CTX PIA and 5 μM 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 was due to age differences, nicotine-evoked [^{3}H]NE release was examined in 2- to 3-week-old rat pups. Similar to adult rats, 1 μM BuIA almost completely and irreversibly inhibited nicotine-evoked [^{3}H]NE release from postnatal rat hippocampal synaptosomes (Fig. 4B). α-CTX MII (100 nM) 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 postna-

tal mice (Fig. 5A). α-CTX AuIB (5 μM) partially but significantly blocked [^{3}H]NE release. We were surprised to find that 10 nM α-CTX PIA potentiated [^{3}H]NE release (Fig. 5B). This was not further investigated.

The α4, β2, and β3 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 lacking a specific nAChR subunit were examined for nicotine modulation of hippocampal [^{3}H]NE release. Nicotine-evoked [^{3}H]NE release was abolished in β2-null mutant mice (β2−/−) and significantly decreased in α4−/− and β3−/− mice (Fig. 6). It is interesting that nicotine-evoked [^{3}H]NE release in β4−/− 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 β4 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 β4−/− pups (Fig. 7B), suggesting the presence of the β2 subunit in all nAChRs. In addition, α-CTX AuIB failed to block [^{3}H]NE release in β4−/− mice (Fig. 7A), consistent with α-CTX AuIB being inactive on β2-containing nAChRs (Luo et al., 1998).

Nicotine-evoked [^{3}H]NE release was pharmacologically examined in synaptosomes from mouse pups that lack either the α4 or the β3 subunit. In α4−/− pups, release was de-

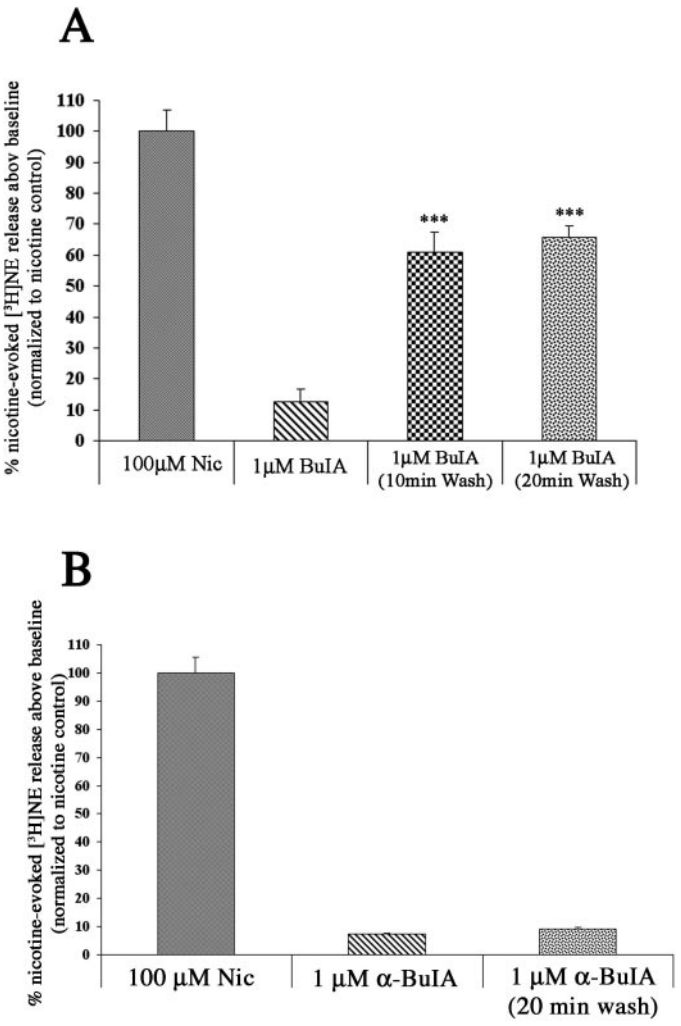

Fig. 4. α-CTX BuIA inhibition of nicotine-evoked [^{3}H]NE release from postnatal mouse and rat hippocampal synaptosomes. Percentage of release above baseline is normalized to 100 μM nicotine control (100%). A, 1 μM α-CTX BuIA blocks [^{3}H]NE release from mouse hippocampal synaptosomes, and its block is partially reversed after 10- and 20-min washes with toxin-free buffer. B, 1 μM α-BuIA almost completely blocks nicotine-evoked [^{3}H]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 post hoc test. Values are mean ± S.E.M. from at least three experiments. Nic, nicotine.

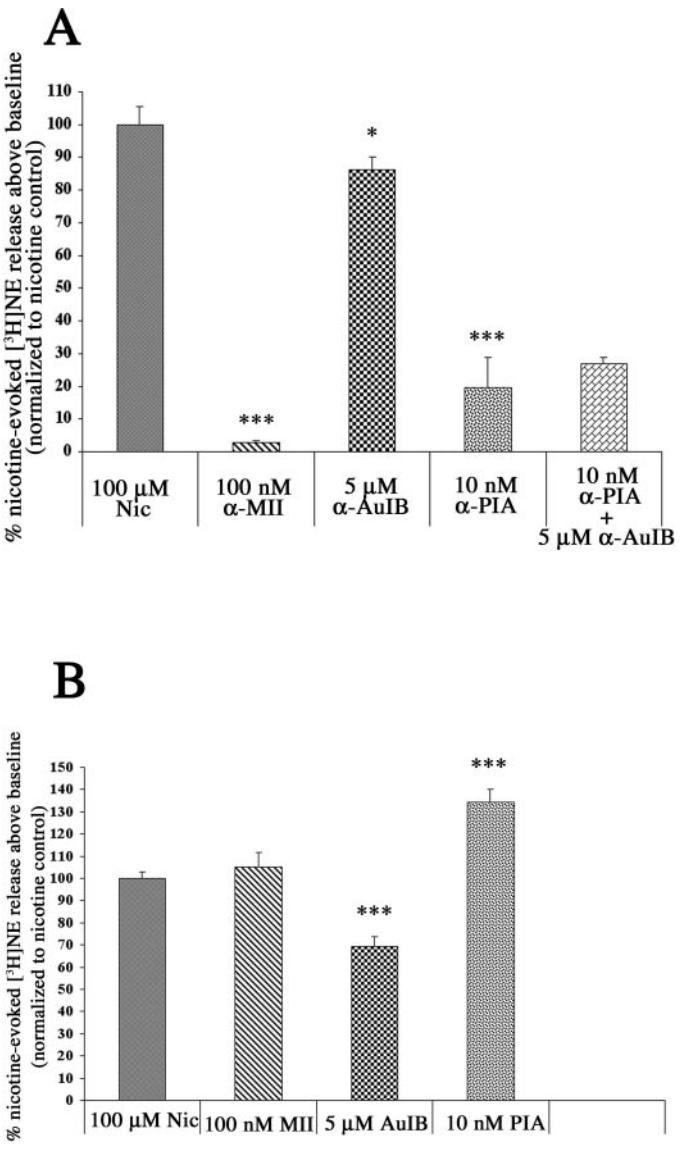

Fig. 5. Effect of selective antagonists on nicotine-evoked [^{3}H]NE release from WT postnatal mouse and postnatal rat hippocampal synaptosomes. Percentage of release above baseline is normalized to 100 μM nicotine control (100%). A, α-CTX MII completely blocks nicotine-evoked [^{3}H]NE release from postnatal mouse hippocampal synaptosomes, whereas α-CTX PIA blocks release by ~80%. α-CTX AuIB blocks release by only approximately 15%. Coapplication 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 μM nicotine control, Dunnett's post hoc test. Values are mean ± S.E.M. from at least three experiments.

creased by almost 80% relative to the WT (Fig. 6, 8A), suggesting that the α4 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 β3−/− 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 β4−/− mice. Similar to WT mouse, β4−/− adult mouse displayed only 16.9 ± 10.5% release above baseline in response to 100 μM nicotine, with no additional release (16.6 ± 4%) at 300 μM nicotine.

Cytisine Is an Efficacious Agonist at β2-Containing nAChRs Present on Mouse Noradrenergic Terminals. Cytisine is a nAChR agonist formerly reported to be highly efficacious at β4-containing nAChRs (Luetje and Patrick, 1991; Chavez-Noriega et al., 1997; Colquhoun and Patrick, 1997) but only poorly efficacious at β2-containing nAChRs (Papke and Heinemann, 1994). In WT mouse hippocampal synaptosomes, 100 μM cytisine was 90 ± 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 ± 3.4% after a 20-min wash with toxin-free buffer (Fig. 9A), suggesting the involvement of mostly β2-containing nAChRs. The high efficacy of cytisine at β2-containing nAChRs was further confirmed in β4−/− mice. In these mice, 100 μM cytisine was 82 ± 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 used a combination of novel subtype-selective ligands and nAChR subunit knockout 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 and pharmacological profiles of nAChRs in mouse versus rat. Although nicotine-evoked [3H]NE release during the second to third postnatal week is similar to adult levels in rats (Leslie et al., 2002; current study), NE release decreases with age in mice. A first major pharmacological difference between the two species is that mouse nAChRs are potently blocked by α6*-selective antagonists, whereas rat nAChRs are not. Second, in addition to a

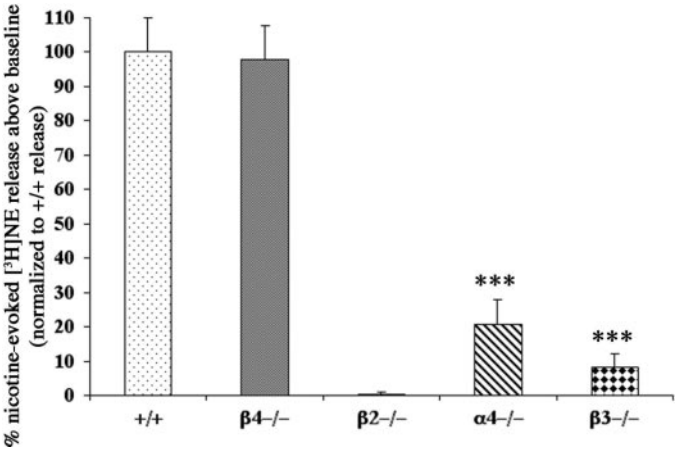

Fig. 6. Nicotine-evoked [3H]NE release from hippocampal synaptosomes of WT (+/+) and nAChR subunit null-mutant mice. Percentage of release above baseline is normalized to release by 100 μM nicotine in WT mice (100%). β4−/− mice show the same amount of [3H]NE release as the WT mice. However, release is abolished in hippocampal synaptosomes of β2−/− mice, whereas it is significantly reduced in α4−/− and β3−/− mice. ***, $p < 0.001$ Dunnett's post hoc test, with WT release as control. Values are mean ± S.E.M. from at least three experiments.

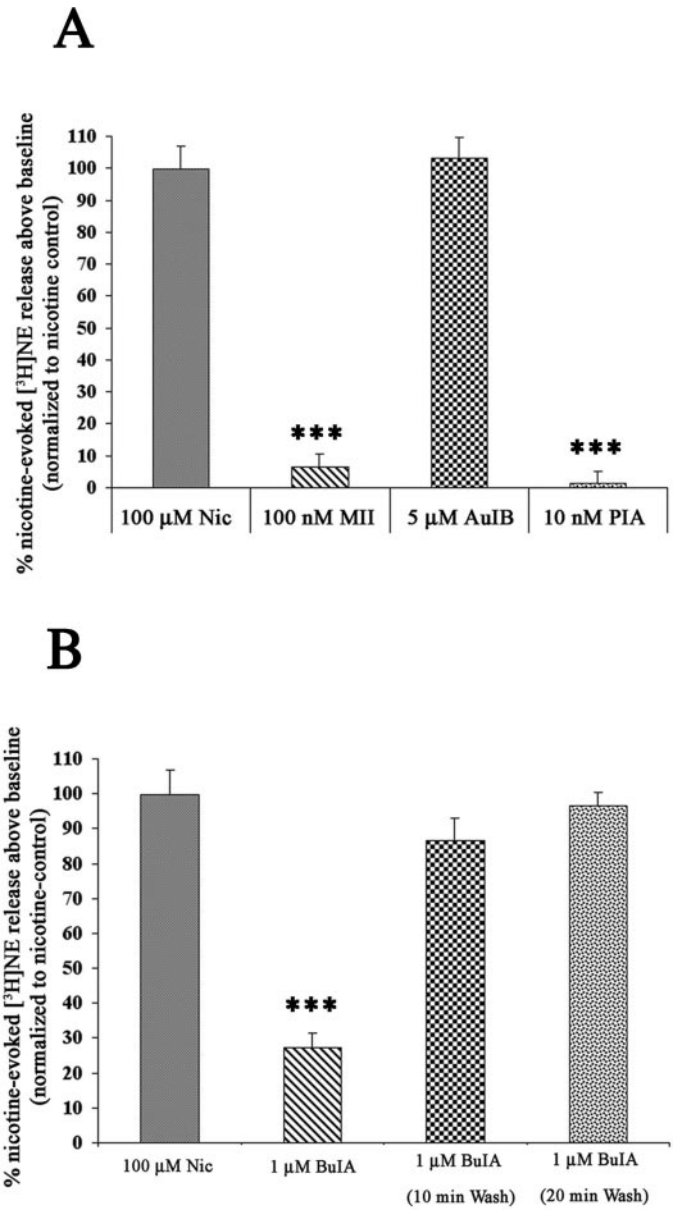

Fig. 7. Effect of selective antagonists on nicotine-evoked [3H]NE release from postnatal β4−/− mouse hippocampal synaptosomes. Percentage of release above baseline is normalized to 100 μM nicotine control (100%). A, both α-CTX MII and α-CTX PIA inhibit nicotine-evoked [3H]NE release, whereas α-CTX AuIB is ineffective in inhibiting this release. B, α-CTX BuIA block of nicotine-evoked [3H]NE release from β4−/− mouse hippocampal synaptosomes is almost completely reversed after 10- and 20-min washes with toxin-free buffer. ***, $p < 0.001$, Dunnett's post hoc test with 100 μM nicotine alone as control. Values are mean ± S.E.M. from three experiments. Nic, nicotine.

population of β2- and β4-containing nAChRs, there is a separate subpopulation of only β2-containing nAChRs on mouse noradrenergic terminals, whereas in the rat, all nAChRs seem to contain a β4 subunit. The results from the present study are summarized in Table 1.

nAChRs on Rat Hippocampal Noradrenergic Terminals. Previous work identified a subpopulation of α3β4-like nAChRs in nicotine-evoked [^{3}H]NE release from adult rat hippocampal synaptosomes (Clarke and Reuben, 1996; Luo et al., 1998). In the present study, it was shown that the β4 subunit is present in most, if not all, nAChRs that modulate nicotine-evoked [^{3}H]NE release in both adult and postnatal rats, as evidenced by the pseudoirreversible block by α-CTX BuIA. In addition, there is an absence of nAChRs that have an α6/βx or α3/β2 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 α3β4* and/or α6β4* nAChRs. However, lack of block by α-CTX MII excludes the α6β4* 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. Coadministration of the two toxins does not produce a greater inhibition than α-CTX MII, suggesting the presence of nAChRs with both the β2 and the β4 subunits (Fu et al., 1999). The greater inhibition by α-CTX MII suggests the possible presence of additional α3β2* and/or α6β2* subtype(s), without β4, 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 α4/β4 or α2/β4 interface. However, in

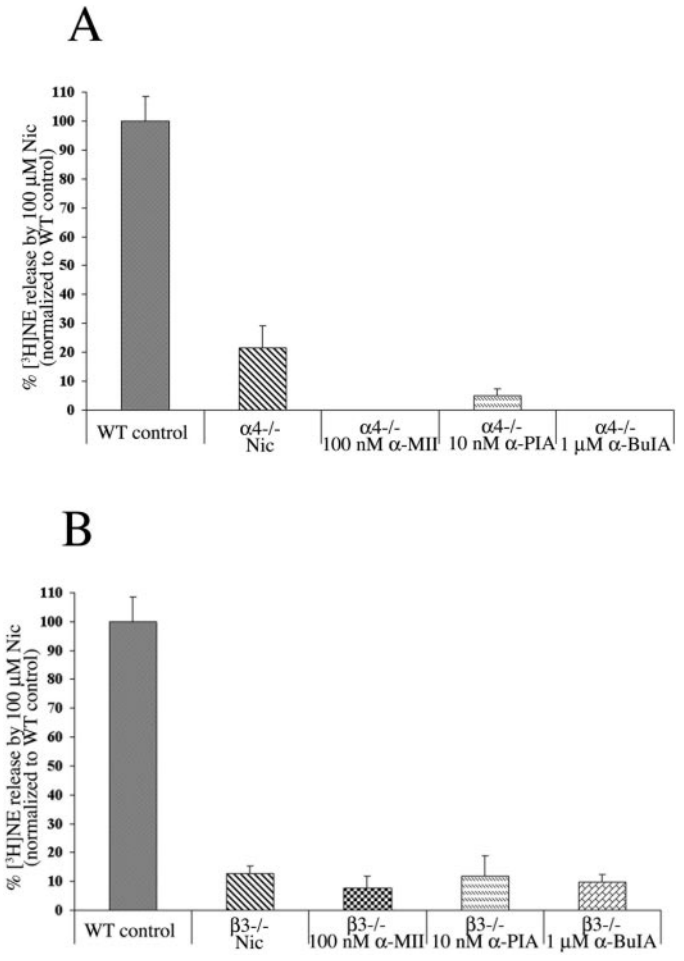

Fig. 8. Effect of α-CTX MII, α-CTX PIA, and α-CTX BuIA on nicotine-evoked [^{3}H]NE release from hippocampal synaptosomes of α4−/− and β3−/− postnatal mice. Percentage of release above baseline is normalized to release by 100 μM nicotine in WT mice (100%). A, α-CTX MII and α-CTX BuIA completely inhibit the residual [^{3}H]NE release in α4−/−mice. Although α-PIA inhibits the release by ~80%, this inhibition does not reach significance. B, nicotine-evoked [^{3}H]NE release is almost completely abolished in β3−/− mice. The residual release is not sensitive to block by any of the toxins. Values are mean ± S.E.M. from three experiments. Nic, nicotine.

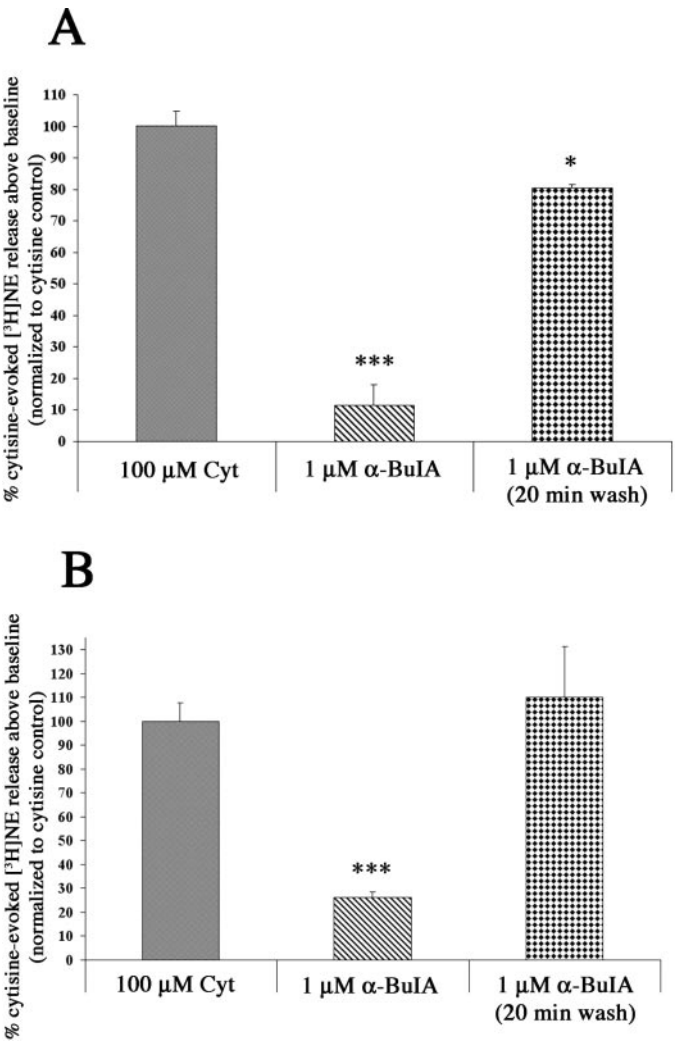

Fig. 9. Cytisine (Cyt) stimulation of [^{3}H]NE release from WT and β4−/− mouse hippocampal synaptosomes. Percentage of release above baseline is normalized to 100 μM cytisine control (100%). A, α-BuIA blocks cytisine-stimulated release in WT mouse by more than 80%; however, this block, although still significant, is mostly reversed after a 20-min toxin washout period. B, α-BuIA inhibits cytisine-stimulated [^{3}H]NE release in β4−/− mouse, but its block is fully reversed after a 20-min washout. *, $p < 0.05$; ***, $p < 0.001$, significantly different from 100 μM cytisine control, Dunnett's post hoc test. Values are mean ± S.E.M. from three experiments.

light of the absence of α2 mRNA in both postnatal and adult LC (Lena et al., 1999; Vincler and Eisenach, 2003), the α2β4* subtype can be excluded. The α4 subunit mRNA and protein have been detected in the rat LC (Lena et al., 1999; Vincler and Eisenach, 2003). The β2 subunit may also be present on the terminals, but the irreversible block by α-CTX BuIA suggests that any β2-containing nAChRs must also contain a β4 subunit at the other ligand binding interface. The presence of the putative structural subunits α5 and β3 in these nAChRs is also a possibility, especially because the mRNA for both subunits has been detected in the LC (Lena et al., 1999).

nAChRs on Mouse Hippocampal Noradrenergic Terminals. We were able to perform much more detailed studies in the mouse hippocampus because of the availability of nAChR subunit-deficient mice. In contrast to postnatal and adult rats, results from the null mutant mice indicated that all nAChRs that regulate nicotine-evoked [^{3}H]NE release contain a β2 subunit. A large proportion of these nAChRs also contain the β3 and/or the α4 subunits (Table 1). In WT mice, partial reversibility of block by α-CTX BuIA indicated the presence of both β4* and β2*(without β4) nAChR subtypes. The β2 subunit, however, seemed to be able to compensate for the β4 subunit in β4−/− 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 α6βx/α3β2-selective α-CTX MII and the α6βx selective α-CTX PIA indicated that most, if not all, receptors also contain an α6 subunit. The lack of coadditivity of inhibition by 10 nM α-CTX PIA and 5 μM α-CTX AuIB suggests a common site of action, possibly the α6β4* rather than an α3β4* subtype. However, the presence of a small population of α6(α3)β4* subtype that is sensitive to block by both α-CTX AuIB and α-CTX PIA cannot be ruled out. This is in contrast to the rat, where approximately half of nicotine-evoked [^{3}H]NE release is modulated by α3β4* but not α6β4* or α6α3β4* nAChRs (Luo et al., 1998).

To further investigate the pharmacology of nAChRs on mouse noradrenergic terminals, cytisine, a ligand formerly believed to only activate β4-containing nAChRs with high efficacy (Luetje and Patrick, 1991; Chavez-Noriega et al., 1997; Colquhoun and Patrick, 1997), was used. More recent studies, however, have shown that cytisine binds to α-CTX MII-sensitive sites, although with low affinity (Whiteaker et al., 2000). In addition, one study has demonstrated that cytisine is as efficacious as nicotine and acetylcholine in activating α-CTX MII-sensitive nAChRs that modulate [^{3}H]DA release from mouse striatal synaptosomes (Salminen et al., 2004). Similar to the latter study, in the present study, cytisine was nearly as efficacious as nicotine in stimulating [^{3}H]NE release from WT hippocampal synaptosomes. In addition, cytisine was also effective in stimulating [^{3}H]NE release from hippocampal synaptosomes of β4−/− mice, where all of the nAChRs contain the α6β2 interface and are α-CTX MII-sensitive. These results confirm that cytisine is a highly efficacious agonist at the α6β2* nAChRs present on mouse noradrenergic terminals.

Nicotine-evoked [^{3}H]NE release was significantly reduced in mice lacking the α4 subunit, suggesting that along with the β2 subunit, the α4 subunit is a critical component of the majority of nAChRs that stimulate [^{3}H]NE release in postnatal mouse hippocampus. The residual release in α4−/− mice is abolished by 100 nM α-CTX MII and by ~80% by 10 nM α-CTX PIA. This suggests that the small residual release in α4−/− mice may be mediated by a population of α6β2β3(β4) receptors that is still functional in the absence of the α4 subunit.

Deletion of the β3 subunit largely eliminates nicotine-evoked [^{3}H]NE release from mouse hippocampal synaptosomes. This result suggests that almost all of the nAChRs on mouse noradrenergic terminals contain a β3 subunit. It has been shown that the α-CTX MII-sensitive component of nicotine-evoked [^{3}H]DA release from striatal synaptosomes is substantially reduced in β3−/− adult mice (Cui et al., 2003; Salminen et al., 2004). In light of the finding that nicotine-evoked [^{3}H]NE release from hippocampus of mouse pups was completely α-CTX MII-sensitive (current study), the loss of the [^{3}H]NE release in β3−/− is consistent with the idea that this subunit is a critical component of native α-CTX MII-sensitive nAChRs that modulate catecholamine release in the central nervous system. It has recently been shown that the deletion of the β3 subunit decreases the number of α6-containing nAChRs on mouse DAergic terminals (Gotti et al., 2005), indicating that α6 and β3 subunits coparticipate in the 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

TABLE 1

Composition of nAChR subtypes expressed on hippocampal noradrenergic terminals of rat and mouse

For each subtype, the percentage 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	Percentage Contributing to NE Release	α-Conotoxin
Adult rat		
α3β4	50%	AuIB-sensitive; slow BuIA reversibility
αxβ4 (not α6)	50%	AuIB- and MII-insensitive; slow BuIA reversibility
Postnatal rat		
α3β4	30%	AuIB-sensitive; slow BuIA reversibility
αxβ4 (not α6)	70%	AuIB- and MII-insensitive; slow BuIA reversibility
Adult mice	—[a]	
Postnatal mice		
α6(α4) β2(β3)	65%	MII- and PIA-sensitive; rapid BuIA reversibility
α6(α4) β2β4(β3)	35%	MII- and PIA-sensitive; slow BuIA reversibility

[a] nAChR-mediated release too small to perform pharmacological studies.

may, in part, be mediated by the release of NE within the hippocampus. SIB-1553A, a nicotinic receptor ligand with selectivity for β4-containing nAChRs, improves attention and working memory in both rats and mice (Bontempi et al., 2001, 2003; Terry et al., 2002). The beneficial effects of SIB-1553A on working memory/attention may be mediated, in part, by release of acetylcholine (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 β4 subunit in nAChRs that modulate [^{3}H]NE release in both rat and mouse hippocampus, provide additional support for the latter.

In addition to its role as a neurotransmitter, NE acts as a neurotrophic factor in the immature central nervous system, regulating cell proliferation, differentiation, and synaptogenesis. In the rat cerebellum, another late-maturing structure, nicotine-evoked [^{3}H]NE release is significantly higher during the second to third postnatal week compared with 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 [^{3}H]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 [^{3}H]NE release is observed.

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