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Acetylcholine-Stimulated [^{3}H]GABA Release from Mouse Brain Synaptosomes is Modulated by α4β2 and α4α5β2 Nicotinic Receptor Subtypes

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Running Title: Nicotinic subunits & GABA release

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Abstract

Nicotinic acetylcholine receptor (nAChR) agonists stimulate the release of GABA from GABAergic nerve terminals, but the nAChR subtypes that mediate this effect have not been elucidated. The studies reported here used synaptosomes derived from the cortex, hippocampus, striatum and thalamus of wild type and α4, α5, α7, β2 and β4 null mutant mice to identify nAChR subtypes involved in acetylcholine (ACh) evoked GABA release. Null mutation of genes encoding the α4 or β2 subunits resulted in complete loss of ACh-stimulated [^{3}H]GABA release in all four brain regions. In contrast, α5 gene deletion exerted a small, but significant decrease in maximal ACh-evoked [^{3}H]GABA release in hippocampus and striatum, with a more profound effect in cortex. Acetylcholine-stimulated [^{3}H]GABA release from thalamic synaptosomes was not significantly affected by α5 gene deletion. No effect was detected in the four brain regions examined in α7 or β4 null mutant mice. Further analysis of ACh-evoked [^{3}H]GABA release revealed biphasic concentration-response relationships in the four brain regions examined from all wildtype animals as well as in α5 null mutant mice. Moreover, a selective reduction in the maximum response of the high-affinity component was apparent in α5 null mutant mice. The results demonstrate that α4β2-type nAChRs are critical for ACh-stimulated [^{3}H]GABA release from all four brain regions examined. In addition, the results suggest that α5-containing receptors on GABAergic nerve terminals comprise a fraction of the high ACh-sensitivity component of the concentration-response curve and contribute directly to the ability of nicotinic agonists to evoke GABA release in these regions.

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Introduction

The nicotinic acetylcholine receptor (nAChR) arguably represents one of the most evolutionarily conserved and well-characterized neurotransmitter receptors, and the majority of high-resolution structural data have been accumulated from studies of the *torpedo*- and muscle-type nAChR (see Millar and Gotti, 2008 for review). It is generally believed that the structures of neuronal nAChRs are very similar to the muscle-type nAChR subunits because the amino acid sequences of neuronal nAChR subunits are similar to the muscle-type subunits (reviewed in Lindstrom, 1998). Assuming that the neuronal nAChRs are pentameric assemblies that resemble the peripheral-type nAChR, the fact that mammalian neurons express mRNAs for nine nAChR genes (designated α2-α7 and β2-β4) suggests that many different nAChR subtypes might be expressed in the central nervous system (CNS).

The importance of identifying the sites of expression and subunit compositions of those nAChRs that are actually expressed in brain is demonstrated by observations that the biophysical and pharmacological properties of nAChRs are affected when different subunit combinations are examined in heterologous expression systems (see for example Zwart and Vijverberg 1998, Moroni et al. 2006, Kuryatov et al. 2008). Of particular importance is the observation that α4α5β2 nAChRs are more cation-permeable than other high-affinity heteromeric nAChRs, and the calcium permeability of these α4α5β2 nAChRs is exceeded only by α7-type nAChRs and N-methyl-D-aspartate (NMDA) glutamate receptors (Kuryatov et al, 2008). Indeed, previous studies performed in our laboratory (Brown et al., 2007) showed that deletion of the α5 subunit decreased maximal agonist-evoked $^{86}Rb+$ efflux in several brain regions without any loss of total receptor binding. These results suggest that the α5 subunit exerts a distinct and neuron-phenotype-specific effect on sequelae of nAChR activation, such as neurotransmitter release.

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The development of genetically engineered (gene knockout or null mutant) mice has provided a vital tool that has facilitated identification and characterization of native neuronal nAChRs. For example, the findings that α4 and β2 mRNAs are found together in many brain regions (Marks et al., 1992, Whiteaker et al., 2006) that also express high affinity [^{3}H]nicotine binding sites (Marks and Collins, 1982, Clarke et al., 1985) led to the suggestion that those nAChRs that bind nicotinic agonists with high affinity are composed of α4 and β2 subunits. Definitive proof that the α4 and β2 subunits are required to form these binding sites was provided by studies that showed the absence of high-affinity [^{3}H]nicotine binding in brain tissue derived from α4 (Marubio et al., 1999) and β2 (Picciotto et al., 1995) null mutant mice. Similarly, the findings that [^{125}I]-α-bungarotoxin (α-Bgt) binding is eliminated in α7 null mutant mice helped establish that α7-type receptors bind α-Bgt with high affinity (Orr-Urtreger et al., 1997). More recent studies using null mutant mice have demonstrated that the α6 (Champtieux et al., 2001), β2 (Salminen et al., 2005), and β3 (Cui et al., 2003) subunits are required to form the [^{125}I]-α-conotoxin MII binding sites expressed in catecholaminergic neurons. In addition, studies from our laboratory (Marks et al. 2007) have used null mutants to identify the subunit compositions of seven different nAChRs that can be measured with the aid of high- and low-affinity [^{3}H]epibatidine binding.

Many nAChRs are expressed on presynaptic nerve terminals where they modulate the release of acetylcholine (ACh), γ-aminobutyric acid (GABA), glutamate, serotonin, and dopamine (reviewed in Wonnacott, 1997). Recently, we (Salminen et al. 2004) used α4, α5, α7, β2, β3, and β4 null mutant mice, along with an assay that measures [^{3}H]dopamine release from striatal synaptosomes, to identify the subunit compositions of the native receptors that are expressed in dopaminergic nerve terminals. Our results indicate that four different nAChR

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subtypes (α4β2, α4α5β2, α4α6β2β3, α6β2β3) are expressed in dopaminergic nerve terminals derived from mouse striatum. The identical conclusion was drawn by Gotti et al. (2005) using immunological methodologies.

The studies reported here used synaptosomes obtained from wildtype as well as α4, α5, α7, β2 and β4 null mutant mice in an attempt to identify the role of these subunits in modulating GABA release. ACh-stimulated [^{3}H]GABA release was studied using synaptosomes prepared from the hippocampus, striatum, cortex, and thalamus. Immunological (Gahring et al, 2004) and RT-PCR (Klink et al. 2001) data have demonstrated that these subunits may be found in GABAergic neurons in several mouse brain regions. The results indicate that ACh-evoked [^{3}H]GABA release in all four brain regions requires α4 and β2 subunits and that the α5 subunit contributes to the formation of functional receptors in three of the four brain regions examined, but to differing degrees.

Methods

Materials. [^{3}H]GABA (33.4 Ci/mmol) was purchased from Perkin-Elmer (Boston, MA). Sucrose and HEPES were obtained from Boehringer-Mannheim (Indianapolis, IN). The following compounds were products of Sigma Chemical Co. (St. Louis, MO): acetylcholine (ACh), atropine sulfate, NO-711(1-[2-[[(Diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride hydrochloride), aminooxyacetic acid (AOAA), GABA, sodium chloride, potassium chloride, calcium chloride, magnesium sulfate, potassium dihydrogen phosphate, D-glucose, and diisopropylfluorophosphate (DFP). Econosafe scintillation cocktail was purchased from Research Products International Corp. (Arlington Heights, IL), and OptiPhase Supermix scintillation cocktail was obtained from PerkinElmer.

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Mice. Male and female mice carrying gene deletions ("knockouts") for Chrna4, Chrna5, Chrna7, Chrnb2, and Chrnb4 which encode α4, α5, α7, β2 and β4 nAChR subunits, respectively, were used in these studies. All nAChR subunit null mutant mice were bred onto the C57BL/6 strain for the indicated number of generations: α4 (Ross *et al*., 2000), 5 generations; α5 (Salas *et al*., 2003), 8 generations; α7 (Orr-Urtreger et al., 1997) 10 generations, β2 (Picciotto *et al*., 1995), 10 generations, and β4 (Xu et al., 1999) 10 generations. All of the animals were produced by heteroygous (+/-) matings maintained at the Institute for Behavioral Genetics (IBG), University of Colorado (Boulder, CO, USA).

The mice were weaned and separated by sex at 25 days of age and were housed in groups of five to a cage. The animals were maintained on a 12-hour light/12-hour dark cycle (lights on 0700 to 1900 hours), were given unlimited access to food (Teklad Rodent Diet) and water, and were used when they were 60-120 days old. All animal care and experimental procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the University of Colorado's animal care and utilization committee.

Genotyping. All genotyping was performed from tail clip samples (~ 1 cm). Qiagen (Valencia, CA) DNEasy Tissue Kits were used to extract DNA from the tail clippings. The α4, α5, α7, β2 and β4 genotypes were determined by PCR with oligonucleotide probes specific for the Chrna4, Chrna5, Chrna7, Chrnb2 and Chrnb4 sequences, respectively as described in Salminen *et al*. (2004). The gene products were electrophoresed on 1.5% agarose gels, and stained with ethidium bromide. Two independent observers then scored the genotypes.

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Synaptosome preparation. Each mouse was sacrificed by cervical dislocation, and the brain was removed from the skull. The cortex, hippocampus, striatum, and thalamus were then dissected on ice. Crude synaptosomes were prepared from each dissected brain region by hand homogenization in 4 volumes of 0.5ml ice-cold (4º C) Percoll medium I (sucrose, 320 mM; HEPES, 5 mM ; pH 7.5) with a glass-Teflon homogenizer. The homogenates were then centrifuged at 12,000g for 20 minutes at 4º C. The pellets of each homogenate were resuspended in uptake buffer (128 mM NaCl, 2.4 mM KCl, 3.2 $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25 mM HEPES, 10 mM glucose, pH 7.5). The volume of perfusion buffer used for resuspending the synaptosomes was 0.8ml for each brain region.

[³H]GABA Release. These experiments used a protocol adapted from that developed by Lu *et al*. (1998) with modifications as specified below. Synaptosomal preparations were incubated for 10 min at 37°C in uptake buffer containing 1.25 mM AOAA, a GABA transaminase inhibitor. [³H]GABA (final concentration 0.3 μM), unlabeled GABA (final concentration 0.25 μM), and DFP (final concentration 10 μM), an irreversible cholinesterase inhibitor, were then added to the suspension, and the suspension was incubated for another 10 min. Aliquots (80 μl) were collected with gentle suction onto 6-mm diameter A/E glass-fiber filters (Gelman Science, Ann Arbor, MI) on the perfusion apparatus, then perfused with buffer containing 1 g/liter bovine serum albumin (1.8 ml/min) for 10 min before fraction collection was started. ACh (0.03-1000 μM) was added to the perfusate for 12 seconds in the middle of each fraction collection run. The amount of radioactivity in each fraction was then determined with scintillation spectrometry (45% counting efficiency). Atropine (1 μM) was included in the perfusion buffer to block any possible muscarinic responses. NO-711 (100 nM), a potent (K_d ~ 6 nM) and selective antagonist

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of the neuronal GABA transporter (GAT-1; Borden, 1996), was also added to the perfusion buffer to prevent calcium independent [^{3}H]GABA release due to reversal of the GABA transporter by large concentrations of ACh (data not shown).

Data Analysis. The amount of ACh-stimulated [^{3}H]GABA release was determined in 12-s fractions. The data were normalized using Sigma Plot 5.0 (Jandel Scientific, San Raphael, CA) as described by Grady et al., (1992). In short, the fractions recorded in the absence of agonist are fit to a single phase exponential decay equation to obtain a measure of baseline release. The stimulated release was divided by the calculated baseline release during the time of stimulation to calculate "response units" where one unit is defined as twice basal release. The responses at each time interval of stimulation were then summed to yield the total response for a given agonist exposure. For comparisons between genotypes, the mean value of a near-maximal concentration of ACh (30μM) for each brain region was used as a further normalizing factor and all responses plotted as a percent of this control value. Concentration-effect curves for [^{3}H]GABA release were plotted and fit with the 4-parameter hyperbolic equation $f=VS/(K+S)+vS/(k+S)$, where S is agonist concentration, maximum response for high and low-sensitivity components are V and v, respectively, and half-maximal agonists concentrations for high- and low-sensitivity components are represented by K and k, respectively. Curve fits were performed using SigmaPlot (SigmaPlot DOS or SigmaPlot 2001), and an F-test was conducted on each data set to ensure that the 4-parameter equation was statistically preferred. To obtain concentration-response parameters, initial curve-fits were calculated from mean concentration-response curves, and the theoretical values applied to each individual experiment in order to generate a series of individual experiment curve-fits. Significant differences ($p< 0.05$) between

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2-group data sets were determined with univariate analysis of variance (SPSS 16.2, Chicago Illinois). Both raw data values and calculated curve-fit parameters were analyzed with ANOVA in order to assess genotype differences that may be evident across a concentration-response curve, but less apparent when calculated from theoretical curve-fit parameters. When EC_{50} values were compared their log values were used. This was done because log(EC_{50}) values are normally distributed but EC_{50} values are not (Hancock *et al*., 1988).

Results

Concentration-dependent nature of ACh-stimulated [³H]GABA Release. Figure 1 presents a typical data trace. The data are presented as counts per minute (cpm) of radioactivity obtained when hippocampal synaptosomes loaded with [³H]GABA were perfused with increasing concentrations of ACh. As higher ACh concentrations were used, more [³H]GABA release (units above baseline) was observed. Similar results were obtained with synaptosomes obtained from the other brain regions. These data are consistent with those reported by Lu *et al*. (1998).

Effects of α4, α5, and β2 gene deletion on ACh-stimulated [³H]GABA Release. Figure 2 illustrates that null mutation of Chrna4, the gene encoding the α4 subunit, resulted in a complete loss of ACh-stimulated GABA release from synaptosomes prepared from all four of the brain regions. Acetylcholine produced a concentration-dependent increase in GABA release in all four of the brain regions, but the maximal responses of the brain regions differed. High concentrations (0.3 and 1 mM) often produced a marked decrease in release from that seen at the determined maximally efficacious concentration (*i.e*. the concentration effects were of the inverted "U" type). These effects were most likely caused by agonist "channel block" in which

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the agonist accumulates in the ion channel and prevents cation flux, even though the receptor is in its active conformation (Zwart and Vijerberg, 1998). In all cases a minimum of 3-4 log units of ACh were required to progress from minimal to maximal release. This suggests that more than one receptor subtype may be mediating this response. Therefore, the data were analyzed to determine whether they fit one- or two-site models. The ACh concentration-effect curves in wildtype animals were best fit by a two-site model in all brain regions examined (F test $p< 0.05$). The calculated curve fit parameters (R_{max} [maximal release] and EC_{50} [concentration that elicited half-maximal release]) for the cortex, hippocampus, striatum, cortex and thalamus are presented in table 1.

Figure 3 shows that null mutation of Chrnb2, the gene encoding the β2 subunit, also resulted in a total elimination of ACh-stimulated [^{3}H]GABA release. The effects of β2 knockout were statistically significant in all four brain regions. The ACh concentration-effect curves for all four brain regions in wildtype animals were best fit by a two-site model (F test $p< 0.05$). The calculated curve fit parameters for the hippocampus, striatum, cortex, and thalamus are presented in table 1.

Figure 4 demonstrates that deletion of Chrna5, the gene encoding the α5 subunit, caused robust decreases in ACh-stimulated [^{3}H]GABA release in the cortex [$F_{(1,118)}=24.95$; $p<0.001$]. Deletion of the α5 subunit also produced a modest, but significant decrease in [^{3}H]GABA release in the striatum and hippocampus [$F_{(1,152)}=7.67$, $p<0.007$; $F_{(1,132)}=17.12$, $p<0.001$, respectively]. No observable effect of α5 gene deletion was observed in thalamic synaptosomes [$F_{(1,90)}=0.76$, $p=0.390$]. The ACh concentration-effect curves for all four brain regions in both wildtype and knockout animals were best fit by a two-site model (F test $p< 0.05$). The calculated curve fit parameters for the cortex, thalamus, striatum and hippocampus are presented in table 1.

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As noted in this table, α5 gene deletion resulted in no change in EC_{50} in cortex, hippocampus or striatum. The Rmax for the high affinity component was significantly reduced in cortex only. The calculated Rmax values for hippocampus and striatum were not significantly different from control but the ANOVAs performed on the entire concentration-response curves did detect a significant effect of genotype, with a trend towards a decrease in the high-affinity component. Deletion of the α5 subunit did not produce any significant changes in the maximal response or EC_{50} values for the low-affinity component.

High- and low-sensitivity α4β2*-type nAChRs differ in their sensitivity to antagonism by the competitive inhibitor dihydro-β-erythroidine (DHβE) (Marks et al. 1999). At low concentrations (≤ 2 μM), only the high-sensitivity fraction is blocked, allowing for a pharmacological isolation of the receptor responses observed. In synaptosomes from α5 null mutant mice, DHβE (2 μM) blocked the high-sensitivity component of ACh-evoked [3H]GABA; removal of the α5 gene did not alter the low-sensitivity α4β2 responses to ACh (Fig. 5).

Deletion of either the β4 or α7 subunit did not result in any measureable changes in [^{3}H]GABA release evoked by ACh (30 μM) in any of the four brain regions screened (Figs. 6A and 6B, respectively). Thus, no further experimentation proceeded with these animals.

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Discussion

Null mutation of either Chrna4 or Chrnb2 totally eliminated ACh-stimulated [³H]GABA release from every brain region studied. These null mutations also eliminate [^{3}H]nicotine binding (Picciotto et al., 1995; Marubio et al., 1999) and cytisine-sensitive [^{3}H]epibatidine binding (Marks et al., 2007) throughout the brain. The binding results have been interpreted as indicating that α4β2*-type receptors are the major nAChR subtype that binds these ligands with high affinity. By analogy, we conclude that α4β2*-type nAChRs modulate GABA release from the presynaptic nerve terminals in the four brain regions that were studied.

The IUPHAR receptor nomenclature committee (Lukas et al., 1999) recommended that nAChRs should be named by including the Greek letters for known subunits along with an asterisk (*) to designate the potential contribution of other subunits. We found that Chrna5 gene deletion resulted in decreased maximal ACh-evoked [³H]GABA release from cortical, striatal and hippocampal synaptosomes with differing degrees of effect. This outcome suggests that α4α5β2-type nAChRs play regionally-distinct modulatory roles in ACh-evoked GABA release. Chrna7 and Chrnb4 gene deletion did not affect ACh-evoked [³H]GABA release. These observations indicate that receptors that include these subunits are not significant contributors to [³H]GABA release from presynaptic nerve terminals in the four brain regions that were studied.

The finding that both α4 and β2 gene deletion eliminated [³H]GABA release from synaptosomes prepared from all four brain regions examined is not surprising given that the mRNAs for both of these subunits are expressed in high concentrations in these brain regions (Marks et al. 1992). Similarly, it is not surprising that Chrna5 deletion resulted in a change in cortical [³H]GABA release given that single-cell reverse transcription (RT-PCR) analyses detected α5 mRNA expression in some, but not all, cortical GABAergic neurons (Porter et al.,

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1999). It should be noted, however, that *in situ* hybridization measures mRNA that is expressed primarily in cell bodies.

Chrna5 gene deletion had a small effect on ACh-stimulated [³H]GABA release from striatal and hippocampal synaptosomes. The *in situ* hybridization experiments indicate that α4, α5 and β2 mRNAs are all expressed in both of these brain regions. However, the expression pattern for α5 mRNA is limited when compared to those observed for α4 and β2 mRNA. These findings may explain why we obtained evidence that suggests both α4β2 and α4α5β2 nAChRs are expressed on GABAergic nerve terminals in three of the four brain regions examined. This correlation between Chrna5 mRNA expression and α5 null mutation effect on ACh-evoked [^{3}H]-GABA release could also explain the lack of α5 subunit removal on [^{3}H]-GABA release measured from thalamic synaptosomes, as there is no appreciable α5 mRNA expressed in that region. Therefore, regional effects of Chrna5 gene deletion could reflect preferential loss of activity in local GABAergic interneuron populations.

Many studies have used electrophysiological methods to study nicotinic modulation of GABA neuron activity, and many of these studies have focused on the hippocampus (see, for examples: Alkondon and Albuquerque, 1993; Alkondon et al., 1997; Kawai et al., 2002), but nicotinic modulation of GABAergic neurons has also been studied in the cortex (Alkondon et al., 2000), thalamus (Lena and Changeux, 1997) and in dopamine-rich regions such as the midbrain (Klink et al., 2001), the ventral tegmental area (Mansvelder and McGehee, 2000), and the nucleus accumbens (Rover et al., 2002). Many of these studies found that pretreatment with the nAChR antagonist, DHβE, blocked cholinergic activation of GABAergic neurons, leading to the conclusion that α4β2-type nAChRs influence the activity of GABAergic neurons. Electrophysiological analyses of the potential roles of nAChRs containing the α5 subunit in

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modulating GABAergic function have not been published. Our results suggest that such studies will yield different results depending on the brain region studied.

Many of the electrophysiological studies also demonstrated a role for α7 containing nAChRs in the modulation of somatodendritic activities of GABAergic neurons. The lack of an effect of α7 subunit gene deletion on [^{3}H]GABA release from synaptosomal preparations suggests that α7-containing nAChRs present on GABAergic neurons are expressed preferentially in somatodentritic locations, whereas the dominant presynaptic nAChR expressed by GABAergic neurons is the α4β2* type.

It is exceedingly likely that our synaptosomal preparation contains a heterogeneous population of nerve terminals with contributions to the overall pool being provided by many potentially distinct subregions. We found that null mutation of Chrna5 significantly decreases the calculated R_{max} for ACh in cortex. The R_{max} for ACh-stimulated GABA release was not significantly altered in striatum and hippocampus, but the ANOVA, which is more sensitive because it analyzes effects over the entire concentration effect curve, did detect a significant effect of gene deletion. Thus, deleting the α5 subunit produces a more subtle effect in striatum and hippocampus. No differences in agonist potency were observed in any brain region studied. In agreement with previously published functional assessment of Chrna5 gene deletion using ^{86}Rb+ efflux, it appears that the loss of the α5 subunit results in a preferential decrease in the maximal response of high ACh-sensitivity nAChRs with no subsequent loss of total receptor number (Brown et al. 2007). In addition, isolation of the low-sensitivity α4β2 functional contribution by examining ACh-evoked [^{3}H]GABA release in the presence of 2 μM DHβE (Marks et al., 1999) showed no differences between α5 wildtype and null-mutant animals in any brain region examined. These results are consistent with studies comparing the functional

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properties of α4β2 and α4α5β2 nAChRs in heterologous expression systems (Kuryatov, 2008) and suggest that the α4α5β2-type nAChR expressed on GABAergic nerve terminals is uniquely capable of influencing neurotransmitter release. This enhanced functionality may be due to distinct biophysical properties, such as the enhanced ion (including Ca^{2+}) conductance of α4α5β2 nAChRs compared to high-affinity nAChRs containing α4 and β2 subunits only.

The biphasic responses for α4β2* nAChRs have been described previously in $^{86}Rb^{+}$ efflux studies (Brown et al. 2007; Marks et al., 1999) and in electrophysiological measurements of heterologous expression systems (Zwart and Vijverburg, 1998; Moroni et al. 2006). It has been suggested that the high- and low-affinity components represent receptors with different stoichiometries: $(\alpha4)_2(\beta2)_3$ (high affinity) and $(\alpha4)_3(\beta2)_2$ (low affinity) (Zwart and Vijverburg, 1998; Marks et al. 2007). It is possible that the loss of the α5 subunit results in the compensatory formation of receptors with the stoichiometry $(\alpha4)_2(\beta2)_3$ that have an equally high affinity for ACh, but are distinctly less efficacious at eliciting neurotransmitter release than the α4α5β2 receptor. This event could explain the selective reduction in the high-sensitivity component of ACh-evoked [³H]GABA release without any significant decline in low-sensitivity α4β2 nAChR function or loss of receptor number as a whole, which was measured by receptor binding assays in α5 null mutant animals (Brown et al, 2006).

Conclusions. The α4 and β2 nAChR subunits are clearly necessary for nicotinic agonist-stimulated GABA release in all four of the brain regions examined. Chrna5 gene deletion does not result in a total loss of ACh-stimulated GABA release in any brain region. However, it is frequently included along with α4 and β2 subunits, and the absence of the α5 subunit decreases overall nAChR-mediated [³H]GABA release that is restricted to an effect on the high-affinity component of the functional profile. These findings suggest that α4α5β2 nAChRs are important

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modulators of [^{3}H]GABA release from striatal, hippocampal, and cortical synaptosomes. Furthermore, α5-containing nAChRs are distinct in their regional distribution and functionality. Thus, nAChRs containing the α5 subunit may serve as therapeutic targets with some selectivity for distinct neuronal pathways.

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Legends for Figures

Figure 1. Representative data of ACh-stimulated [^{3}H]GABA release from hippocampal synaptosomes. A total of 23 10-second fractions were taken from each synaptosomal sample, and the radioactivity in each fraction was measured in a scintillation spectrometer. Thus, these data are presented in counts per minute. The synaptosomes were stimulated with a range of ACh concentrations (0.03-1000 μM) for 12 seconds in the middle of each run (hash marks beneath each trace). The fractions before and after the stimulation were then used to determine the baseline [^{3}H]GABA release, which was subsequently used to determine the size of the response (see materials and methods for details). The size of the responses increased in a concentration-dependent manner.

Figure 2. Effect of α4 nAChR subunit gene deletion on ACh-evoked [^{3}H]GABA release. Chrna4 gene deletion resulted in a total loss of ACh-stimulated [^{3}H]GABA release from synaptosomes made from the **A)** cortex, **B)** hippocampus, **C)** striatum, and **D)** thalamus. Animals lacking the α4 nAChR subunit did not exhibit a concentration-dependent response to ACh in any of the four brain regions. N= 2-8 for each data point in each graph. Error bars represent the standard error of the mean (S.E.M.).

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Figure 3. Effect of β2 nAChR subunit gene deletion on ACh-evoked [³H]GABA release. ACh-stimulated [³H]GABA release was not detected in synaptosomes made from the **A)** cortex, **B)** hippocampus, **C)** striatum, or **D)** thalamus of Chrnb2 null mutant mice. As with α4 null mutants, animals lacking the β2 nAChR subunit did not exhibit a concentration-dependent GABA release response to ACh in any of the four brain regions studied. N= 1-3 for each data point in each graph. Error bars represent the S.E.M.

Figure 4. Effect of α5 nAChR subunit gene deletion on ACh-evoked [³H]GABA. Chrna5 gene deletion generally resulted in a decrease of ACh-stimulated [³H]GABA release from synaptosomes made from the **A)** Cortex, **B)** hippocampus, **C)** striatum, and **D)** thalamus. The decreased response in the α5 null mutants was readily observable in the cortex, diminished in the striatum and hippocampus, and was not significant in the thalamus. N= 2-11 for each data point in each graph. Error bars represent the S.E.M.

Figure 5. Effect of α5 nAChR subunit gene deletion on DHβE-resistant nAChR function measured with ACh-evoked [³H]GABA release. ACh-stimulated [³H]GABA release in the presence of 2μM DHβE from cortex, hippocampus, striatum and thalamus (graphs A, B, C, and D, respectively) shows no effect of Chrna5 gene deletion ($p>0.05$). Error bars represent SEM.

Figure 6. Effets of β4 and α7 subunit null mutation on 30μM ACh-evoked [³H]GABA release from cortex, hippocampus, striatum and thalamus. No statistically significant effect of gene deletion was observed

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Table 1: Effects of gene deletion on high and low-sensitivity components of ACh-evoked [^{3}H]GABA release.

Cortex

Genotype	Rmax (HS)	logEC50 (HS)(μM)	Rmax (LS)	logEC50 (LS) (μM)
α4 +/+	100.31 ±11.80	-0.17±0.098	39.78 ±25.21	1.40±0.480
α4 -/-	-	-	-	-
β2 +/+	80.58±29.56	-0.44±0.381	15.06±15.05	1.35±0.66
β2 -/-	-	-	-	-
α5 +/+	84.45±13.16	-0.32±0.171	50.10±10.36	0.93±0.45
α5 -/-	40.15±6.48	-0.66 ±0.14	56.37±17.85	1.31±0.52

Hippocampus

Genotype	Rmax (HS)	logEC50 (HS) (μM)	Rmax (LS)	logEC50 (LS) (μM)
α4 +/+	81.28±16.13	-0.34±0.199	53.03±16.72	2.14±0.28
α4 -/-	-	-	-	-
β2 +/+	77.98±6.46	-0.44±0.13	105.28±39.65	2.20±0.23
β2 -/-	-	-	-	-
α5 +/+	69.40±10.92	-3.42±2.70	61.24±7.11	1.27±0.53
α5 -/-	51.30±14.31	-1.98±1.43	51.23±9.84	1.20±0.20

Striatum

Genotype	Rmax (HS)	logEC50 (HS) (μM)	Rmax (LS)	logEC50 (LS) (μM)
α4 +/+	42.83±4.31	-1.12±0.15	64.98±24.01	1.00±0.55
α4 -/-	-	-	-	-
β2 +/+	48.10±8.03	-1.67±0.62	60.35±9.02	0.60±0.21
β2 -/-	-	-	-	-
α5 +/+	51.79±6.96	-0.73±0.18	63.17±12.12	0.998±0.12
α5 -/-	51.18±9.37	-0.46±0.18	41.61±13.68	0.82±0.47

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Thalamus

Genotype	Rmax (HS)	logEC50 (HS) (μM)	Rmax (LS)	logEC50 (LS) (μM)
α4 +/+	54.30±10.31	-2.59±2.10	62.98±25.91	1.16±0.62
α4 -/-	-	-	-	-
β2 +/+	40.58±8.24	-2.37±1.29	75.72±17.67	0.94±0.44
β2 -/-	-	-	-	-
α5 +/+	41.54±5.23	-0.33±0.20	82.71±22.09	1.46±0.21
α5 -/-	47.17±12.00	-2.75±2.13	47.10±17.77	1.00±0.40

R_{max} (maximal ACh-stimulated [^{3}H]GABA release) and EC_{50} (ACh concentration producing 50% maximal response) values were calculated from concentration-response curves as described in the methods. Each value represents the mean +/- standard error of the mean as compiled from 4-8 individual concentration-effect curves for each genotype. The two components of the concentration-response curve are designated as high-ACh sensitivity (HS) and low-ACh sensitivity (LS).

Figure 1

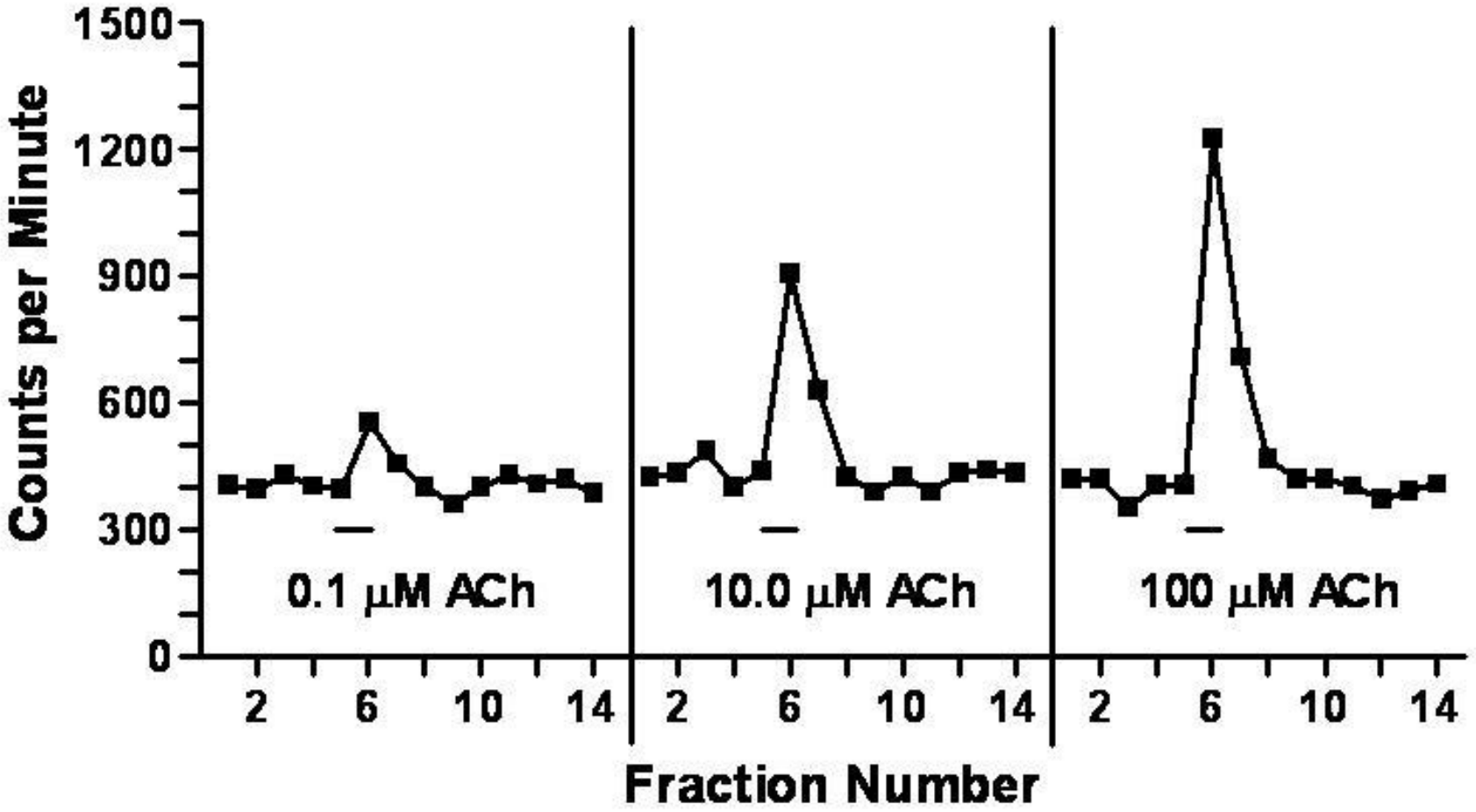

Figure 2

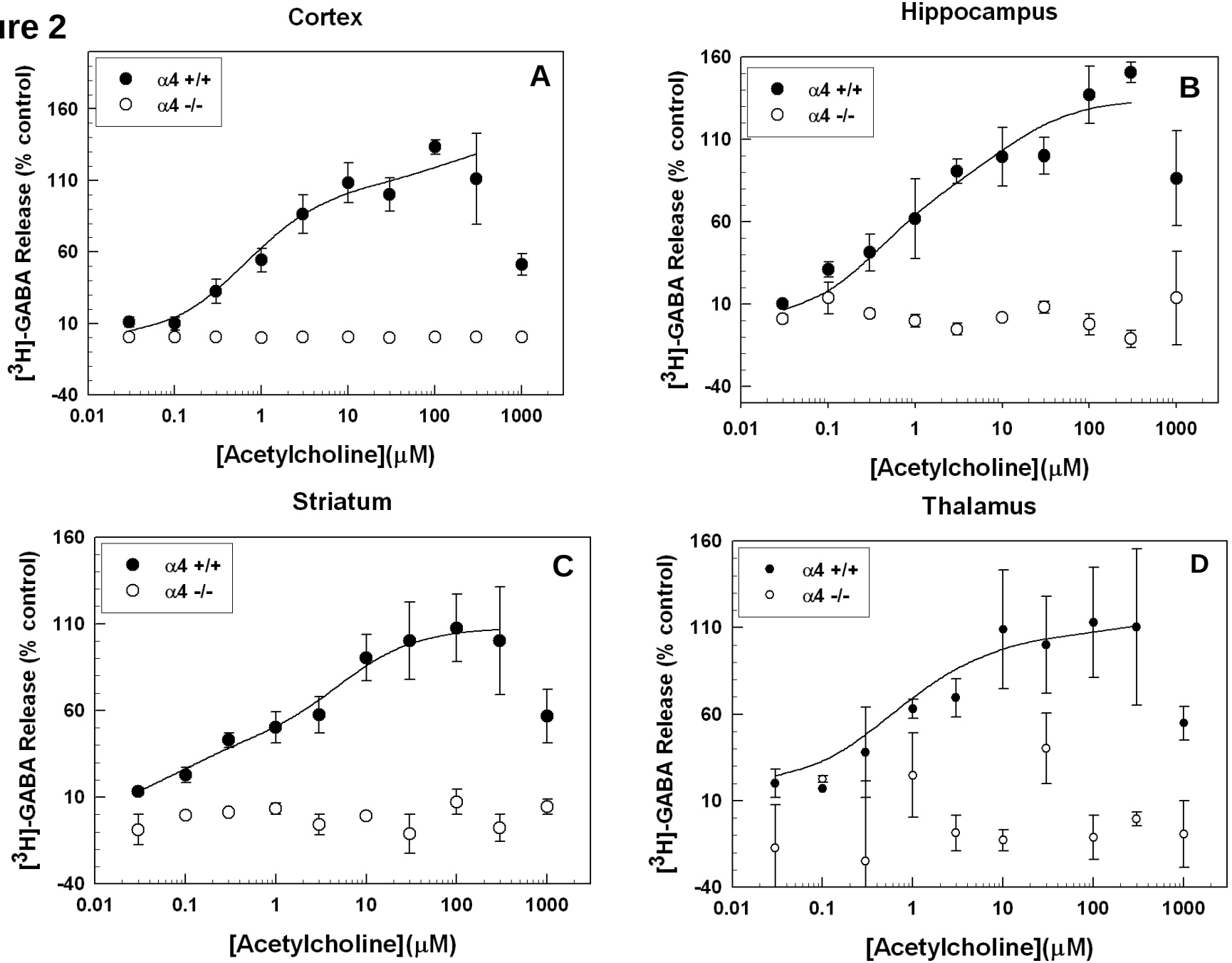

Figure 3

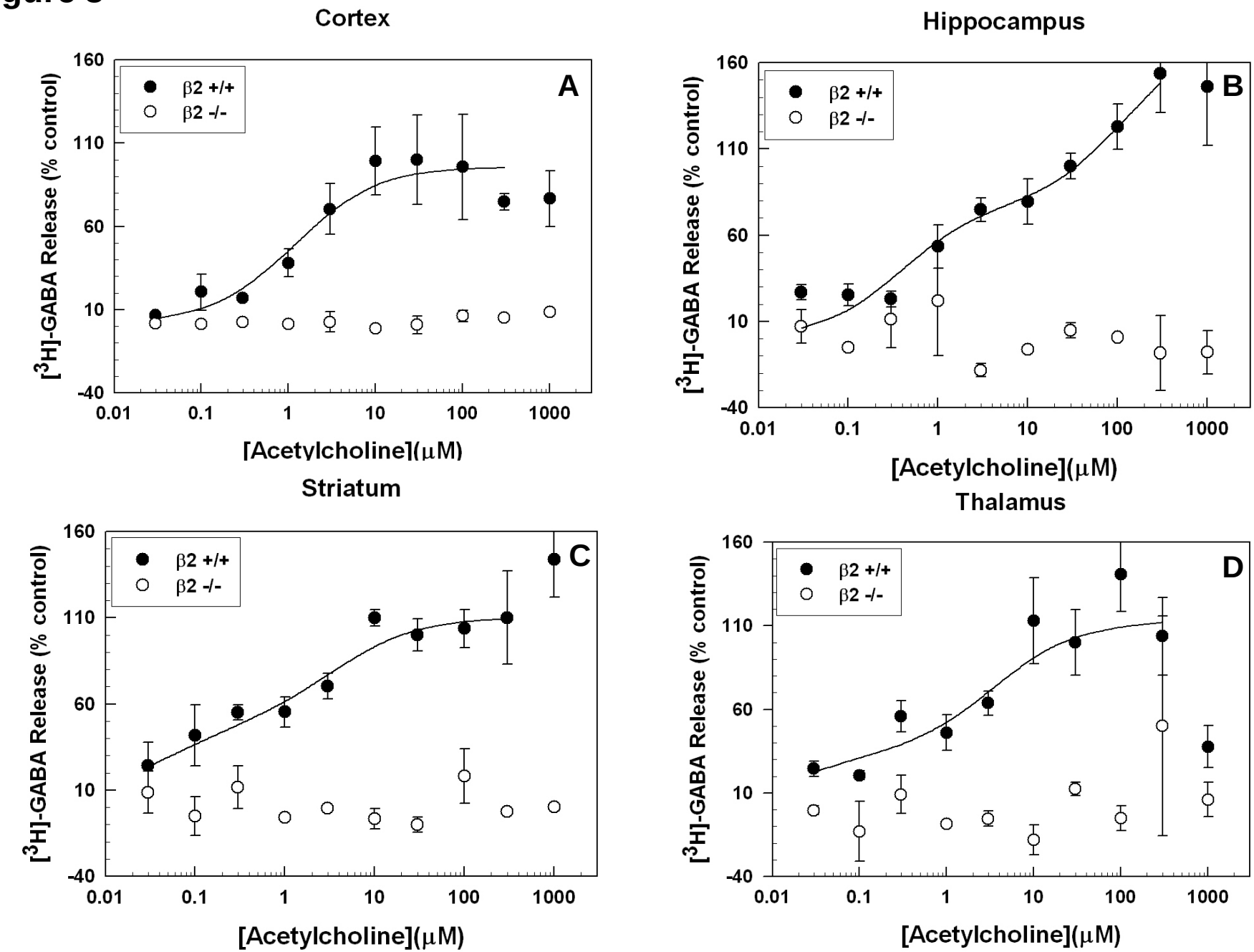

Figure 4

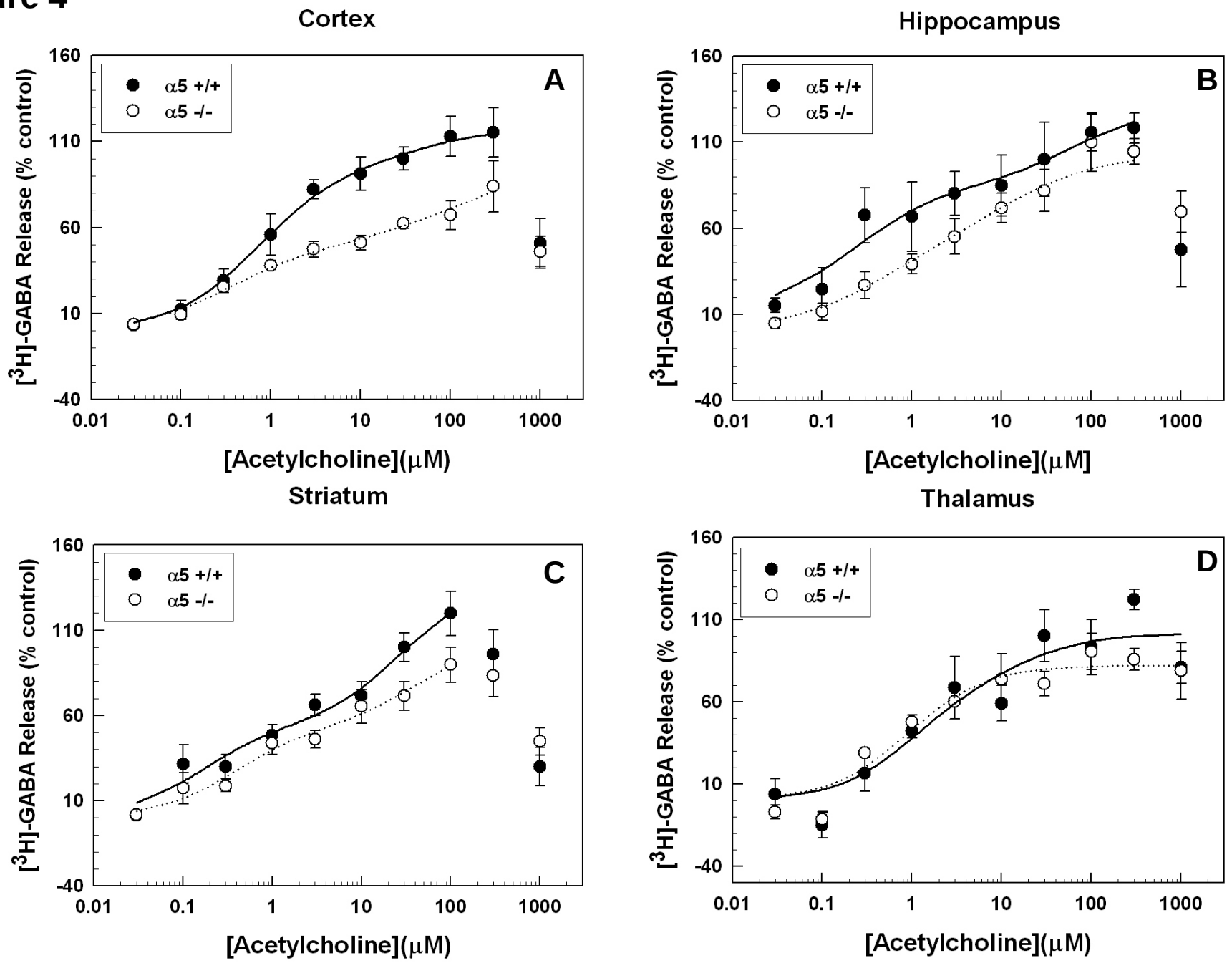

Figure 5

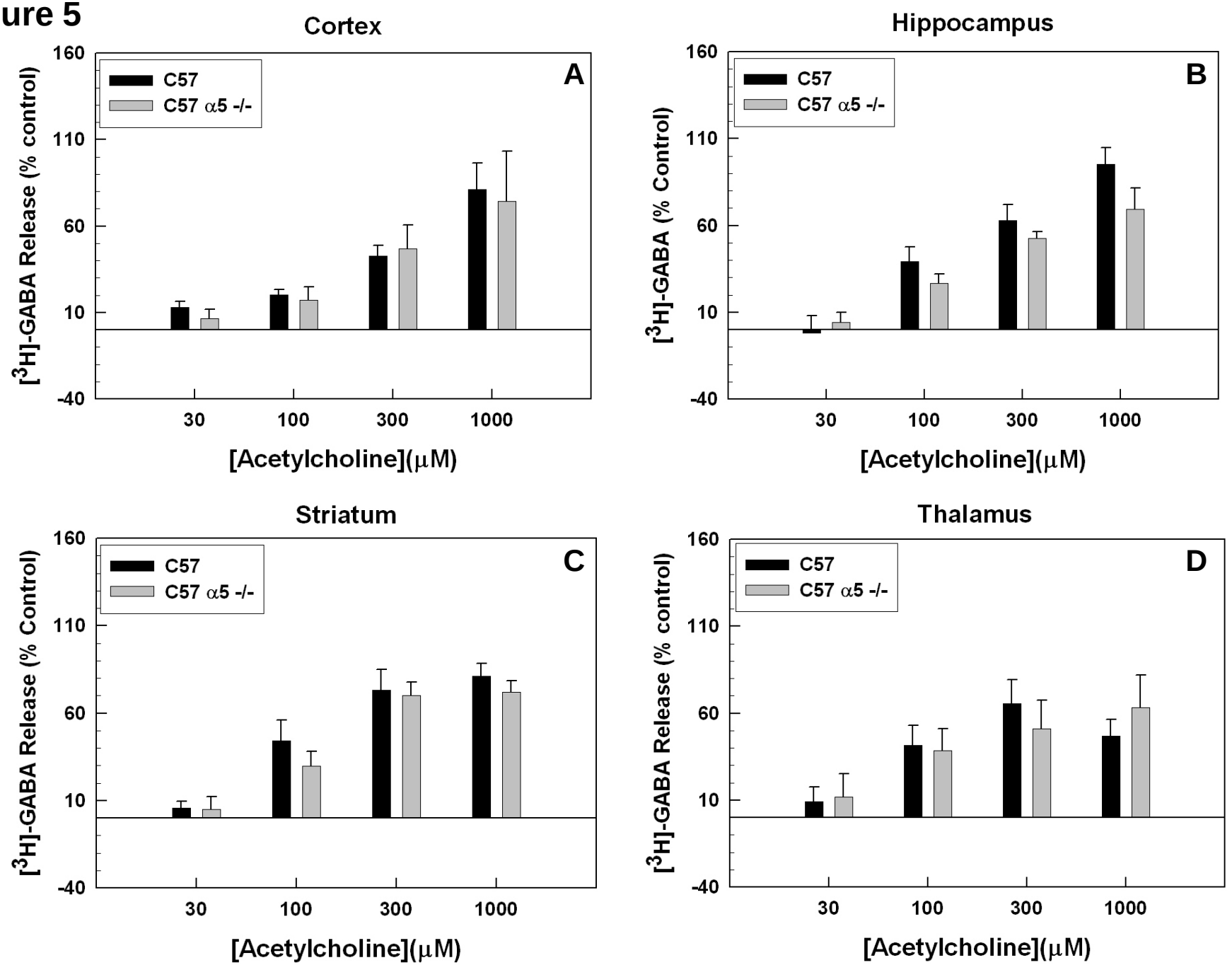

Figure 6