ABSTRACT

Several recent electrophysiological studies have demonstrated that nicotinic agonists stimulate the release of \( \gamma \)-aminobutyric acid (GABA) from rodent brain tissue. Our studies used a neurochemical approach to characterize nicotinic receptor-stimulated \(^{3}H\)-GABA release from mouse brain synaptosomes. Nicotine increased \(^{3}H\)-GABA release from synaptosomes preloaded with \(^{3}H\)-GABA in a concentration-dependent manner. This release appeared rapidly, was Ca\(^{++}\) dependent, and was partially (about 50\%) blocked by 100 nM tetrodotoxin and totally blocked by mecamylamine and dihydro-\( \beta \)-erythroidine. \( \alpha \)-Bungarotoxin had no effect. Twelve nicotinic agonists were compared for their effects on \(^{3}H\)-GABA release. The agonists differed in potency (EC\(_{50}\)) and efficacy (E\(_{\text{max}}\)). The EC\(_{50}\) and E\(_{\text{max}}\) values were significantly correlated (\( r = 0.95, P < .001 \) for EC\(_{50}\); \( r = 0.93, P < .001 \) for E\(_{\text{max}}\)) to values obtained for these same agonists when \(^{86}\text{Rb}^+\) efflux was determined. A significant correlation (\( r = 0.84, P < .01 \)) was found when the EC\(_{50}\) values for agonist-stimulated \(^{3}H\)-GABA release and IC\(_{50}\) values for agonist inhibition of \(^{3}H\)-nicotine binding were compared. Differences in \(^{3}H\)-GABA release were detected in 12 brain regions and maximal release was significantly correlated with \(^{3}H\)-nicotine binding. The pharmacological and regional comparisons suggest that the nAChR that stimulates \(^{3}H\)-GABA release is the one that binds \(^{3}H\)-nicotine with high affinity (\( \alpha4\beta2 \)). Unequivocal evidence that the receptor that modulates nicotine-stimulated \(^{3}H\)-GABA release contains a \( \beta 2 \) subunit was obtained in a study using wild-type, heterozygous and homozygous \( \beta 2 \) null mutant mice. \(^{3}H\)-GABA release and \(^{3}H\)-nicotine binding decreased along with the number of copies of the null mutant gene.

The broad array of behavioral and physiological effects produced by nicotine are presumably initiated by binding to nAChRs that are located throughout the peripheral and central nervous systems. The postsynaptic nAChR found on electric organ and skeletal muscle is the best described of all neurotransmitter receptors (Galzi and Changeux, 1995; Karlin and Akabas, 1995) but motor neurons also seem to have presynaptic autoreceptors that modulate ACh release (Riker et al., 1957). Activation of these autoreceptors decreases ACh release under some circumstances but increases release under other circumstances (Tian et al., 1994; Domet et al., 1995).

Presynaptic nAChRs in brain apparently modulate release of several neurotransmitters (see Wonnacott, 1997, for a recent review). Nicotinic agonists stimulate the release of dopamine (Grady et al., 1992, 1997; Marshall et al., 1996; Rowell et al., 1987; Wonnacott et al., 1989), ACh (Beani et al., 1985; Lapchak et al., 1989; Meyer et al., 1987), and norepinephrine (Clarke and Reuben, 1996) from brain slice and/or synaptosomal preparations. These processes are Ca\(^{++}\) dependent and are blocked by nicotinic antagonists such as mecamylamine.

Several neurochemical studies suggest that nicotine also stimulates GABA release, but it is not clear whether this is a direct or indirect effect. Wonnacott et al. (1989) reported that nicotine directly stimulates \(^{3}H\)-GABA release from rat hippocampal synaptosomes. This effect was blocked by the nAChR antagonist DH\(\beta\)E but not by \( \alpha \)-BTX. In contrast, Kayadjanian et al. (1994) reported that nicotine produces a transient increase in \(^{3}H\)-GABA release from slices obtained from rat globus pallidus and substantia nigra, but this effect

ABBREVIATIONS: Ach, acetylcholine; nAChR, nicotinic cholinergic receptors; GABA, \( \gamma \)-aminobutyric acid; DH\(\beta\)E, dihydro-\( \beta \)-erythroidine; TTX, tetrodotoxin; DMPP, dimethylphenyl piperazinium; DFP, diisopropyl fluorophosphate; \( \alpha \)-BTX, \( \alpha \)-bungarotoxin; HEPES, N-[2-hydroxyethyl]-piperazine-N’-[2-ethanesulfonate] hemisodium salt; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; R(+) SCH23390, R(+) 7-chloro-8-hydroxy-3-methyl-1-phenyl-2.3.4.5-tetrahydro-1H-3-benzazepine hydrochloride, MDL-72222, 3-tropanyl-3.5-dichlorobenzoate; AP-5, DL-2-amino-5-phosphono-pentanoic acid.
was blocked by dopamine receptor antagonists, suggesting that GABA release is a secondary response that follows nicotine-induced dopamine release. Bianchi et al. (1995) also concluded, from a study done with guinea pig cortical slices, that nicotine stimulates GABA release but only as a consequence of stimulating serotonin release that then stimulates GABA release.

Several electrophysiological studies indicate that nicotine stimulates GABA release directly via activation of nAChRs found on, or near, GABA nerve terminals. Léna et al. (1993) concluded that nicotinic agonists stimulated GABA release by activating preterminal nAChRs, because pretreatment of rat interpeduncular nucleus slices with TTX, the Na⁺ channel blocker, blocked nicotinic agonist-evoked increases in postsynaptic GABAergic currents. In contrast, Léna and Changeux (1997) concluded that nicotine-stimulated GABA release from mouse thalamic slices occurs via activation of receptors found on the nerve terminal. This conclusion was drawn, in part, because GABA release was not blocked by TTX.

Direct evidence that supports a presynaptic localization for nAChRs that modulate GABA release comes from the studies of Alkondon et al. (1996) who measured the effects of focal application of ACh on whole cell currents recorded from cultured, dissociated hippocampal pyramidal and bipolar cells; the latter presumably make up the majority of hippocampal GABAergic interneurons. These investigators reported that ACh-induced increases in current density increased with distance from the center of the cell soma suggesting that the nAChRs are at or near the nerve ending.

Mammalian brain contains many nAChR subunits (α2–α7, β2–β4) (reviewed in Lindstrom, 1996) and, assuming that brain nAChRs are pentameric, many different types of receptors might exist. However, in situ hybridization studies have shown that the mRNAs for some of the receptor subtypes are found in only a few brain regions whereas others, such as the α4, β2 and α7 subunits, are widespread leading to the postulate that receptors made up of these subunits should be most frequently encountered (reviewed in Lindstrom, 1996). Studies done using expression systems, primarily Xenopus oocytes, have demonstrated that both α and β subunits affect rank order of agonist potency and efficacy (Luejtje and Patrick, 1991; Wheeler et al., 1993) as well as sensitivity to antagonists (Harvey et al., 1996; Luejtje et al., 1990). These findings suggest that pharmacological approaches may be useful in establishing the subunit composition of receptors that modulate nicotinic agonist-evoked neurotransmitter release.

Molecular genetic strategies might also be useful in determining the functional roles of nAChR subunits. In the last few years transgenic mice have been developed where the β2 nAChR gene has been successfully “knocked out” resulting in mice that lack high affinity nicotine binding sites (Picciotto et al., 1995, 1998). Léna and Changeux (1997) argued, based in part on the observation that β2 null mutant mice do not show nicotinic agonist-induced changes in thalamic GABAergic miniature synaptic currents, that the nAChR that modulates GABA release in mouse thalamus is made up of α4 and β2 subunits.

Our studies comprise a pharmacological assessment of nicotinic agonist-induced [3H]-GABA release from mouse brain synaptosomes. The results suggest that the same receptor that binds [3H]-nicotine with high affinity (α4β2) may be critically involved in modulating GABA release from synaptosomes prepared from many, if not all, mouse brain regions.

### Methods

**Materials.** [3H]-GABA (84–90 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL. DMPP was obtained from Aldrich Chemical Co., Milwaukee, WI. (+)-Anatoxin-a hydrochloride, methylcarbachol chloride, R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride, sulpiride, 3-tropanol-3,5-dichlorobenzonate, 6-cyano-7-nitroquinoline-2,3-dione and DHβE were purchased from Research Biochemicals International, Natick, MA. Mecamylamine was a gift from the Merck Research Laboratories, Rahway, NJ. Sucrose and HEPES were obtained from Boehringer-Mannheim, Indianapolis, IN. The following compounds were products of Sigma Chemical Co., St. Louis, MO: nicotine hydrogen (−)-tartrate (l-nicotine), (+)-nicotine (+)-di-p-toluyltartrate (d-nicot ine), ACh, cytisine, (−)-epibatidine-l-tartrate, carbachol iodide, tetra methylammonium iodide, atropine sulfate, α-BTX, (−)-anabasine, (−)-nor nicotinamide aminoxy acetic acid, GABA, sodium chloride, potassium chloride, calcium chloride, magnesium sulfate, potassium dihydrogen phosphate, veratridine, TTX, d(+)-glucose, DL-2-amino-5-phosphonopentaanoic acid and DFP. Econo-safe scintillation cocktail was purchased from Research Products International Corp., Arlington Heights, IL.

**Animals.** Female C57BL/J6 and β2 null mutant (wild-type, heterozygotes and homozygous null mutant) mice were used in this study. Animals were 60 to 90 days old and were bred at the Institute for Behavioral Genetics, Boulder, CO. The β2 null mutants were originally derived from a C57BL/6-JDBA hybrid (Picciotto et al., 1995). The animals used in this study had been backcrossed onto a C57BL/J6 background for six generations. Mice were housed five per cage and were allowed free access to food and water. The animal colony room was maintained on a 12 hr light/12 hr dark cycle with lights on between 7:00 A.M. and 7:00 P.M. All procedures were in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the University of Colorado animal care committee.

**Synaptosome preparation.** Crude synaptosomes were prepared by hand homogenization of the mouse brain tissue in 0.32 M sucrose buffered with 5 mM HEPES (pH 7.5) in a glass-Teflon homogenizer. The homogenate was centrifuged at 1000 g for 20 min. The supernatant was then centrifuged at 12,000 g for 10 min. The resulting pellets were resuspended in the buffer containing 1 g/liter bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO). The following compounds were purchased from Sigma Chemical Co., St. Louis, MO: (+) nicotine (−)-tartrate (l-nicotine), (+)-nicotine (+)-di-p-toluyltartrate (d-nicotine), ACh, cytisine, (−)-epibatidine-l-tartrate, carbachol iodide, tetramethylammonium iodide, atropine sulfate, α-BTX, (−)-anabasine, (−)-nor nicotine, aminoxy acetic acid, GABA, sodium chloride, potassium chloride, calcium chloride, magnesium sulfate, potassium dihydrogen phosphate, veratridine, TTX, d(+)-glucose, DL-2-amino-5-phosphonopentaanoic acid and DFP. Eco-no-safe scintillation cocktail was purchased from Research Products International Corp., Arlington Heights, IL.

**[3H]-GABA uptake.** The crude synaptosomes were incubated for 10 min at 37°C in perfusion buffer containing 1 mM aminoxyacetic acid, an inhibitor of GABA transaminase. [3H]-GABA and unlabeled GABA were then added to final concentrations of 0.1 and 0.25 μM, respectively, 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) and washed twice with 0.5 ml perfusion buffer. These filters were then transferred to the perfusion apparatus. Samples to be used with ACh were incubated with 10 μM DFP, an irreversible cholinesterase inhibitor, during uptake.

**Perfusion and release.** The perfusion apparatus and procedure have been described in detail previously (Grady et al., 1992). Briefly, each 6-mm filter containing the synaptosomes was placed on a 13-mm glass-fiber filter mounted on a polypropylene platform and perfused with the buffer containing 1 gliter bovine serum albumin at a rate of 1.8 ml/min for 10 min before fraction collection was started. All fractions were collected for 12 sec. In most experiments,
with the exception of the time course experiment, agonists were added to the perfusate for 12 sec. This time period was selected simply because the fractions were collected every 12 sec. Atropine (1 μM) was included in the perfusion buffer for experiments with ACh and carbachol.

Data analysis. To correct for differences in total synaptosomal \[^{[3]H}\]-GABA content within and between experiments, the amount of \[^{[3]H}\]-GABA release induced by an agonist stimulation was normalized as follows. The fractions before and after the stimulation that represent basal release were identified and were then fit as the first-order process \(E_t = E_0 e^{-kt}\), where \(E_0\) is the actual data obtained at each time, \(t\); \(E_t\) is the initial basal release and \(k\) is the rate of decrease of release. This calculation yielded the theoretical basal release for each fraction. The release of \[^{[3]H}\]-GABA exceeding baseline, which represents the agonist-stimulated release, was then calculated by subtracting the theoretical basal release from the actual data and was finally divided by the average baseline underlying the peak. The data are expressed as "units" (U) of release where one unit represents a doubling of the release above baseline. Release traces were constructed using this normalization (e.g., fig. 1). Total release for any stimulation was the sum of the counts exceeding baseline for all fractions after agonist treatment. The inset to figure 1 provides a concentration response curve for total release expressed in units relative to baseline.

Results for each agonist were initially calculated by fitting data to the Hill equation: \(E = A S^n/(K^n + S^n)\), where \(S\) is the agonist concentration, \(E\) is the observed response, \(A\) is the maximum release, and \(n\) is the Hill coefficient. Inasmuch as no Hill coefficient was significantly different from 1, the \(EC_{50}\) and \(E_{max}\) values obtained for the 12 agonists tested using whole brain synaptosomes, for analyses of brain regional differences, for analyzing effects of nonnicotine antagonists on \[^{[3]H}\]-GABA release and for analyzing effects of genotypes of \(\beta\) null mutant on \[^{[3]H}\]-GABA release and \[^{[3]H}\]-nicotine binding. Post hoc comparisons were done using Tukey’s test with the significance level set at 0.05. Values of \(n\) reported in the figure legends represent the number of synaptosomal preparations each of which was derived from a different animal.

Results

Characterization of nicotine-stimulated \[^{[3]H}\]-GABA release. Initial studies assessed the effects of nicotine on \[^{[3]H}\]-GABA release from thalamic synaptosomes. This brain region was chosen because it has the highest levels of \[^{[3]H}\]-nicotine binding of any of 12 brain regions that we routinely study (Marks et al., 1996). A concentration-dependent release of \[^{[3]H}\]-GABA was observed following a 12-sec stimulation period (fig. 1). The \(EC_{50}\) for nicotine-stimulated \[^{[3]H}\]-GABA release calculated from these data is 5.60 ± 1.97 μM with a Hill coefficient of 0.88 ± 0.26. This Hill coefficient is not significantly different from 1.0.

Nicotine produced a rapid increase in \[^{[3]H}\]-GABA release (fig. 2a), but \[^{[3]H}\]-GABA release did not persist in the continued presence of agonist (fig. 2b). The total amount of \[^{[3]H}\]-GABA release increased with the time of exposure to nicotine, but the rate of release decreased with exposure time (\(k = 0.062 \pm 0.021\) sec\(^{-1}\), \(t_{1/2} = 11.5\) sec). The rate constant of the declining phase of release was \(k = 0.02 \pm 0.006\) sec\(^{-1}\) which yields a \(t_{1/2}\) of 34 sec. This decrease in response presumably arose because of receptor desensitization.

Effects of \(Ca^{2+}\) and TTX on \[^{[3]H}\]-GABA release. The \(Ca^{2+}\) dependence of the release process was determined by
measuring [3H]-GABA release stimulated by 30 mM nicotine added to perfusion buffer containing 3.2 mM Ca$^{2+}$ or nominally calcium-free buffer (4.8 mM NaCl was substituted for the 3.2 mM CaCl$_2$). Results of a typical experiment are shown in figure 3. No release (0.06 ± 0.03 U) was seen in calcium-free media.

Inasmuch as TTX has been reported to partially inhibit nicotine-induced $^{86}$Rb$^+$ efflux (Marks et al., 1993) and [3H]-dopamine release (Marshall et al., 1996) from brain synaptosomes, the effects of TTX (100 nM) on [3H]-GABA release stimulated by nicotine, veratridine and K$^+$ were measured (table 1). TTX treatment completely inhibited veratridine-stimulated release, but had no effect on K$^+$-stimulated release, indicating that this concentration of TTX was adequate to block Na$^+$ channels but did not directly affect the release mechanism. TTX treatment inhibited nicotine-induced [3H]-GABA release by 50%.

Effects of nicotinic receptor antagonists on nicotine-stimulated [3H]-GABA release. The effects of three nAChR antagonists (DHβE, mecamylamine, α-bungarotoxin) on nicotine-stimulated [3H]-GABA release were examined using whole brain synaptosomes (fig. 4). DHβE and mecamylamine produced concentration-dependent inhibition of nicotine-evoked [3H]-GABA release with IC$_{50}$ values of 0.34 and 1.23 μM, respectively. α-BTX did not affect [3H]-GABA release even at the highest concentration tested (1 μM). This concentration of α-bungarotoxin completely inhibits the binding of [$^{125}$I]-α-bungarotoxin to membranes prepared from mouse brain (Marks and Collins, 1982).

Effects of neurotransmitter antagonists on nicotine-stimulated [3H]-GABA release. Bianchi et al. (1995) noted that the 5-HT$_3$ receptor antagonist, MDL-72222, inhibited nicotine-stimulated GABA release from guinea pig cortical slices, and Kayadanian et al. (1994) reported that the D1 antagonist, (+)-SCH-23390, blocked [3H]-GABA release from rat substantia nigra slices. Consequently, the potential effects of MDL-72222 and (+)-SCH-23390 treatment on [3H]-GABA release were measured using synaptosomes prepared from whole brain. Potential effects of atropine (muscarinic antagonist), sulpiride (D2 antagonist), 6-cyano-7-nitroquinolxaline-2.3-dione (glutamate antagonist) and AP5 (NMDA antagonist) were also determined and compared with the effects produced by DHβE. All seven antagonists were present during the 10-min prewash period as well as during and after the 12-sec stimulation of 30 μM nicotine. The results of these experiments are depicted in figure 5. None of the antagonists, with the exception of DHβE, altered the [3H]-GABA release elicited by 30 μM nicotine. This suggests that [3H]-GABA release is a direct consequence of nAChR activation.

Effects of nicotinic agonists on [3H] GABA release. The effects of perfusion for 12 sec with varying concentrations of 12 agonists on [3H] GABA release from whole brain synaptosomes are illustrated in figure 6. All 12 agonists stimulated a concentration-dependent increase in [3H]-GABA release. The Hill coefficients calculated from these data were not significantly different from 1.0 for any of the agonists. Therefore, the Michaelis-Menten equation was used to fit the data:

\[
\frac{A_t}{A_0} = \frac{1}{1 + e^{-k(t-t_0)}}
\]

where $A_t$ is total release at time, $t$; $A_0$ is the maximal release; $k$ is the rate of activation of release.
<p>Fig. 4. Concentration-response curves for inhibition of nicotine-stimulated [3H]-GABA release by DHβE, mecamylamine and α-BTX. Synaptosomes from whole brain were used and stimulated with 30 μM nicotine for 12 sec in the presence of various concentrations of each antagonist. DHβE and mecamylamine were only present during the 12-sec exposure to nicotine. α-BTX was incubated with synaptosomes for 60 min at 37°C before perfusion was begun and was not present during the perfusion (10 min). Each point represents the mean ± S.E.M. of four to six separate stimulations of synaptosomes. Open circles are 30 μM nicotine in the absence of antagonists. Lines are theoretical curve fit for inhibition (see “Methods” for equation).

Fig. 5. Effects of (+)-SCH-23390, sulpiride, MDL-72222, CNQX, AP-5, atropine and DHβE on [3H]-GABA release stimulated by 30 μM nicotine. Whole brain synaptosomes were exposed to the antagonists for 10 min before 12-sec stimulation of nicotine and antagonists continuously presented during and after stimulations of nicotine. Data are the mean ± S.E.M. calculated from at least four separate stimulations of synaptosomes. * P < .05 vs. control. Analysis of variance with post hoc multiple comparison (Tukey’s test) was performed.

used to calculate EC<sub>50</sub> and E<sub>max</sub> values (table 2). Significant differences in agonist potency (F<sub>11,72</sub> = 14.88, P < .01) were observed. The response showed stereoselectivity since the naturally occurring isomer, l-nicotine, was more potent than D-nicotine (EC<sub>50</sub> = 1.6 and 12.8 μM, respectively).

The maximal [3H]-GABA release (E<sub>max</sub>) stimulated by the 12 agonists also differed significantly (F<sub>11,72</sub> = 45.4, P < .001). Not only was l-nicotine more potent, it was also more efficacious than D-nicotine; the maximal [3H]-GABA release stimulated by D-nicotine was 60% of that elicited by l-nicotine. All of the quaternary ammonium compounds (acetylcholine, DMPP, carbachol, methylocarbachol and TMA) appeared to have similar, high maximal release (1.16–1.37 U). Epibatidine also elicited a high maximal GABA release (1.35 U). With the exception of l-nicotine (0.83 U) and epibatidine, the other nonquaternary compounds (D-nicotine, cytisine, (+)-anatoxin-a, nornicotine and (±)-anabasine) produced low maximal GABA release (E<sub>max</sub> values ranged from 0.27–0.5 U).

Studies using β2 null mutants. Figure 7 presents the results of experiments where nicotine- and K<sup>+</sup>-stimulated [3H]-GABA release were measured in synaptosomes (whole brain) prepared from homozygous wild type (+/+), heterozygote (+/−) and homozygous null (−/−) β2 mutant mice (Picciotto et al., 1995, 1998). Figure 7a shows release traces obtained following stimulation for 12 sec with 30 μM nicotine for one mouse of each genotype. Figure 7b provides the overall results: Genotype exerted a significant overall effect on nicotine-stimulated [3H]-GABA release (F<sub>2,14</sub> = 11.55, P < .01). There was virtually no release obtained in synaptosomes prepared from the homozygous β2 null mutants. The [3H]-GABA release seen in heterozygotes was intermediate between the wild-type controls and the homozygous null mutants. No differences were seen among the three genotypes after 20 mM K<sup>+</sup> stimulation (fig. 7c) indicating that the [3H]-GABA release mechanism was not disrupted by the null mutation. [3H]-Nicotine binding was also measured in membrane fractions prepared from the whole brain synaptosomes (fig. 7d). A significant effect of genotype on [3H]-nicotine binding was observed (F<sub>2,14</sub> = 43.72, P < .001). As was the case for the [3H]-GABA release data, a gene dose effect was seen: [3H]-nicotine binding was virtually absent in the β2 null mutants and the heterozygotes were midway between the mutant and wild-type. [3H]-Nicotine binding and nicotine-stimulated [3H]-GABA release were significantly correlated (r = 0.76, P < .001) across the three genotypes (+/+, +/- and −/−).

Regional comparison of nicotine-stimulated [3H]GABA release. Figure 8 illustrates the results of experiments where concentration-effect curves for nicotine-stimulated GABA release were determined in 11 brain regions of C57BL/6 mice; whole brain data are included for comparison. Nicotine stimulated concentration-dependent increases in [3H]-GABA release in every brain region studied. Hill coefficients calculated for each of these curves were not significantly different from 1.0. The EC<sub>50</sub> and E<sub>max</sub> values were calculated using the Michaelis-Menten equation and are presented in table 3. The EC<sub>50</sub> values for nicotine-stimulated release ranged between (1.43–19.9 μM). The EC<sub>50</sub> value in cerebellum is significantly different from those obtained in any other regions (F<sub>10,58</sub> = 2.53, P < .05). The E<sub>max</sub> values differed significantly among the brain regions (F<sub>10,58</sub> = 21.22, P < .001) with the release observed in striatum being...
the highest (1.41 U) and the release observed in olfactory bulb being the lowest (0.34 U).

The pattern of regional stimulation of maximal $[^{3}H]$-GABA release was compared to $L$-$[^{3}H]$-nicotine binding (Marks et al., 1992; Marks, unpublished data) and analyzed by correlation analysis (fig. 9). Maximal $[^{3}H]$-GABA release induced by nicotine was significantly correlated with the regional distribution of nicotine binding ($r = 0.69$, $P < .01$).

**Discussion**

Our data demonstrate that application of nicotinic agonists to synaptosomes that have been preloaded with $[^{3}H]$-GABA results in a concentration-dependent release of $[^{3}H]$-GABA. GABA release occurs rapidly after agonist application and decreases to zero in the continued presence of agonist, suggesting that desensitization occurs. Agonist-stimulated release is Ca$^{2+}$ dependent and is blocked by classical nAChR antagonists. Nicotine stimulated the release of $[^{3}H]$-GABA from synaptosomes prepared from every brain region tested. This finding argues that nicotine-evoked GABA release may play a major role in regulating behavioral and centrally mediated physiological responses to nicotine.

Nicotinic-receptor-mediated GABA release has previously been observed using electrophysiological and biochemical techniques. Electrophysiological studies with slices prepared from rat interpeduncular nucleus (Léna and Changeux, 1997), and rat hippocampus (Alkondon et al., 1997) have detected a nicotine-stimulated increase in miniature inhibitory postsynaptic currents that were blocked by GABA antagonists. Nicotine also promotes the release of GABA in vivo. Iontophoretic application of nicotine to the rat medial septum results in a decrease in neuronal firing rate which seems to be due to GABA release (Yang et al., 1996), and nicotine promotes the release of GABA in the rat dorsal motor nucleus of the vagus (Bertolino et al., 1996). Using biochemical methods, Wonnacott et al. (1989) observed nicotine stimulated release of $[^{3}H]$-GABA from rat hippocampal synaptosomes, and Kyadjanian et al. (1994) and Bianchi et al. (1995) reported nicotine stimulated release from tissue slices. On the basis of pharmacological data, the latter two studies suggested that GABA release may be secondary to nicotine-stimulated dopamine or serotonin release, respectively. However, such secondary effects seem unlikely in our study because synaptomes were used, a rapid response was observed and the sample perfusion rate was rapid, reducing neurotransmitter accumulation. Furthermore, antagonists of dopamine, serotonin, glutamate and muscarinic receptors had no effect on nicotine-stimulated GABA release, indicating that activation of these receptors was not involved in the release. Consequently, it is likely that the GABA release that was measured in our studies occurs

**Table 2**

Agonist-stimulated $[^{3}H]$-GABA release from whole brain synaptosomes

<table>
<thead>
<tr>
<th>Agonists</th>
<th>$EC_{50}$ ($\mu M$)</th>
<th>$E_{max}$ (U)</th>
</tr>
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<tbody>
<tr>
<td>a. Cytisine</td>
<td>0.005 ± 0.007$^c$</td>
<td>0.29 ± 0.04$^{a,c,h,k,l}$</td>
</tr>
<tr>
<td>b. Epibatidine</td>
<td>0.009 ± 0.006$^{d,l}$</td>
<td>1.35 ± 0.07$^{a,c,d,f,g,j}$</td>
</tr>
<tr>
<td>c. (+)-Anatoxin-a</td>
<td>0.28 ± 0.2$^{e,f}$</td>
<td>0.55 ± 0.04$^{b,e,f,h,i,k,l}$</td>
</tr>
<tr>
<td>d. (-)-Abasine</td>
<td>0.39 ± 0.20$^{e,f}$</td>
<td>0.35 ± 0.02$^{a,c,d,f,g,j}$</td>
</tr>
<tr>
<td>e. Acetylcholine</td>
<td>1.61 ± 0.17</td>
<td>1.16 ± 0.03$^{a,c,d,f,g,j}$</td>
</tr>
<tr>
<td>f. L-Nicotine</td>
<td>1.63 ± 0.59$^{a,c}$</td>
<td>0.83 ± 0.05$^{a,c,d,f,j}$</td>
</tr>
<tr>
<td>g. Nornicotine</td>
<td>2.83 ± 0.96$^{a,c}$</td>
<td>0.27 ± 0.08$^{a,c,d,f,h,k,l}$</td>
</tr>
<tr>
<td>h. DMPP</td>
<td>6.75 ± 1.79$^{a,c}$</td>
<td>1.16 ± 0.05$^{a,c,d,f,g,j}$</td>
</tr>
<tr>
<td>i. Methylcarbachol</td>
<td>6.89 ± 2.38$^{a,c}$</td>
<td>1.37 ± 0.14$^{a,c,d,f,g,j}$</td>
</tr>
<tr>
<td>j. D-Nicotine</td>
<td>12.8 ± 2.92$^{a,c}$</td>
<td>0.5 ± 0.05$^{a,c,d,f,h,j}$</td>
</tr>
<tr>
<td>k. Carbachol</td>
<td>32.4 ± 12.1$^{a,c}$</td>
<td>1.27 ± 0.05$^{a,c,d,f,g,j}$</td>
</tr>
<tr>
<td>l. TMA</td>
<td>42.26 ± 15.31$^{a,c}$</td>
<td>1.20 ± 0.08$^{a,c,d,f,g,j}$</td>
</tr>
</tbody>
</table>

$EC_{50}$ values and the maximal release of $[^{3}H]$-GABA ($E_{max}$) were calculated using the Michaelis-Menten equation. Data are presented as mean ± S.E.M. Each agonist was assigned a lowercase letter. These letters, when placed above a value, indicate significant differences from the relevant agonist (Tukey b test, $P < .05$).
the ob-
argued that the nAChR that modulates GABA release from
$[3H]$-GABA release elicited by 20 mM K$^+$

In contrast, veratridine-stimulated release

mice by TTX. In contrast, TTX did not block nicotine-evoked $[3H]$-GABA release from mouse brain synapto-

because of a direct activation of nAChRs found on GABAergic neurons. It is possible, however, that other neurotransmitters modulate nicotinic activation-induced GABA release in vivo.

Nicotinic-receptor-mediated GABA release has been re-

Fig. 7. Comparison of $[3H]$-GABA release and $[3H]$-nicotine binding in β2 nAChR receptor null mutant mice. The effects of 30 μM nicotine and 20 mM K$^+$ on simulation of $[3H]$-GABA release, and $[3H]$-l-tocinonic binding, were examined in synaptosomes (whole brain) prepared from wild type (control $+/+$, $n = 4$), heterozygote (+/−, $n = 7$) and homozygous β2 null mutant (−/−, $n = 5$) mice. Panel a provides representative normalized $[3H]$-GABA release traces for 30 μM nicotine. Figure 7b provides total $[3H]$-GABA release stimulated by 30 μM nicotine. Figure 7c presents the $[3H]$-GABA release elicited by 20 mM K$^+$. Figure 7d presents $[3H]$-l-

these findings suggest that approximately half of the total release that we measured resulted from a cascade where nAChR stimulation produced enough voltage change to activate TTX-sensitive Na$^+$ channels that, in turn, generated enough voltage change so that voltage-gated Ca$^{2+}$ channels were activated leading to transmitter release. This might occur if a significant fraction of the synaptosomes contained preterminal elements where the nAChRs were not in close proximity to the relevant Ca$^{2+}$ channels. However, the finding that TTX did not block approximately 50% of the nicotine-evoked $[3H]$-GABA release suggests either that the Ca$^{2+}$ permeability of the relevant nAChRs is sufficient to stimulate the release process directly or the nAChRs are in close proximity to voltage-gated Ca$^{2+}$ channels that are activated by the voltage change produced by nAChR activation. This might occur if approximately half of the synaptosomes were derived from neurons where the nAChRs are directly associated with the terminal.

Although definitive assignment of a distinct nicotinic receptor as the mediator of nAChR-stimulated GABA release is not yet possible, the abolition of the response in β2 null mutants indicates that this subunit is present in the nAChR subtype that mediates GABA release from mouse brain synap-

The $[3H]$-nicotine efflux assay uses methods nearly identical to those used for GABA release, a direct comparison of the results for these two responses is possible. Figure 10a presents dose-response curves for the effects of four agonists (ACh, cytisine, DMPP, nicotine) on $[3H]$-GABA and $[3H]$-Rb$^+$ efflux from mouse brain synaptosomes. As is readily evident from the concentration effect curves shown for the four agonists, virtually identical EC$_{50}$ and E$_{max}$ values were obtained for the four agonists in the two assays. Figure 10b provides a direct comparison of the EC$_{50}$ and E$_{max}$ values for all 12 of the agonists. Significant correlations for the EC$_{50}$ (r = 0.95, P < .001) and E$_{max}$ (r = 0.93, P < .01) values were obtained when agonist effects on the two assays were compared. This finding suggests that the two assays are measuring the same receptor(s).

Figure 11 presents a comparison of the relationship between the EC$_{50}$ values for agonist-stimulated release of $[3H]$-GABA and the IC$_{50}$ values of these same agonists for inhibition of $[3H]$-l-nicotine binding. The binding data are those presented in Marks et al. (1993 and 1996). The potency of agonist-stimulated $[3H]$-GABA release was highly correlated to the IC$_{50}$ values of agonist inhibition $[3H]$-nicotine binding (r = 0.84, P < .01).

The potencies of agonist stimulation of $[3H]$-GABA release (EC$_{50}$ values) were highly correlated with the potencies of these 11 compounds with respect to stimulation of $[3H]$-nicotine binding (IC$_{50}$ values). These findings argue that the receptor that modulates nicotine-evoked GABA release is very similar, if not identical, to
the receptor that modulates \(^{86}\)Rb\(^+\) release at low \(\mu\)M concentrations and binds \(^{3}H\)-nicotine with high affinity. Immunological evidence (Whiting and Lindstrom, 1988) and evidence obtained with the \(\beta_2\) null mutants (Picciotto et al., 1995; our data) argue that the high affinity \(^{3}H\)-nicotine binding site includes a \(\beta_2\) subunit. Because antibodies directed against the \(\alpha_4\) subunit precipitate more than 90% of rat brain high affinity nicotine binding sites (Flores et al., 1992), it seems highly likely that this binding site is made up of \(\alpha_4\) and \(\beta_2\) subunits. These considerations suggest that \(\alpha_4\beta_2\)-containing nAChRs account for a major percentage of the nAChRs that modulate GABA release. This conclusion agrees with the

![Fig. 8. A regional comparison of nicotine-stimulated \(^{3}H\)-GABA release from synaptosomes. Synaptosomes were stimulated for \(^{3}H\)-GABA release by 12-sec exposure of various concentrations of nicotine. Each point represents the mean ± S.E.M. calculated from four to eight separate stimulations of synaptosomes. The curves were obtained by fitting the data to the Michaelis-Menten equation with a nonlinear least-squares algorithm.](image)

![Fig. 9. Correlation between maximal \(^{3}H\)-GABA release and maximal \(^{3}H\)-nicotine binding in 11 brain regions. The abbreviations for brain regions are as following, OB, olfactory bulb; HT, hypothalamus; CB, cerebellum; MB, midbrain; HB, hindbrain; HP, hippocampus; IC, inferior colliculi; SC, superior colliculi; CX, cortex; TH, thalamus; and ST, striatum. The E\(_{\text{max}}\) (maximum of \(^{3}H\)-GABA release) data are summarized in table 3 and the \(^{3}H\)-nicotine binding (B\(_{\text{max}}\)) data were obtained from P2 synaptosomal preparation (Marks et al., 1993).](image)

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>(E_{\text{C50}}) ((\mu)M)</th>
<th>(E_{\text{max}}) (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Olfactory bulb</td>
<td>1.40 ± 4.50</td>
<td>0.34 ± 0.66</td>
</tr>
<tr>
<td>b. Hippocampus</td>
<td>8.15 ± 4.20</td>
<td>0.75 ± 0.13</td>
</tr>
<tr>
<td>c. Striatum</td>
<td>1.48 ± 0.53</td>
<td>1.41 ± 0.10</td>
</tr>
<tr>
<td>d. Cortex</td>
<td>8.09 ± 4.30</td>
<td>1.09 ± 0.12</td>
</tr>
<tr>
<td>e. Thalamus</td>
<td>5.60 ± 1.97</td>
<td>1.27 ± 0.05</td>
</tr>
<tr>
<td>f. Midbrain</td>
<td>2.88 ± 1.16</td>
<td>0.54 ± 0.02</td>
</tr>
<tr>
<td>g. Superior. colliculi</td>
<td>5.60 ± 2.40</td>
<td>1.23 ± 0.13</td>
</tr>
<tr>
<td>h. Inferior. colliculi</td>
<td>2.19 ± 0.69</td>
<td>0.74 ± 0.03</td>
</tr>
<tr>
<td>i. Hypothalamus</td>
<td>1.91 ± 1.45</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td>j. Cerebellum</td>
<td>19.99 ± 8.60</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td>k. Hindbrain</td>
<td>3.12 ± 0.69</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td>Whole brain</td>
<td>1.63 ± 0.50</td>
<td>0.83 ± 0.05</td>
</tr>
</tbody>
</table>

\(E_{\text{C50}}\) and \(E_{\text{max}}\) values were calculated using the Michaelis-Menten equation and are presented as mean ± s.e.m. Each brain region was assigned a lowercase letter. These letters, when placed above a value, indicate that the \(E_{\text{C50}}\) or \(E_{\text{max}}\) values of the relevant brain regions are significantly different from the listed region as determined using a Tukey b test (\(P < .05\)).

A significant association (\(r = 0.67\), \(P < .01\)) was found between maximal \(^{3}H\)-GABA release and \(^{3}H\)-nicotine binding when these parameters were measured in 11 brain regions. This finding also supports the argument that the re-
receptor that binds nicotine with high affinity is also the one that modulates GABA release. However, when we made a similar comparison for nicotine-stimulated $^{3}H$-GABA release a higher correlation ($r = 0.93$) was obtained between these measures across the same brain regions (Marks et al., 1993). One potential explanation for this difference is that $\alpha 4\beta 2$-containing receptors modulate nicotine-induced GABA release in most brain regions, but in some regions another receptor, or additional receptors may modulate GABA release. In addition, it seems likely that the receptor that binds $[3]H$-nicotine with high affinity ($\alpha 4\beta 2$) has functions in addition to modulating GABA release. These other functions may well vary across brain regions.

One potential explanation for regional differences in nicotine-stimulated $[3]H$-GABA release is brain regions clearly vary in numbers of GABA neurons. This, obviously, should result in regional variability in $[3]H$-GABA uptake into synaptosomes. This variability does not, however, explain the regional variability in nicotine-stimulated $[3]H$-GABA uptake although the nicotine-stimulated $[3]H$-GABA release of these three regions was virtually the same.

Alkondon et al. (1996, 1997) have obtained data that argue that nicotinic agonist-induced GABA release may be modulated by $\alpha 7$-containing nAChRs that bind $\alpha$-bungarotoxin with high affinity. Our data do not support this argument because $\alpha$-bungarotoxin did not block release. Similarly, $\alpha$-bungarotoxin does not block $[3]H$-GABA release from rat hippocampal synaptosomes (Wonnacott et al., 1989). It is probably unwise, however, to conclude that $\alpha 7$-containing nAChRs do not modulate GABA release because $\alpha 7$-containing nAChRs desensitize very quickly (Couturier et al., 1990), and synaptosomal perfusion studies may not be capable of detecting $[3]H$-GABA release produced by a receptor that desensitizes quickly. Thus, $\alpha 7$-containing nAChRs may modulate some GABA release but it is highly unlikely that $\alpha 7$-modulated release contributes substantially to the release that we measured.

The results of the experiments reported here clearly demonstrate that activation of presynaptic nAChRs results in concentration-dependent release of GABA. Because this effect is not seen in synaptosomes prepared from whole brain of $\beta 2$ null mutant mice, it seems very safe to conclude that the $\beta 2$ subunit is a component of all of the receptors that modulate this response. The pharmacological approach, primarily the agonist studies, argue that an $\alpha 4$ subunit is also involved, at least in most brain regions.

References
Alkondon M, Pereira EFR, Barbosa CT and Albuquerque EX (1997) Neuronal nicotinic acetylcholine receptor activation modulates $\gamma$-aminobutyric acid release from CA1 neurons of rat hippocampal slices. J Pharmacol Exp Ther 283:1396-1411.

Fig. 11. Relationship between $EC_{50}$ values of the 12 agonists for stimulations of $[3]H$-GABA release and $IC_{50}$ values of these same agonists for inhibition of L-$[3]H$-nicotine binding. The binding data are those presented in Marks et al. (1996 and 1993). The correlation coefficient is significant ($P < .01$).


