Nicotinic Agonist Modulation of Neurotransmitter Levels in the Rat Frontoparietal Cortex

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ABSTRACT—Anabaseine is a naturally occurring toxin that stimulates a variety of neuronal and muscle nicotinic receptors. GTS-21 [3-(2,4-dimethoxybenzylidene)anabaseine], an anabaseine derivative, selectively stimulates α7-containing nicotinic receptors. Here we report the first in vivo study of the effects of these two nicotinic agonists on cortical extracellular acetylcholine (ACh), dopamine (DA), norepinephrine (NE) and serotonin (5-HT) levels, measured with a microdialysis probe placed within the frontoparietal cortex in the absence of a cholinesterase inhibitor. At 3.6 μmol/kg, s.c., anabaseine increased cortical ACh and NE above baseline values without significantly affecting DA and 5-HT. The ACh and NE elevations were inhibited by i.p. pre-administration (4.9 μmol/kg) of the nicotinic antagonist mecamylamine (Mec). In contrast, GTS-21 (3.6 μmol/kg, s.c.) significantly increased NE and DA without affecting ACh and 5-HT levels. Following Mec injection, GTS-21 increased ACh 25-fold and 5-HT 13-fold, while NE and DA levels were slightly decreased in comparison with GTS-21 alone. We suggest that at the dose used, Mec may preferentially block high affinity nicotinic receptors which normally provide an inhibitory influence upon ACh release, thereby permitting expression of the complete stimulatory effect of GTS-21 on neuronal α7-receptors. GTS-21 and other receptor subtype-selective nicotinic agonists should be helpful in clarifying the roles of particular nicotinic receptors in modulating cortical neurotransmitter levels.

Keywords: Acetylcholine, Alzheimer's therapy, Mecamylamine, Microdialysis, Nicotinic agonist

Several laboratories are currently synthesizing and testing a variety of nicotinic agonists that may have therapeutic use for the treatment of Alzheimer's disease and other dementias, Parkinson's disease, Tourette's syndrome, tobacco dependence, and other disorders (1, 2). Anabaseine (Fig. 1), a neurotoxin present in certain animal venoms, has been a useful molecular model for nicotinic drug design (3, 4). It is a potent agonist at certain peripheral and central nicotinic receptors (5, 6; W.R. Kem et al., submitted). GTS-21 [3-(2,4-dimethoxybenzylidene)anabaseine] (Fig. 1), a benzylidene derivative of anabaseine that selectively activates central nicotinic receptors, is currently undergoing clinical tests as a possible means of ameliorating the cognitive impairment associated with Alzheimer's disease (7). This experimental drug possesses mixed agonist/antagonist properties. It is a partial agonist at α7-homo-oligomeric receptors but probably acts as a relatively weak antagonist on high affinity nicotinic receptors, since its maximum effect on $\alpha 4\beta 2$-receptors expressed in the *Xenopus* oocyte was only about 4% that of acetylcholine (ACh) (8). At much higher concentrations than are necessary to enhance cognition, GTS-21 acts as a weak antagonist at peripheral nervous system nicotinic receptors (W.R. Kem et al., unpublished results). GTS-21 was found to improve several types of cognitive behavior in experimental mammals (6, 9, 10).

The actions of nicotine and several related nicotinic agonists on cortical neurotransmitter levels have recently been investigated by in vivo microdialysis methods. Toide and Arima (11) were the first to show that nicotine elevated ACh in the rat frontal cortex. Toth et al. (12), who locally infused 1 mM nicotine through a microdialysis tube into the rat frontal cortex, observed significant increases in dopamine (DA), serotonin (5-HT) and gluta-

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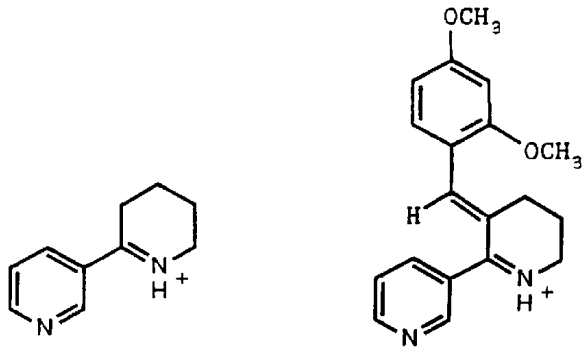

Fig. 1. Structures of anabaseine and GTS-21 in their monocationic form.

mate, but not norepinephrine (NE); in their study, ACh was not measured. Ribeiro et al. (13) found that 4 mg/kg subcutaneous (s.c.) nicotine bitartrate elevated 5-HT for 120 min; this was blocked by prior administration of 5 mg/kg mecamylamine (Mec). Summers and Giacobini (14), using a much lower dose, found that s.c. administered nicotine increased ACh, NE and DA levels but not 5-HT levels. Pretreatment with Mec prevented the nicotine stimulation of ACh, DA and NE levels. ACh and DA levels also increased significantly when nicotine was administered locally through the microdialysis probe (15). Several chemically related nicotinic agonists had different actions on the levels of these four neurotransmitters, perhaps by preferentially stimulating certain nicotinic receptor subtypes (16).

While the pharmacological properties of anabaseine have been investigated using a variety of in vitro preparations, its ability to alter neurotransmitter release in vivo has not been previously investigated. In the present study, we show that the broad spectrum nicotinic agonist anabaseine and the α7-selective agonist GTS-21 affect cortical neurotransmitter levels in very different ways. Some possible mechanisms that might explain these observations are presented in order to stimulate further investigation.

MATERIALS AND METHODS

Materials

Anabaseine dihydrochloride (MW 251) was synthesized according to Bloom (17) and GTS-21 dihydrochloride (MW 381) according to a method previously reported for the synthesis of related 3-substituted anabaseines (18, 19). (*S*)-Nicotine bitartrate and Mec hydrochloride were purchased from Sigma Chem. Co. (St. Louis, MO, USA).

Microdialysis surgery and experiments

Surgical procedures and experimental protocols closely followed those described by Summers et al. (14, 16). Microdialysis probes (AN69HF) were inserted transversely into the cortices (A +1.0, V −2.0 measured from the bregma) of male Sprague-Dawley rats weighing 250–400 g. Rats were allowed to recover overnight from surgery before the experiment. Injected compounds were dissolved in saline and subcutaneously (anabaseine and GTS-21) or intraperitoneally (Mec) administered in a volume of 2 ml/kg animal weight. The animals were awake and able to move within their respective cages during the experiment.

Measurements of neurotransmitter levels

Fractions of dialysate were collected every 30 min into vials containing 1.0 N acetic acid to prevent oxidation of catecholamines. Samples (4–6) were collected to establish basal neurotransmitter levels before administration of drug. Samples were split and run on two separated HPLC systems. One was coupled to a post-column immobilized enzyme reactor for ACh detection that contained acetylcholinesterase to convert ACh to choline and choline oxidase to convert the choline to betaine and electrochemically active hydrogen peroxide. The three biogenic amines were determined with a Coulochem II electrochemical detector (ESA, Bedford, MA, USA). Quantification was achieved by comparing peak areas of the samples to a standard curve. See the previous paper (14) for a more detailed description of these methods.

Statistical analyses

Paired *t*-tests were used to assess the differences between baseline and post-injection time points within rats. Results are expressed as the mean ± S.E.M. and asterisks indicate points that are significantly ($P<0.05$) different from the baseline. Each mean value was obtained from six rats run in parallel.

RESULTS

Anabaseine effects

This toxin (Fig. 2) significantly enhanced the extracellular levels of both ACh (50% increase from baseline) and NE (62%). It affected these two neurotransmitters in a manner similar to nicotine when applied in an equimolar (3.6 μmol/kg) dose (16). DA and 5-HT concentrations were unaffected.

When administered 1.5 hr before anabaseine, Mec (4.90 μmol/kg or 1 mg/kg) blocked elevations of both ACh and NE, as was previously observed with nicotine (16). The 5-HT level was not affected by anabaseine after Mec preadministration. DA increased 85% after sequen-

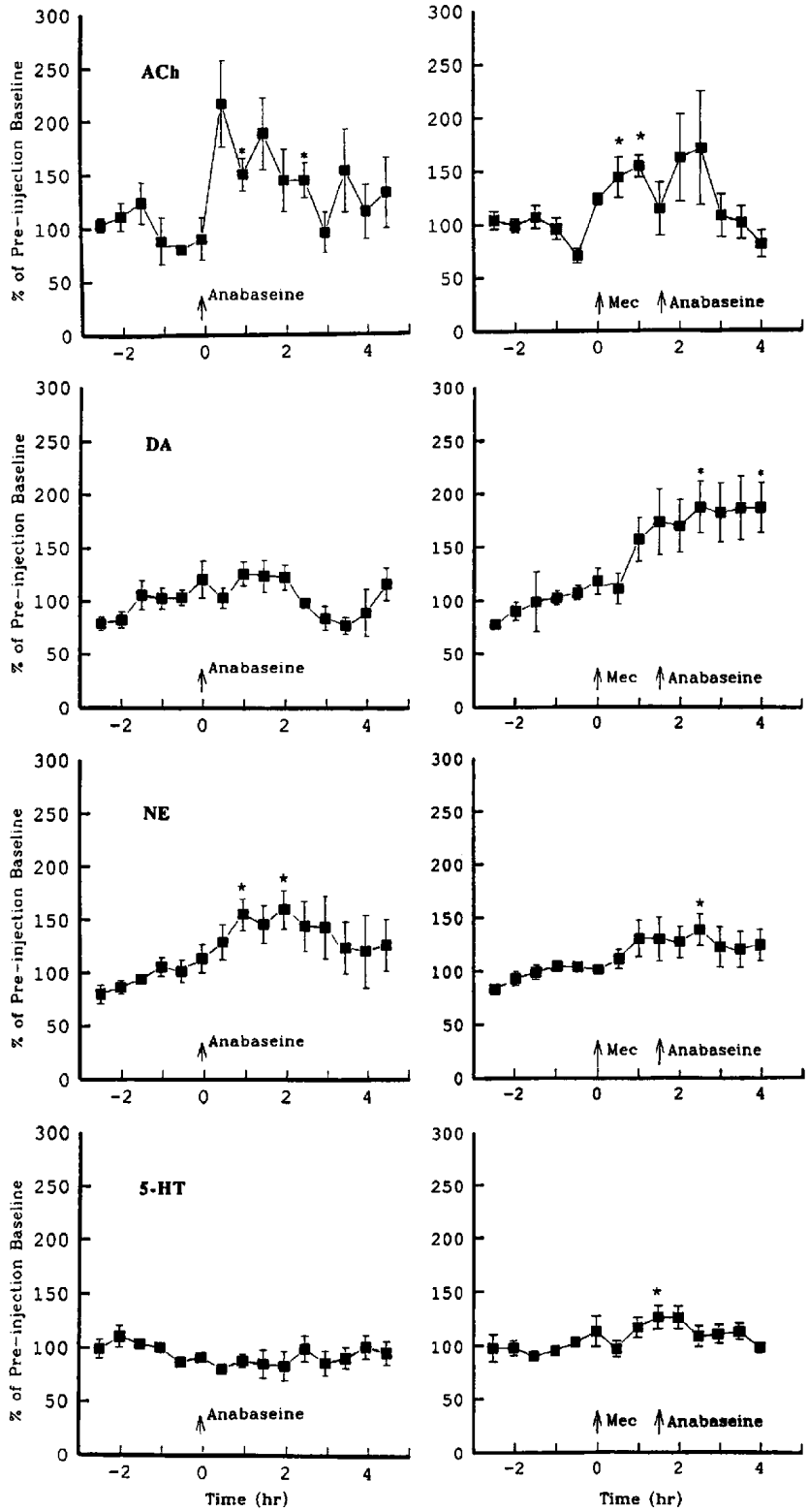

Fig. 2. Effects of systemic anabaseine (3.6 μmol or 0.90 mg/kg, s.c.) on extracellular levels of ACh, DA, NE and 5-HT in the rat frontoparietal cortex. Data are expressed as a percentage of the pre-injection control levels (average of the six samples prior to injection=100%); mean±S.E.M., n=6, *P<0.05 by paired Student's *t*-test analysis. Effects of Mec pre-administration (4.90 μmol or 1 mg/kg, i.p.) 1 hr before administration of anabaseine are shown on the right.

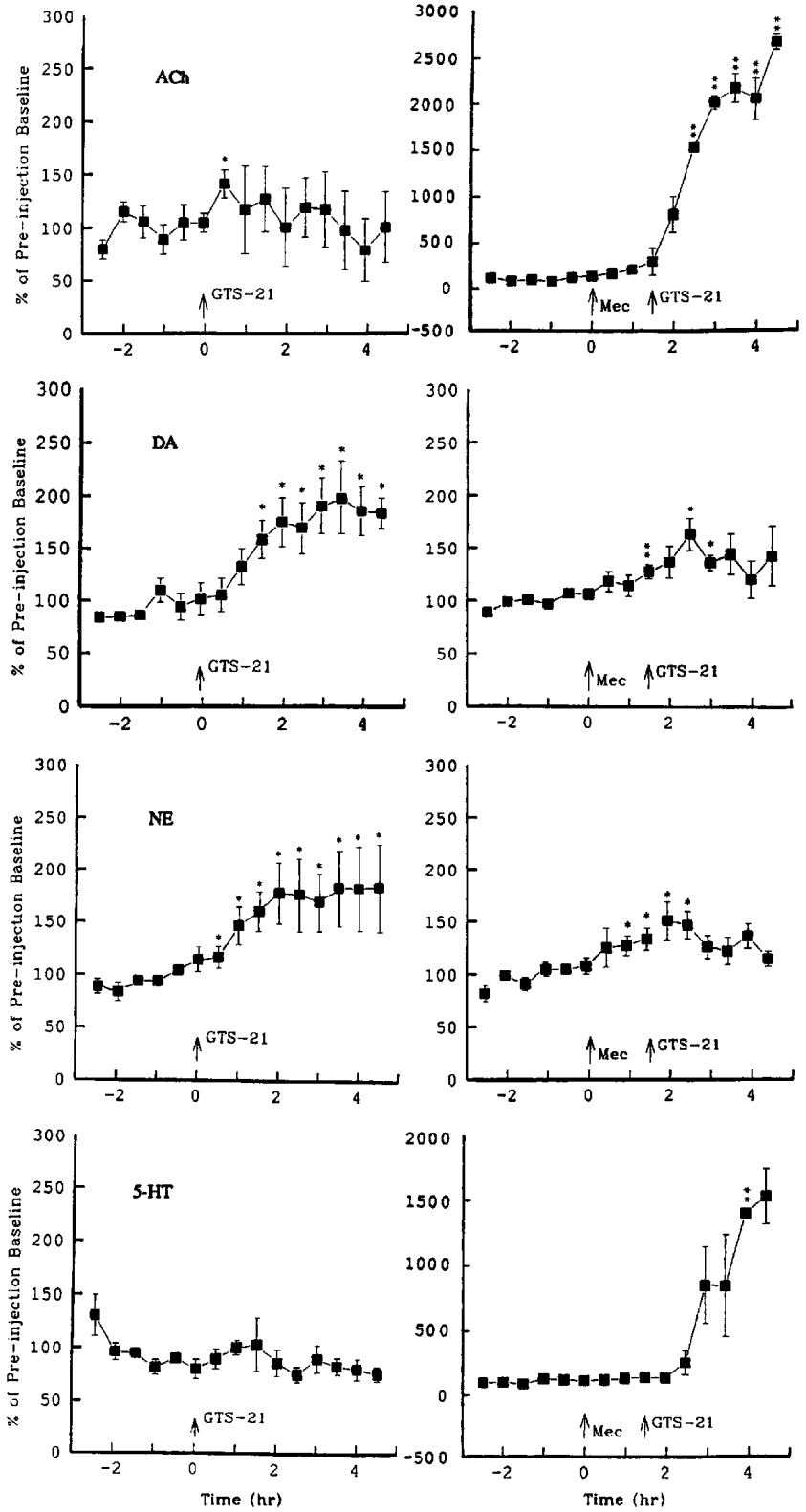

Fig. 3. Effects of systemic GTS-21 (3.6 μmol or 1.37 mg/kg, s.c.) on extracellular levels of ACh, DA, NE and 5-HT in the rat frontoparietal cortex. Data are expressed as a percentage of the pre-injection control levels (average of the six samples prior to injection = 100%); mean ± S.E.M., n = 6, *$P < 0.05$ and **$P < 0.01$ by paired Student's t-test analysis. Effects of Mec pre-administration (4.90 μmol/kg, i.p.) 1 hr before administration of GTS-21 are shown on the right.

tial administration of Mec and anabaseine. Some of this increase apparently was in response to Mec alone.

GTS-21 effects

This 3-substituted anabaseine acted quite differently from anabaseine (Fig. 3). ACh levels were not altered (except possibly at 30 min) when GTS-21 was administered at the same 3.6 μmol/kg (1.37 mg/kg) dose. 5-HT concentration was also unaffected by GTS-21, as was previously observed with anabaseine and nicotine. However, NE (83% increase) and DA (96% increase) levels were significantly elevated by GTS-21, in contrast to the results with equimolar doses of anabaseine and nicotine.

When 1 mg/kg Mec was administered 1.5 hr before GTS-21 injection, remarkable increases in both ACh (25-fold) and 5-HT (13-fold) concentrations were observed (Fig. 3). The two compounds together caused neurotransmitter alterations that were not predicted from the data available on their actions when administered separately.

DISCUSSION

Anabaseine actions

Our microdialysis measurements showing anabaseine enhancement of extracellular ACh levels are the first in vivo evidence for an ACh releasing action of anabaseine, which was initially observed in vitro using an isolated rat cortex mince preparation (20). A number of laboratories have previously reported that nicotine and related compounds release ACh from cortical slices, minces and synaptosomes (see ref. 14 for pertinent references). Araujo et al. (21) showed that the ACh releasing action of the nicotinic agonist methylcarbamylcholine on a slice preparation was inhibited by some nicotinic antagonists known to affect $\alpha4\beta2$- and $\alpha3\beta2$-receptors, but was not inhibited by α-bungarotoxin, which inhibits $\alpha7$-subunit-containing receptors. Anabaseine, like nicotine, is an agonist at these high affinity nicotinic receptors. In contrast, GTS-21 (see below) is an antagonist at these receptors but a partial agonist at $\alpha7$-type receptors. That it did not increase extracellular ACh (Fig. 3) is consistent with the hypothesis that cholinergic neurons are primarily modulated by the high affinity nicotinic receptors.

In contrast with nicotine, anabaseine failed to elevate DA levels (14). Other experiments utilizing higher doses of anabaseine would be required to eliminate the possibility that this merely reflects a quantitative difference in sensitivity of this dopaminergic pathway to these nicotinic agonists.

Mec preadministration inhibited the ACh and NE elevating actions of anabaseine. It was previously shown that Mec administration alone at the dose employed here had little or no effect upon extracellular concentrations of the four neurotransmitters measured in the present study (14). However, Toide et al. (22) have found that 5 mg/kg Mec can elevate ACh levels. In the present experiments, Mec also produced some elevation in ACh, NE and DA levels (Figs. 2 and 3).

Comparison of anabaseine and nicotine actions

The similar ACh and NE elevating actions of equimolar doses of anabaseine and nicotine (14) suggest that these nicotinic agonists may be affecting levels of these two neurotransmitters through identical nicotinic receptors. We suggest that these elevations are mediated by stimulation of high affinity nicotinic receptors, which in the rat cerebral cortex are predominantly (>90%) composed of $\alpha4\beta2$ subunits (23). Cholinergic neurons innervating the frontal cortex project from the nucleus basalis of Meynert. Thalamic neurons with unidentified neurotransmitters which project to the cortex also possess nicotinic receptors (24). Theoretically, nicotinic agonists could act upon nicotinic receptors on the cell bodies of the projecting cholinergic neurons as well as locally within the cortex upon nerve terminals. Although the affinity of anabaseine for $\alpha4\beta2$-receptors is approximately sevenfold less than of nicotine (5, 6; W.R. Kem et al., submitted), anabaseine should penetrate the blood-brain barrier much more readily than nicotine because it is a significantly less polar compound. This may explain why an equimolar dose of anabaseine produced changes in transmitter concentrations very similar to that of nicotine. In addition, the 3.6 μmol/kg dose of nicotine was expected to produce a nearly maximal effect (15).

Nicotinic receptors located on cell bodies or terminals of non-cholinergic neurons represent other possible sites of action for nicotinic receptor agonists. Thus, actions of the two anabaseine compounds on NE and DA levels could be directly on neurons secreting these neurotransmitters. Since neither anabaseine nor nicotine (14) affected serotonin levels, serotoninergic neurons projecting from the raphe nuclei apparently lack sufficient numbers of nicotinic receptors to modulate the secretion of 5-HT.

GTS-21 actions

In contrast with anabaseine, GTS-21 probably acts essentially as an antagonist on the $\alpha4\beta2$ subtype, since its ability to stimulate these receptors is quite limited (7). However, like anabaseine, it possesses a high efficacy at homomeric $\alpha7$-type receptors and a similar affinity for binding to $\alpha7$-containing receptors in rat brain (6, 25). Thus, our failure to observe elevation of ACh with GTS-21, when administered alone, is consistent with the hypothesis stated above that $\alpha4\beta2$-type receptors are

dominant in mediating the enhancement of ACh release by nicotine, anabaseine and some other nicotinic agonists in the frontal cortex (16).

Other nicotinic receptor subtypes may also exist on adrenergic neurons, but the fact that GTS-21 (Fig. 3) enhances NE levels suggests that α7-receptors are involved. DA levels were elevated by GTS-21, in contrast with anabaseine, and this was reduced by preadministration of Mec.

Non-nicotinic sites of GTS-21 action

GTS-21 may act at sites other than nicotinic cholinergic receptors. It has been found to interfere with the binding of several neurotransmitter radioligands to their respective receptors, but only at concentrations much higher ($>50\times$) than are expected to occur in the present experiments (6, 9, 10). Recent electrophysiological experiments (26) have also demonstrated that GTS-21 is also a weak antagonist at 5-HT_3 receptors, which are homologous with nicotinic receptor subunits. Several 5-HT_3 antagonists have been reported to stimulate the cortical release of ACh (27). However, it seems rather unlikely that the cause of the massive elevation in 5-HT we observed after administration of both Mec and GTS-21 is due to its inhibition of 5-HT_3 receptors because GTS-21 is only an effective antagonist at this receptor at concentrations (>20 μM, ref. 26) greatly exceeding the submicromolar peak plasma concentrations predicted to occur in the present experiments (V.M. Mahnir et al., unpublished results). The IC_{50}'s of GTS-21 inhibition of rat brain receptor binding were as follows: α7-nicotinic, 400 nM; $\alpha4\beta2$, 200 nM; 5-HT_3, 30,000 nM; muscarinic, β-adrenergic, and $GABA_A$, >50,000 nM (V.M. Mahnir et al., unpublished results). At the present time, a likely non-nicotinic site for GTS-21 action has yet to be identified.

Possible basis for the pronounced elevation of ACh and 5-HT in the presence of GTS-21 and mecamylamine

Since GTS-21 did not affect ACh and 5-HT levels when administered alone, we were surprised that in the presence of Mec, GTS-21 caused a massive (25- and 13-fold, respectively) elevation in ACh and 5-HT levels. Although even a hypothetical basis for this phenomenon is currently unavailable, we wish to briefly consider some factors that could contribute to this synergistic action.

In addition to their excitatory synaptic effects on neurotransmitter release, some nicotinic agonists may also reduce release by stimulating inhibitory inputs to the neurons releasing the neurotransmitter. If Mec preferentially blocks nicotinic receptors that reside on the inhibitory neurons, it could greatly enhance the release of neurotransmitters whose secretion is normally limited by inhibitory interneuron modulation. GABAergic neurons are known to innervate and inhibit cholinergic neurons in the nbM which project to the cortex. It has recently been found that GABAergic interneurons in the interpeduncular nucleus and hippocampus possess high nicotine affinity receptors which when activated cause an increase in GABA secretion (28–30). We suggest that Mec, at the dose employed in our study, preferentially reduced nicotinic activation of GABAergic neuron high affinity nicotine receptors, without greatly affecting the α7-containing receptors. At the dose we used, GTS-21 was only expected to block some of the high affinity receptors.

Although there is still little published data concerning the relative susceptibility of different nicotinic receptor channels to Mec blockade, current data on the human receptors suggests that there are large differences in sensitivity between nicotinic receptors for this compound. The sensitivity of rat PC12 cell autonomic nicotinic receptors (mostly composed of $\alpha3$ and $\beta4$ subunits) to Mec was over $100\times$ higher than that of human neuromuscular nicotinic receptors (31). This is consistent with its previous use as an anti-hypertensive drug. While the sensitivities of rat nicotinic receptors to Mec are still unknown, the human α7-receptor (32) has recently been reported to have a Mec IC_{50} of 1.8 μM, which is about tenfold lower sensitivity than the PC12 (rat) receptor and about a tenfold higher sensitivity relative to the human neuromuscular nicotinic receptor (31). It is known that high concentrations of Mec will block both α-bungarotoxin-inhibitable (probably α7) and high affinity (probably $\alpha4\beta2$) nicotinic receptors residing on cultured hippocampal neurons (33). Several laboratories have also reported that nicotine stimulated behaviors and physiological effects were blocked by quite different concentrations of Mec (34). For instance, sensory gating in rats was only affected by Mec doses of 5 mg/kg and higher (35, 36). The NMDA-type glutamate channel is also inhibited by relatively large concentrations of Mec, which further complicates the interpretation of in vivo data (37, 38). Quantitative comparisons of the ability of Mec to block different rat nicotinic receptor channels in vitro are needed to determine whether our assumption that the α7-containing receptors are less susceptible than the $\alpha4\beta2$-receptors is valid.

For some time it has been known that 5-HT_2 receptors are located near or on cholinergic nerve terminals in the rat cortex (39). It has very recently been shown that ACh release in the rat frontal cortex can be enhanced by stimulation of the 1B and 2A subtypes of 5-HT receptors, as well as by inhibition of 5-HT_3 receptors (40, 41). Thus it is possible that enhancement of 5-HT levels by the GTS-21 plus Mec combination would further enhance ACh release. If this were the case, then selective antagonists for the 1B and 2A serotonin receptors would be ex-

pected to diminish the massive ACh increase we observed with these two compounds.

Large increases in frontoparietal cortex ACh levels have also been observed after administration of muscarinic antagonists alone (42) or concomitantly with nicotine (43). Quirion et al. (43) have suggested that blockade of muscarinic M_2 type autoreceptors on cholinergic nerve terminals and concomitant stimulation of neuronal nicotinic receptors may be a useful therapeutic strategy for ameliorating a decreased cholinergic function in Alzheimer's dementia. It is also conceivable that co-administration of GTS-21 and Mec might significantly enhance cognition by simultaneously increasing both ACh and 5-HT levels.

More intensive microdialysis investigations of the actions of these and other nicotinic compounds upon neurotransmitter levels in relevant regions of the brain could shed new light on the mechanisms by which nicotinic receptors influence neurochemical mechanisms involved in cognition.

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