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Cotinine, a Neuroactive Metabolite of Nicotine: Potential for Treating Disorders of Impaired Cognition

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ABSTRACT

The pharmacological effects of the tobacco-derived alkaloid nicotine have been widely studied in humans and animals for decades. However, relatively little attention has been given to the potential actions of its major metabolite, cotinine. After nicotine consumption the duration of cotinine's presence in blood and brain greatly exceeds that of nicotine. Therefore, cotinine could mediate the more protracted pharmacological effects of nicotine. The studies described in this report were thus designed to further investigate certain neuropharmacological actions of cotinine. Behavioral tests (e.g., delayed matching-to-sample) were conducted in aged rhesus monkeys to assess the effects of cotinine on working memory and attention. In rats a prepulse inhibition (PPI) procedure was used to assess the effects of the compound on auditory gating — a method for predicting the potential antipsychotic properties of drugs. Cotinine exhibited significant effectiveness in these tasks. The drug was also cytoprotective in differentiated PC-12 cells with a potency equivalent to that of nicotine. The effects of chronic cotinine treatment on the expression of nicotinic and muscarinic acetylcholine receptors in rat brain were measured by [^{125}I]epibatidine, [^{125}I]α-bungarotoxin ([^{125}I]BTX), [^{3}H]pirenzepine ([^{3}H]PRZ), and [^{3}H]AFDX-384 ([^{3}H]AFX) autoradiography. Unlike nicotine, cotinine failed to upregulate the expression of brain nicotinic receptors. Based on its relative safety in man, cotinine should prove useful in the treatment of diseases of impaired cognition and behavior without exhibiting the toxicity usually attributed to nicotine.

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INTRODUCTION

Cotinine is a primary metabolite of nicotine that can exert measurable effects on certain types of behavior, working memory and cognition. The metabolite enjoys a long pharmacological half-life (15– 19 h) relative to nicotine (2–3 h). Indeed, high plasma cotinine levels (as often observed in smokers) originally were considered to be a factor contributing to the expression of smoking- or nicotine-induced withdrawal symptoms (49). However, cotinine was subsequently shown not to induce nicotine-like withdrawal, and the compound was reported to be relatively safe in humans at doses up to 10 times that usually obtained during cigarette smoking (26). For example, (in contrast to nicotine) such high doses of cotinine were not associated with significant effects on blood pressure, heart rate or the electrocardiogram. On rare occasion cotinine has been evaluated for its effects on human memory processing (16) and shown to exhibit both positive and negative effects (29). A similar profile of cotinine effects was reported in experiments on animal behavior, particularly operant food-motivated responding (43). In each case the effects of cotinine was shown to be biphasic: at doses in the range of 0.1–0.5 mg/kg cotinine had a positive effect on task performance, and at doses from 1–30 mg/kg it produced a decrement in task performance.

The behavioral evidence in support of the bioactive potential for cotinine is supported by slightly more substantial work performed at the cellular level. The family of brain nicotinic acetylcholine receptors is comprised of a variety of pentameric ligand-gated ion channels with slightly different ion conducting properties, different levels of expression and anatomical distribution (4,15,18,35,37). Receptor subtypes are composed of different α and β subunits. The more highly expressed subunits include $\alpha_4\beta_2$, $\alpha_3\beta_4$, and homo-oligomeric α_7 nicotinic receptors. In studies in which conditions favored the binding of [^{3}H]epibatidine to heteropentameric nicotinic receptors on membranes derived from rat cortex and hippocampus, nicotine was about 100 fold more potent than cotinine in displacing [^{3}H]epibatidine binding (51). Data derived from functional assays (7,8) indicated that cotinine exhibits the properties of a weak nicotinic agonist. Moreover, pretreatment with cotinine inhibited the subsequent response to acetylcholine — an effect explained by partial receptor desensitization. The ability of cotinine to partially desensitize nicotinic receptors was confirmed in studies with adrenal chomaffin cells (51). Again the initial activation due to cotinine was attenuated by classical nicotinic receptor antagonists. In other experimental situations virtually no pharmacological action was attributed to cotinine (36,49,53). Also, Audesirk and Cabell (2) reported that in cultured hippocampal cells, nicotine and cotinine produced virtually opposite effects in terms of cell survival and axon or dendrite branching. Clearly there is a need to study the effects of cotinine in a variety of behavioral paradigms and in models that have proven useful for studying the varied effects of nicotine; and there is a need to expand dose-response studies in each of these situations.

Our interest in the pharmacological actions of cotinine was originally derived from the observation that nicotine and certain of its analogs are capable of inducing a protracted degree of improvement in tasks that involve cognition (see refs. 9,11,42). The finding that the effects of nicotine could outlast its presence in blood and brain suggested the possibility of a long-lived metabolite and/or the induction of some prolonged pharmacodynamic action induced by the parent compound or one or more of its metabolites. Whereas a role for each of these possibilities has not been ruled out, pharmacokinetic-pharmacodynamic discordance in the cognition-enhancing effects of other nicotinic drugs, some without

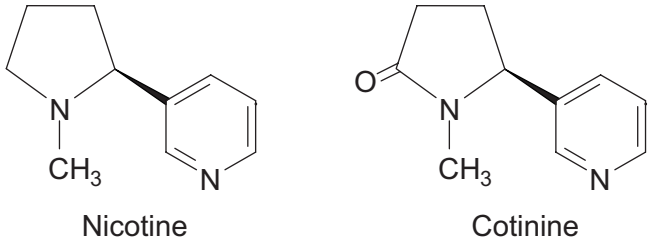

Fig. 1. The chemical structures of cotinine and nicotine.

long-lived metabolites, as well as other memory-enhancing agents without nicotinic properties, has been reported (11).

There often exists a mismatch between potency measured as the binding K_d (generally measured in the low nM range) and potency as measured by the EC_{50} for neurotransmitter release (generally measured in the low μM range). These differences have been partly attributed to the ability of nicotine to shift nicotinic receptors into a desensitized, but high affinity state (45). However, even during periods of receptor desensitization nicotinic receptor-mediated activity is far from completely eliminated (24,44). Because of the long half-life of cotinine, relative to nicotine, it is conceivable that even during desensitization and after the elimination/metabolism of nicotine the metabolite plays a role in modulating neural activity.

Preliminary studies from our laboratory confirmed that cotinine possesses at least 3 nicotine-like properties (10): (1) In the rat, the motor response to acoustic startle can be inhibited by the presentation of a low-level acoustic prepulse presented just in advance of the high-level acoustic pulse, thereby providing a measure of sensory gating. Disruption of sensory gating can be produced by drugs like apomorphine that can induce a schizophreniform action. Under the conditions established at baseline, apomorphine treatment suppresses the ability of the pre-pulse to inhibit acoustic startle. Many drugs with potential antipsychotic actions reverse the effects of apomorphine. Treatment with cotinine significantly reversed the effects of apomorphine on acoustic startle, supporting the possibility that the metabolite may share antipsychotic properties with nicotine. (2) Cotinine was effective in preventing the cytotoxicity associated with growth factor withdrawal in differentiated PC-12 cells. In this regard cotinine was slightly more potent than nicotine. (3) Cotinine produced a dose-dependent increase in accuracy in an automated delayed matching-to-sample task by macaques. Below we describe new experiments in support of a significant pharmacological profile of activity for cotinine consistent with the potential of the drug for use in the treatment of disorders of cognition and behavior. The chemical structures of cotinine and nicotine are presented in Fig. 1.

COGNITION IN AGED NON-HUMAN PRIMATES

Eight young adult rhesus monkeys (*Macaca mulatta*), well trained (>100 individual sessions) in a delayed matching to sample task (DMTS, see below), were used as test subjects in this study (Table 1). The monkeys were maintained on a 12 h light-dark cycle and were tested each weekday between 09:00 and 14:00 h. Room temperature and humidity were maintained at 72.0 ± 1.0°C and 52.0 ± 2.0%, respectively. Each subject in this study

had participated in one or more pharmacological studies of drugs that were considered to have fully reversible actions. At least a 4-week washout period preceded the initiation of this study. All procedures employed during this study were reviewed and approved by the Medical College of Georgia Institutional Animal Care and Use Committee and are consistent with AAALAC guidelines.

Delayed Matching-to-Sample (DMTS) Task

Subjects had unlimited access to tap water and standard laboratory monkey chow (Harlan Teklad Laboratory 20% monkey diet, Madison, WI, USA) supplemented with fruits and vegetables. On weekdays during behavioral testing the feeding schedule for each animal was modified such that their standard daily ration was withheld until after testing was completed. The subjects were weighed on a monthly basis to insure maintenance of normal body weight. Test panels attached to each animal's home cage presented the task by using a computer-automated system. A touch-sensitive screen (15′ AccuTouch LCD Panelmount TouchMonitor)/pellet dispenser units (Med Associates) mounted in light-weight aluminum chassis was attached to the home cage. The stimuli included red, blue, and yellow rectangles. A trial was initiated by presentation of a sample rectangle composed of one of the three colors. The sample rectangle remained in view until the monkey touched within its borders to initiate a pre-programmed delay (retention) interval. Following the delay interval, the two choice rectangles located below where the sample had been, were presented. One of the two choice rectangles was presented with its color matching the stimulus color, whereas the other (incorrect) choice rectangle was presented as one of the two remaining colors. A correct (matching) choice was reinforced. Non-matching choices were neither reinforced nor punished. The inter-trial interval was 5 sec and each session consisted of 96 trials. The presentation of stimulus color, choice colors, and choice position (left or right on the screen) were fully counterbalanced so as to relegate non-matching strategies to chance levels of accuracy. Five different presentation sequences were rotated through each daily session to prevent the subjects from memorizing the first several trials. Delay intervals were established during numerous non-drug or vehicle sessions prior to initiating the study. The duration for each delay interval was ad-

TABLE 1. Rhesus monkeys (females) used as subjects in the studies

			Delay interval (sec)		
Subject I.D.	Age (years)	Weight (kg)	Short	Medium	Long
446	29	4.9	10	15	40
507	24	7.6	10	25	70
517	24	6.3	10	20	30
667	31	8.2	3	5	7
671	31	4.4	5	10	15
683	31	7.8	20	50	80
C4N	24	7.6	7	10	15
8LY	25	13.6	5	7	10
Mean	**27.4**	**7.6**	**8.8**	**17.8**	**33.4**
S.E.M.	**1.3**	**1.1**	**2.0**	**5.5**	**10.6**

justed for each subject until three levels of group performance accuracy were approximated: Zero delay interval (85–100% of trials answered correctly); Short delay interval (75–84% correct); Medium delay interval (65–74% correct); and Long delay interval (55–64% correct). Values obtained for each difficulty level (delay interval) were averaged and recorded as the mean percent correct. Drug effects were calculated as the absolute change from vehicle-associated accuracy. Statistical comparisons between vehicle and treated (drug) groups were performed using a repeated measures analysis of variance (JMP Statistical Discovery Software v. 4.0, SAS Institute, Inc., Cary, NC, USA). Post hoc analysis was performed using a multiple comparison procedure with orthogonal contrasts and statistical significance was assessed at an α level of 0.05 (2-tailed).

Three vehicle sessions were interspersed among the cotinine sessions for both the standard DMTS distractor-DMTS studies. Distractor sessions (interference sessions, see below) were run no more than 3 times every 2 weeks, and a minimum washout period of at least 2 days was maintained between doses. Subsequent drug doses were administered only if a monkey's performance returned to baseline levels during that period. Cotinine (Sigma-Aldrich, St. Louis, MO, USA) was administered in a series of ascending doses. Cotinine was weighed to the nearest 0.1 mg and dissolved in sterile normal saline within a total injection volume of 0.035 mL/kg. Injections were given in the thigh muscle 15 min prior to testing.

The test subjects in the study approximated the baseline performance levels according to the criteria as described above (Fig. 2, "Vehicle" plot). A highly significant decrement in performance with increasing delay interval was observed in both study groups (delay effect, $p < 0.001$) and post hoc analyses indicated that accuracy at each delay interval was significantly different ($p < 0.05$) from other delay intervals.

Cotinine produced statistically significant improvement in DMTS task accuracies that was dependent upon delay interval, $F_{(12,160)} = 2.62$, $P = 0.0032$. The data are presented in Fig. 2. Post hoc analysis indicated that improvement in task accuracy was primarily relegated to Medium ($t = 2.7$, $P = 0.008$) and Long ($t = 3.88$, $P = 0.001$) delay trials after the 10 mg/kg dose, and to Long delay trials after the 0.3 mg/kg dose ($t = 3.82$, $P = 0.0002$). There was also a nearly significant improvement following the 3 mg/kg dose during Long delay intervals ($t = 1.81$, $P = 0.072$). During sessions run 24 h after cotinine administration (in the absence of vehicle or drug) there continued to be an improvement in DMTS task accuracy relative to the original vehicle baseline, $F_{(12,160)} = 1.96$, $P = 0.031$. These data are presented in the inset of Fig. 2. Post hoc analysis indicated that improvement in task accuracy was primarily relegated to Long delay trials following the 3 mg/kg dose ($t = 3.21$, $P = 0.0016$) and there was a nearly significant increase in accuracy following the 10 mg/kg dose during Medium delay intervals ($t = 1.85$, $P = 0.067$).

Distractor-DMTS (D-DMTS)

Distractor stimuli were presented in a non-predictable manner to the test subject on 24 of the 96 trials completed during distractor DMTS sessions. The stimuli were initiated 1 sec into the delay interval and remained active for 3 sec. They consisted of a random pattern of three colored rectangles (identical to the rectangles that served as sample and choice stimuli in the standard DMTS task) that were caused to flash in an alternating manner. The total onset time for a given colored rectangle was 0.33 sec. Immediately, as one colored rectangle was extinguished, a different colored rectangle was presented. Thus, during presentation of the distractor, each color was presented in random order 3 separate

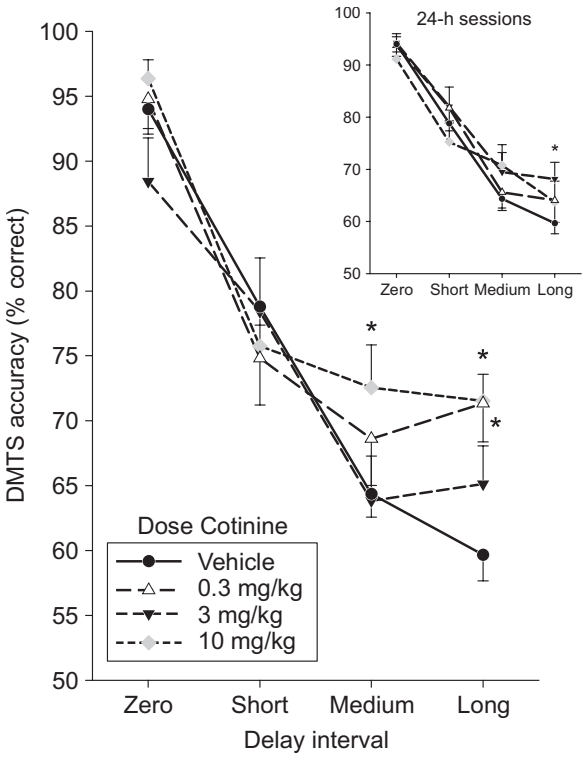

Fig. 2. The effect of cotinine on standard DMTS (delayed matching-to-sample) task accuracy in monkeys ($n = 8$) when the test was administered 10 min after dosing. Inset: the effect of cotinine on standard DMTS accuracy when the test was administered 24 h after dosing. *Significantly different ($P < 0.05$) from vehicle.

times. Distractor stimuli were present an equal number of times on trials with Short, Medium, and Long delay intervals. The remaining trials were non-distractor trials and the data for distractor and non-distractor trials underwent separate statistical treatments.

Because of our limited experience with the distractor paradigm in aged animals an additional, lower (0.1 mg/kg) dose of cotinine was included in the series. In this study data are presented as non-distractor and distractor trials within each session. In each case the mean accuracies associated with each delay interval during a series of standard DMTS sessions are presented for comparison (unfilled bars) as shown in Fig. 3. During non-distractor trials there was a carryover effect from the distractor trials that reduced accuracies relative to those obtained during standard DMTS sessions. Cotinine did not significantly improve accuracies during non-distractor trials although examination of Fig. 3 (upper panel) reveals that the 3 and 10 mg/kg doses were associated on average with improved accuracies during Short and Medium delay trials relative to vehicle non-distractor trials. In fact, during Medium delay trials task accuracies after cotinine administration increased beyond those associated with even the standard DMTS task.

Task distractors significantly impaired accuracies relative to the standard DTMS task (Fig. 3, lower panel). In the case of Short delay intervals accuracies declined to chance levels. Administration of cotinine significantly improved task accuracies during distractor trials, $F_{(5,30)} = 3.09$, $P = 0.0123$), though the effect was independent of delay interval. Post hoc analysis indicated that improvement was relegated to the 0.1 mg/kg ($t = 3.24$, $P = 0.0016$) and the 3 mg/kg ($t = 2.47$, $P = 0.0154$) doses. There was also a nearly signif-

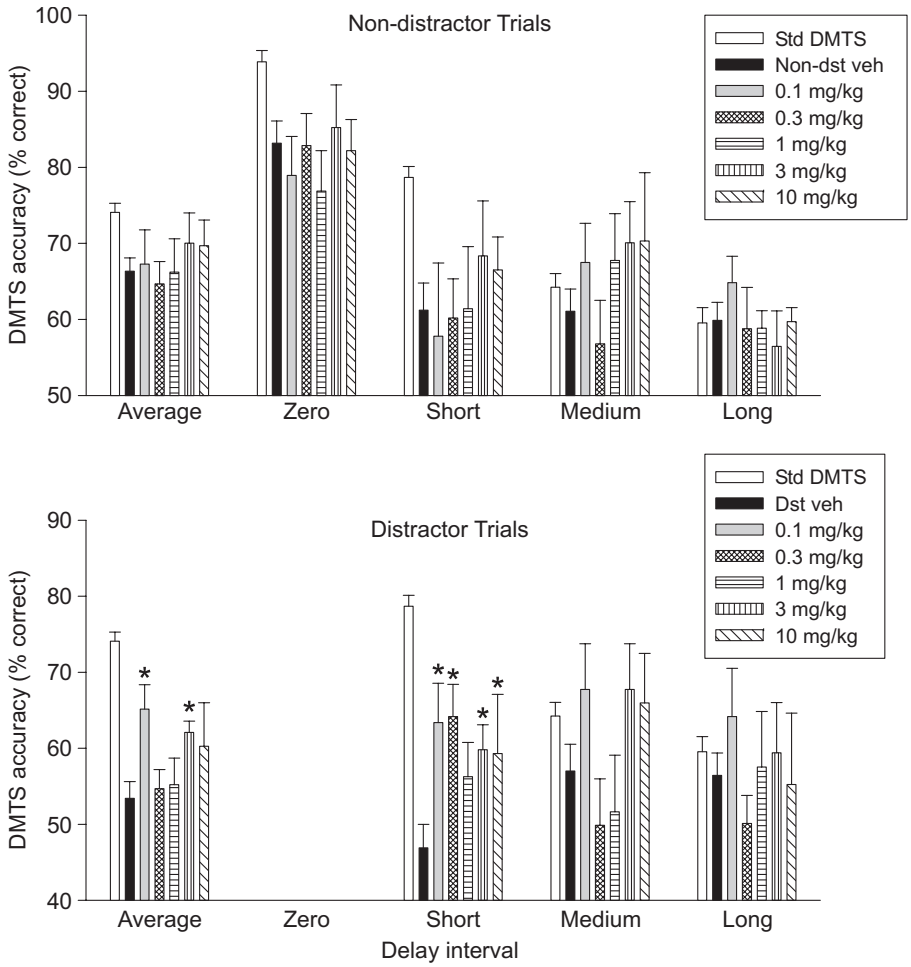

Fig. 3. Effect of cotinine on DMTS task accuracy in monkeys ($n = 8$) during non-distractor (upper panel) and distractor/interference (lower panel) trials within sessions. Mean accuracies from separate standard DMTS sessions (open bars) are provided for comparison with the distractor task accuracies. Non-dst veh, Non-distractor vehicle session; Dst veh, distractor vehicle session. *Significantly different from vehicle ($P < 0.05$).

icant ($t = 1.87$, $P = 0.065$) improvement in accuracies following the 10 mg/kg dose. Examination of Fig. 3 (lower panel) reveals that most of the improvement was associated with Short delay trials. In fact, during Short delays the 0.1, 0.3, 3, and 10 mg/kg doses of cotinine were associated with significant improvements in accuracy relative to vehicle distractor baseline.

PREPULSE INHIBITION IN RATS

Based on the evidence that sensory gating abnormalities in schizophrenia contribute to deficits in attention, cognitive impairment, and possibly even hallucinations (1), and on

the fact that a very high percentage of schizophrenics smoke cigarettes (and, therefore, have sustained circulating levels of cotinine) we were interested in the effects of cotinine on sensory gating. Prepulse inhibition (PPI) of the acoustic startle reflex is a well characterized model for identifying sensory information-processing deficits in a number of neurologic and psychiatric conditions, including schizophrenia (6). PPI is defined as the reduction in startle response produced by a low-intensity stimulus presented before a high-intensity, startle-producing stimulus (25). It is a cross-species phenomenon that has been hypothesized to serve as a mechanism for the gating or filtering of irrelevant or distracting stimuli. In a rodent PPI task (used extensively to characterize compounds with potential antipsychotic activity) a weak acoustic stimulus (the prepulse) decreases the reflexive flinching response (startle) produced by a second, more intense, stimulus (the pulse). The role of a number of neurotransmitter systems in modulating PPI (23) has been reviewed and there is considerable evidence to indicate that stimulation of dopamine receptors, activation of serotonin receptors, or blockade of NMDA or cholinergic receptors results in disruption of the PPI (see also 30, 48). Thus, effective antipsychotic agents have been frequently reported in animal studies to block disruptions in PPI induced by antagonists or agonists of the aforementioned receptors. Probably the most frequently used pharmacological model for such studies is the apomorphine-reversal model. Potencies of drugs at reversing the negative effects of this dopamine agonist on PPI, appear to correlate well with their clinical potencies in schizophrenia. Other commonly used approaches include reversal of NMDA antagonists (e.g., phencyclidine, MK-801), and muscarinic antagonists (e.g., scopolamine) on PPI.

In the present study, dose-response curves were established for evaluating cotinine in each of the models described above (i.e., apomorphine, MK801 and scopolamine antagonism of PPI). Male 2–3 months old albino Wistar rats (Harlan Sprague–Dawley, Inc.) were housed individually in a temperature- controlled room (25°C), maintained on a 12-h light/dark cycle with free access to food (NIH-07 formula) and water. Individual subjects were behaviorally tested in one of four startle chambers (San Diego Instruments, San Diego, CA, USA) consisting of a Plexiglas tube (diameter 8.2 cm, length 25 cm), placed in a sound–attenuating chamber. The tube was mounted on a plastic frame with an attached piezoelectric accelerometer that recorded and transduced the motion in the tube. Two days before PPI testing the experimental subjects were each placed in one of the startle test chambers for a period of 10 min (without any startle stimuli) as an initial period of acclimation and habituation to the apparatus. One day before PPI testing the animals were again placed in the test chamber and exposed to 12 startle stimuli and to each prepulse level 3 times (see below). This procedure was done to reduce the highly variable responses to the initial exposures to the startle stimuli as well as to ensure that the prepulse stimuli (alone) had no significant effect on the startle response. On the day of PPI testing, experimental subjects were transported to the startle chamber room and left undisturbed for at least 30 min. Afterwards, the rats were placed in the chamber, and then allowed to habituate for a period of 5 min, during which a 70 dB background white noise was present. After habituation, the rats received 12 startle trials, 12 no-stimulus trials, and 12 trials of each of the prepulse/startle trials for a total of 60 trials. The intertrial interval ranged from 10 to 30 sec, and the total session lasted 25–30 min. The startle trials consisted of 120 dB white noise bursts lasting 20 msec. The prepulse inhibition trials consisted of a prepulse (20 msec burst of white noise with intensities of 75, 80, or 85 dB) followed, 100 msec later, by a startle stimulus (120 dB, 20 msec white noise). During the no-stimulus trial, no startle noise was presented, but the movement of the rat was re-

corded. This represented a control trial for detecting differences in overall activity. The 60 different trials were presented pseudorandomly, ensuring that each trial was presented 12 times and that no two consecutive trials were identical. The resulting movement of the rat in the startle chamber was measured for 100 msec after startle stimulus onset (sampling frequency 1 kHz), rectified, amplified, and fed into a computer that calculated the maximal response over the 100-msec period. Basal startle amplitude was determined as the mean amplitude of the 12 startle trials. Prepulse inhibition was calculated according to the formula 100–100% *x* (PPx/p120), in which PPx is the mean of the 12 prepulse inhibition trials (i.e., for each individual prepulse level), and p120 was the basal startle amplitude. The average level of PPI was also calculated (average of the responses to pp75, pp80, or pp85) and analyzed separately.

For the studies in which the effects of cotinine alone on PPI were evaluated, cotinine (one of several doses) was dissolved in vehicle (sterile saline) and injected i.p., 30 min before testing followed by vehicle s.c., 10 min before testing. For the apomorphine and MK801 reversal studies, test subjects were administered vehicle (normal saline) or cotinine dissolved in vehicle i.p., 30 min before testing followed by either vehicle, apomorphine 0.5 mg/kg or MK-801 0.1 mg/kg (dissolved in vehicle) s.c., 10 min before testing. For the scopolamine-reversal studies, scopolamine 0.33 mg/kg dissolved in vehicle was administered 40 min before testing followed by either vehicle or cotinine (dissolved in vehicle) 20 min before testing. Reference doses of haloperidol, clozapine and donepezil (administered i.p.) were used to reverse the effects of apomorphine, MK801, or scopolamine, respectively.

In all of the prepulse inhibition (PPI) studies described below, under vehicle conditions, there was a highly significant reduction in the startle response when the various prepulse stimuli preceded the startle stimulus under vehicle conditions. (i.e., prepulse level difference $p < 0.001$ in all studies — see the open bars in Figs. 4–7). As indicated in Fig. 4A, there were no significant effects of cotinine on PPI across the 5 doses evaluated (treatment effect, $F_{(5,39)} = 1.3$, p = 0.29), nor was there a significant treatment *x* prepulse level interaction $F_{(78,134)} = 1.2$, $p = 0.32$. Similar effects were obtained when the data were averaged across prepulse level. In addition, there were no significant effects of cotinine on the startle response, $F_{(5,39)} = 0.3$, $p = 0.90$ (see Fig. 4B). As indicated in Fig. 5A, in the cotinine-apomorphine reversal study, there were significant differences in responses to the various drug treatments (treatment effect, $F_{(7,70)} = 12.5$, $p < 0.001$; the effects of the prepulse levels, $F_{(2,14)} = 135.7$, $p < 0.001$), and the treatment *x* prepulse level interaction was highly significant, $F_{(140,230)} = 3.6$, $p < 0.001$. Post hoc analyses indicated that apomorphine significantly ($p < 0.05$) diminished PPI at all three prepulse levels when the effect was compared to the vehicle-associated response. At a reference dose of 0.3 mg/kg haloperidol significantly antagonized (i.e., almost completely reversed) the effects of apomorphine on PPI at all three prepulse levels. Post hoc analyses (Fisher's LSD Test) further indicated that cotinine at doses of 0.033–1.0 mg/kg improved the deficits in PPI produced by apomorphine at the 75 and 80 dB prepulse levels. While the higher doses of cotinine (0.33 and 1.0 mg) antagonized the actions of apomorphine on PPI at the 85 dB prepulse level, the effects did not reach the required level of significance (i.e., $p > 0.05$). These dose-related effects of cotinine were also apparent when the data were averaged across the prepulse levels (i.e. treatment effect, $p < 0.001$, see Fig. 5B).

As indicated in Fig. 6A, in the cotinine MK-801 reversal study, there were significant differences in responses to the various drug treatments, treatment effect ($F_{(6,60)} = 7.8$, $p < 0.001$); the effects of the prepulse levels ($F_{(2,12)} = 134.9$, $p < 0.001$), although the

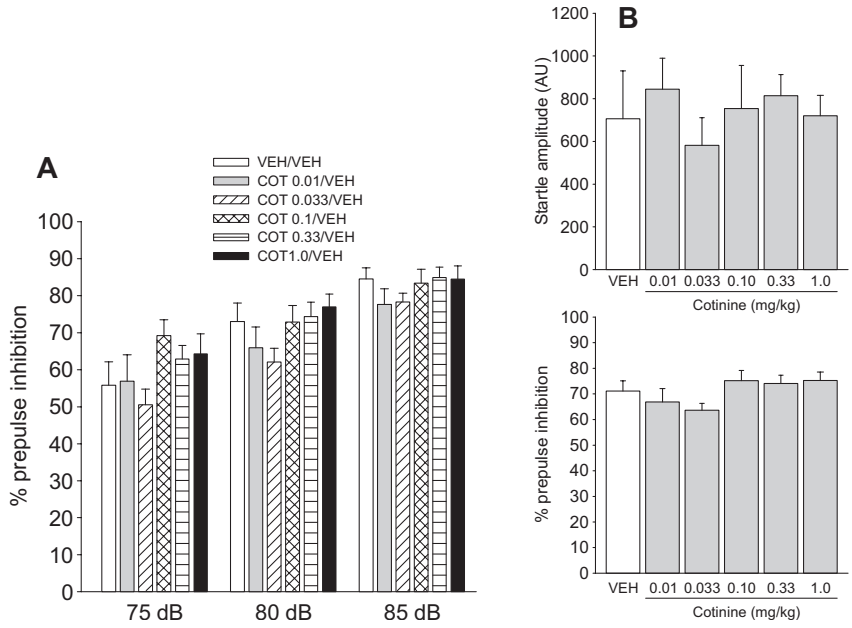

Fig. 4. A. Effects of several doses of cotinine on the percentage of prepulse inhibition in rats (for the three prepulse intensities: 5, 10, and 15 dB above background). **B.** Effects of cotinine on mean startle amplitude to 120-dB, 20-msec noise burst. **C.** Effects of cotinine on the percentage of prepulse inhibition averaged across prepulse level. Bars represent mean ± S.E.M. for each treatment ($N = 6–8$). VEH, vehicle; COT, cotinine. There were no significant treatment-related effects (i.e., all p values were >0.05).

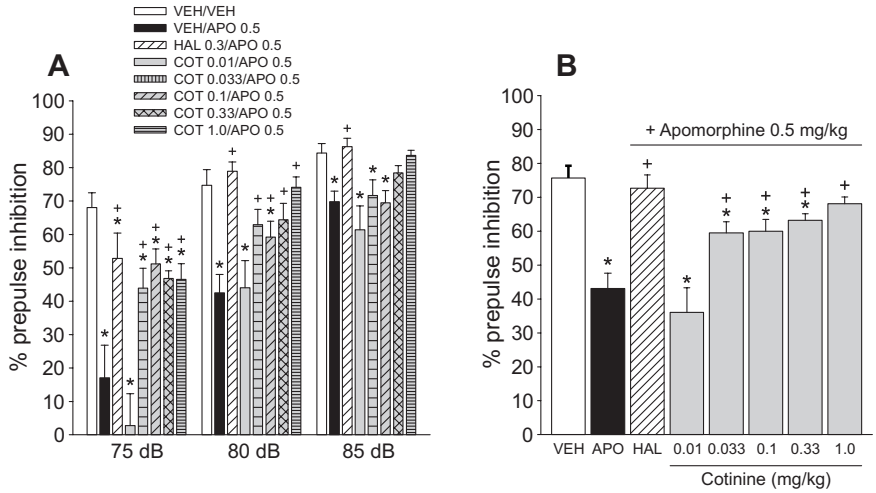

Fig. 5. A. Effects of apomorphine (0.5 mg/kg) and several doses of cotinine (and a reference dose of the typical antipsychotic haloperidol) on apomorphine-induced deficits in prepulse inhibition in rats associated with three prepulse intensities (5, 10, and 15 dB above background). **B.** Effects of cotinine (and a reference dose of HAL) on apomorphine-induced deficits in prepulse inhibition averaged across prepulse level. Bars represent mean ± S.E.M. for each treatment ($N = 9–10$). VEH, vehicle; COT, cotinine; APO, apomorphine, HAL, haloperidol. *Significantly different ($p < 0.05$) from the vehicle associated response; $^{+}$significantly different ($p < 0.05$) from the apomorphine-associated response.

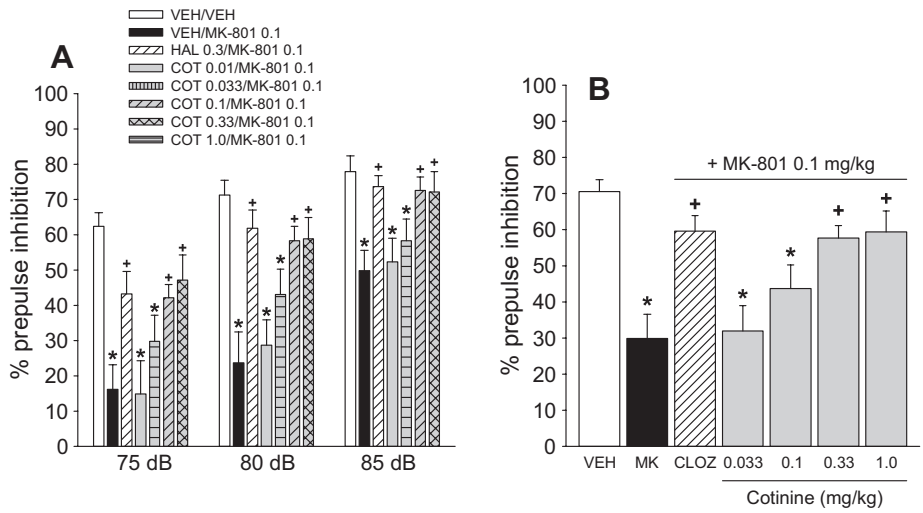

Fig. 6. A. Effects of MK801 (0.1 mg/kg) and several doses of cotinine (and a reference dose of the atypical antipsychotic clozapine) on MK-801-induced deficits in prepulse inhibition in rats associated with three prepulse intensities (5, 10, and 15 dB above background). **B.** Effects of cotinine (and a reference dose of clozapine) on MK801-induced deficits in prepulse inhibition averaged across prepulse level. Bars represent mean ± S.E.M. for each treatment ($N = 9–10$). VEH, vehicle; COT, cotinine; CLOZ, clozapine. *Significantly different ($p < 0.05$) from the vehicle associated response; $^{+}$significantly different ($p < 0.05$) from the MK-801-associated response.

treatment *x* prepulse level interaction was not significant ($F_{(120,200)} = 1.7$, $p = 0.067$). Post hoc analyses indicated that MK-801 significantly ($p < 0.05$) diminished PPI (at all prepulse levels) when compared to the vehicle-associated response. Clozapine at a reference dose of 5.0 mg/kg significantly antagonized the effect of MK-801 at all prepulse levels. Post hoc analyses further indicated that cotinine at doses 0.33 and 1.0 mg/kg improved the deficits in PPI produced by MK-801 at all three prepulse levels. These positive effects of cotinine were also apparent when the data were averaged across the prepulse levels (i.e., treatment effect, $p < 0.001$, see Fig. 6B).

As indicated in Fig. 7A, in the scopolamine-cotinine reversal study, there were significant differences in responses to the various drug treatments, treatment effect ($F_{(6,60)} = 2.8$, $p < 0.02$) and the effects of the prepulse levels ($F_{(2,12)} = 116.5$, $p < 0.001$). The treatment × prepulse level interaction was not significant ($F_{(120,200)} = 0.9$, $p = 0.54$), however. Post hoc analyses indicated that scopolamine significantly ($p < 0.05$) diminished PPI at the 5 and 10, but not at the 15 dB prepulse level. Donepezil at a reference dose of 1.0 mg/kg fully reversed the effects of scopolamine on PPI at all 3 prepulse levels. In addition, two of the four doses of cotinine (i.e., 0.33, and 1.0 mg/kg) significantly improved the deficits in PPI produced by scopolamine either at the individual prepulse levels (Fig. 7A) or when the data were averaged across prepulse level (Fig. 7B).

In summary, cotinine (particularly at doses approaching 0.33 to 1.0 mg/kg) was associated with positive effects in all three PPI pharmacologic models (i.e., it significantly improved or completely reversed the antagonist effects on PPI in each study). These results support the premise that cotinine could serve as a prototypical antipsychotic agent, and further, that schizophrenics may in fact be self medicating the auditory gating deficits (and associated cognitive deficits) by exposing themselves to cotinine (and nicotine) via cigarette smoke.

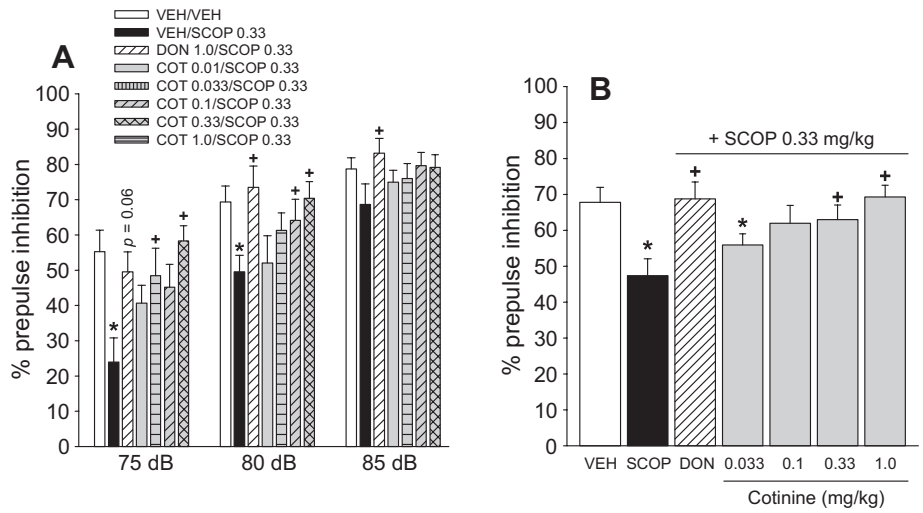

Fig. 7. A. Effects of scopolamine (0.33 mg/kg) and several doses of cotinine (and a reference dose of the acetylcholinesterase inhibitor donepezil) on scopolamine-induced deficits in prepulse inhibition in rats associated with three prepulse intensities (5, 10, and 15 dB above background). **B.** Effects of cotinine (and a reference dose of donepiezil) on scopolamine-induced deficits in prepulse inhibition across prepulse level. Bars represent mean ± S.E.M. for each treatment ($N = 8–10$). VEH, vehicle; COT, cotinine; SCO*P*, scopolamine; DON, donepezil. *Significantly different ($p < 0.05$) from the vehicle associated response; $^{+}$significantly different ($p < 0.05$) from the scopolamine-associated response.

CELL VIABILITY

The potential neuroprotective action produced by cotinine was assessed by its ability to prevent the cytotoxicity induced by growth factor withdrawal in differentiated PC-12 cells. The cells were dissociated by trituration and plated at 10,000 cells per well on poly-L-lysine coated 96 well plates containing DMEM.NGF media. Next, the differentiated cells were incubated with vehicle or with a test drug (prepared in serum-free DMEM media with no exogenous NGF) for 24 h. A parallel set of control cells were maintained in DMEM.NGF medium in each experiment. Cell viability (cytotoxicity) was determined by using the Cell Titer 96 cell proliferation/cytotoxicity assay kit (Promega), which is based on the cellular conversion of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenltetrazolium bromide (MTT) into a formazan product that could be detected spectrophotometrically. At the completion of the incubation period, the culture medium was aspirated and 15 mL of dye solution in DMEM was added. After 4 h at 37°C, 100 mL of solubilization/stop solution was added and the absorbance of solubilized MTT formazan products was measured at 579 nm. All data were normalized to untreated control cells in each plate. The data represent the average of least 3 experiments (plates) each performed with 4 replicates.

Differentiated PC-12 cells in culture are well-suited for this study. PC-12 cells express functional α_7 nicotinic receptors (5,41) with properties that are very similar to the α_7 receptors expressed within the central nervous system (CNS) (20). PC-12 cells express mRNAs that encode α_3, α_5, α_7, β_2, and β_4 subunits that comprise subtypes of brain nicotinic receptors (27). PC-12 cells also constitutively express β-amyloid precursor protein. Like basal forebrain cholinergic neurons, differentiated PC-12 cells are dependent on nerve growth factor for survival (3,13,47), and proliferation (3,13). The importance of

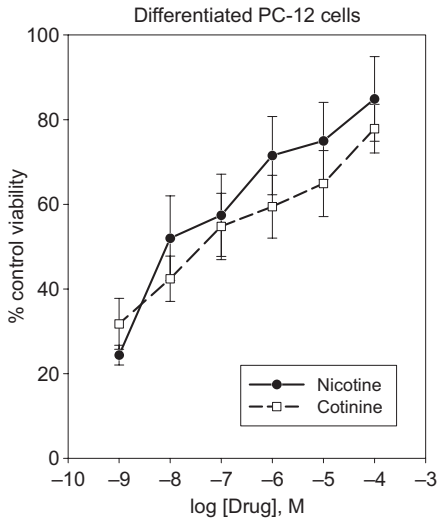

Fig. 8. Comparison of nicotine (filled circles) and cotinine (squares) in their ability to protect differentiated PC-12 cells from cytotoxicity related to growth factor deprivation. Each compound was incubated with the cells 24 h prior to growth factor withdrawal.

nicotinic receptors, particularly the α_7 subtype, resides partly in their participation in the cytoprotective or neuroprotective effects of nicotine and other nicotinic receptor agonists (12,17,19,31–34,46).

In this experimental series we compared the relative cytoprotective action of nicotine and cotinine in NGF-differentiated PC12 cells. Cells were plated and induced to differentiate over a 5-day period in 96 well plates. The cells then were incubated for 24 h with varying concentrations of nicotine or cotinine. Subsequently, the cells were washed and placed into serum-free medium, without NGF. The data are presented in Fig. 8. Growth factor withdrawal induced a $58.3 \pm 3.0\%$ loss in cell viability. Nicotine treatment produced a level of cytoprotection that almost completely restored the cells to control (growth factor maintained) levels of viability. Over the same concentration range cotinine treatment was also associated with cytoprotection, and in fact the potency of cotinine was similar to that for nicotine.

AUTORADIOGRAPHY

In order to assess the effects of chronic cotinine administration on cholinergic receptor densities, autoradiographic analyses of brain tissues harvested from rats previously exposed to cotinine were conducted with subtype specific cholinergic radioligands to nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs), i.e., receptors that have been found to play important roles in learning and memory processes (52,42). High-affinity (heteromeric α/β subunit complexes) and low-affinity (homomeric α_7) nAChRs were labeled with [^{3}H]epibatidine ([^{3}H]EPB) and [^{125}I]α-bungarotoxin ([^{125}I]BTX), respectively. Of the high-affinity nAChRs, the $\alpha_4\beta_2$ subtype predominates in density and distribution in the mammalian central nervous system (39). The density of M_1 and M_2 mAChRs, i.e., the mAChRs expressed in highest quantities in mammalian brain (52), were quantified using [^{3}H]pirenzipine ([^{3}H]PRZ) and [^{3}H]AFDX 384 ([^{3}H]AFX), respectively.

Rats ($n = 6$) were injected s.c. with cotinine 1.0 mg/kg every 12 h (along with paired saline controls) for 14 consecutive days. This dosing approach was based on a previous study in our laboratory (28) in which nicotine (0.7 mg/kg/day) produced notable effects on cholinergic receptors (e.g., unregulated high affinity nAChRs). Our rationale was that if some of these effects were actually due to the cotinine, generated via nicotine metabolism, then we would certainly expect to detect significant effects of cotinine on cholinergic receptor density (particularly since the selected dose of cotinine was even higher than that of nicotine). Twenty four hours following the last injection, the rats were sacrificed by decapitation, whole brain tissue was removed and flash frozen in 2-methylbutane for storage at –70°C. Using a Microm® HM cryostat (–18°C), the left hemisphere ($n = 6$ per group) of each brain was serially sectioned (16 μM) up to the midline onto gelatin-coated slides. To define the response of the radiosensitive films to increasing amounts of radioactivity, tissue paste standards containing increasing amounts of radioactivity were prepared as described previously (see ref. 28) and included in all film exposures. The tissue paste standards were prepared from whole rat brains homogenized in ice-cold phosphate buffer. Depending on the radioligand being evaluated, increasing amounts of [^{3}H]choline chloride or [^{125}I]BTX were added to individual aliquots of homogenized whole brain tissue. The specific activity range of each set of tissue paste standards was 0.5–30.0 nCi/mg, as determined by a liquid scintillation or gamma counter (depending on the radioisotope).

For all autoradiography experiments, slides were incubated with radioligands diluted in Kreb-Ringers-Hepes buffer (nAChR radioligands) or 50 μM Tris-HCl buffer (mAChR radioligands). For radioligand concentration and incubation times, types of buffers used, duration of film exposure, etc. please refer to reference 28. Nonspecific binding was determined by the addition of 300 or 1 μM nicotine hydrogen tartrate to the buffer prior to incubation with [^{3}H]EPB and [^{125}I]BTX, respectively. Nonspecific binding was determined by the addition of 10 μM atropine sulfate to the buffer prior to the addition of [^{3}H]PRZ or [^{3}H]AFX.

After incubation with designated radioligands, slides were stored overnight in a vacuum desiccator at room temperature. Autoradiograms were prepared by exposing the slides to radiosensitive film (Hyperfilm-3H or Bmax, Amersham Bioscience, Piscataway, NJ, USA) for 1 to 10 weeks depending on the radioligand. All films were manually processed after exposure to slides with Kodak® D-19 Developer (5-min), Indicator Stop Bath (30-sec), and Rapid Fixer with hardener (10-min).

Images of each section were captured from autoradiograms for the densitometry of individual brain regions using NIH Image Software and an imaging station (Macintosh PowerPC 8100/100I computer, Data Translation QuickCapture imaging board, Sony SC-77 CCD camera, and a Northern Lights Precision Desktop Illuminator). Receptor binding was quantified as optical density in all brain areas that had a signal that was greater than non-specific binding and the background of the film. Optical densities of the tissue paste standards (with known nCi/mg concentrations) were obtained and a sigmoidal calibration curve (standard [nCi/mg] vs. optical density) was generated using Table Curve 2D software (Systat, Richmond, CA, USA). After films were developed, sections were stained with 0.5% cresyl violet (pH 4.0) in order to better visualize and discriminate between the structures and boundaries of individual brain structures. Brain nuclei were identified using Paxinos and Watson's *Rat Atlas*, 4th Edition (38).

Autoradiographic results are depicted in Tables 2–4 and in Fig. 9. For each of the ligands employed in this study, the pattern of binding site distribution was similar to that observed in previous studies (14,21,22,28)

TABLE 2. Comparison of [^{3}H]pirenzepine or [^{3}H]AFDX-384 binding densities in selected brain regions in 16 weeks-old male Wistar rats treated with vehicle (saline) or cotinine (2.0 mg/kg/day) for 14 days

	Vehicle	Cotinine	Vehicle	Cotinine
Brain area	[^{3}H]pirenzepine		[^{3}H]AFDX-384	
Centrobasolateral amygdaloid nucleus	6.125 ± 0.185	6.071 ± 0.155	2.947 ± 0.0143	3.048 ± 0.203
Basolateral amygdaloid nucleus	11.234 ± 0.160	11.421 ± 0.110		
Anterior amygdaloid area	9.780 ± 0.124	9.727 ± 0.149		
Amygdaloid hippocampal area			3.196 ± 0.238	3.402 ± 0.270
Lateral globus pallidus	2.231 ± 0.022	2.221 ± 0.060		
Caudate putamen	10.736 ± 0.136	10.826 ± 0.088	9.587 ± 0.127	9.862 ± 0.254
Accumbens nucleus	11.348 ± 0.131	11.425 ± 0.062	10.0 ± 0.16	10.2 ± 0.19
Substantia innominata	3.197 ± 0.037	3.241 ± 0.035	9.997 ± 0.155	10.179 ± 0.189
Bed nucleus of stria terminalis	4.458 ± 0.055	4.317 ± 0.109	2.681 ± 0.102	2.773 ± 0.058
Olfactory bulbs	7.226 ± 0.575	8.100 ± 0.127		
External plexiform layer			10.706 ± 0.155	10.732 ± 0.161
Granular cell layer			9.045 ± 0.087	9.302 ± 0.088
Anterior olfactory nucleus	11.015 ± 0.126	11.128 ± 0.065	5.562 ± 0.511	5.989 ± 0.742
Olfactory tubercle	11.383 ± 0.158	11.246 ± 0.105	10.355 ± 0.159	10.538 ± 0.170
Lateral septal nucleus	3.603 ± 0.093	3.796 ± 0.174	2.982 ± 0.102	3.060 ± 0.181
Medial septal nucleus			2.440 ± 0.059	2.218 ± 0.100*
Cerebral cortex	9.868 ± 0.190	10.117 ± 0.113	4.352 ± 0.224	5.098 ± 0.496
Lamina I, cortex	11.256 ± 0.117	11.278 ± 0.042	6.798 ± 0.686	7.850 ± 0.714
Lamina II–VI, cortex	9.691 ± 0.212	9.567 ± 0.100	4.065 ± 0.253	4.291 ± 0.332
Piriform, cortex			3.842 ± 0.212	4.259 ± 0.316
Cingulate, cortex	10.691 ± 0.132	10.825 ± 0.082		
Insular, cortex	10.878 ± 0.080	10.671 ± 0.123		
Retrosplenial, cortex	7.538 ± 0.311	7.669 ± 0.177		
Piriform, cortex	10.874 ± 0.119	10.683 ± 0.168		
Visual, cortex	10.608 ± 0.096	10.618 ± 0.087		
Entorhinal cortex	10.218 ± 0.158	10.249 ± 0.108	3.511 ± 0.116	3.798 ± 0.272
CA1 hippocampus	12.068 ± 0.140	12.034 ± 0.131	6.734 ± 0.671	7.133 ± 0.661
CA2/3 hippocampus	10.068 ± 0.122	10.133 ± 0.061	3.093 ± 0.093	3.037 ± 0.158
Dentate gyrus			3.908 ± 0.198	4.000 ± 0.239
Polymorph layer, dentate			3.635 ± 0.108	3.674 ± 0.223
Inner blade dentate gyrus	9.897 ± 0.129	9.987 ± 0.079		
Outer blade dentate gyrus	11.962 ± 0.129	11.975 ± 0.119		
Presubiculum	8.182 ± 0.215	7.888 ± 0.224		
Subiculum	6.929 ± 0.190	7.245 ± 0.173	2.917 ± 0.098	2.779 ± 0.169
Parasubiculum	6.989 ± 0.269	7.439 ± 0.168		
Postsubiculum	5.536 ± 0.273	5.664 ± 0.269		
Thalamus	3.426 ± 0.113	3.421 ± 0.092		
Thalamic nucleus, anterior	2.806 ± 0.147	2.876 ± 0.161	4.293 ± 0.165	4.128 ± 0.219
Thalamic nucleus, lateral	3.537 ± 0.075	3.482 ± 0.070		
Thalamic nucleus, laterodorsal			2.745 ± 0.090	2.755 ± 0.148
Thalamic nucleus, lateroposterior			2.648 ± 0.145	2.713 ± 0.187
Thalamic nucleus, mediodorsal	3.764 ± 0.109	3.873 ± 0.096	2.691 ± 0.078	2.869 ± 0.186
Medial geniculate nucleus	3.174 ± 0.100	3.317 ± 0.084	2.234 ± 0.052	2.294 ± 0.139

TABLE 2 (continued)

Brain area	Vehicle	Cotinine	Vehicle	Cotinine
	[^{3}H]pirenzepine		[^{3}H]AFDX-384	
Dorsolateral geniculate nucleus	2.955 ± 0.051	2.941 ± 0.096	2.245 ± 0.171	2.223 ± 0.236
Ventrolateral geniculate nucleus			2.400 ± 0.131	2.435 ± 0.192
Thalamic nucleus, posterior	3.720 ± 0.115	3.512 ± 0.081	2.768 ± 0.076	2.836 ± 0.174
Thalamic nucleus, ventral	2.633 ± 0.123	2.522 ± 0.032		
Thalamic nucleus, ventroposterior			1.989 ± 0.044	2.021 ± 0.093
Hypothalamus	1.737 ± 0.033	1.692 ± 0.056		
Tuberomammillary nucleus	3.260 ± 0.217	2.867 ± 0.140		
Periaqueductal gray	1.611 ± 0.055	1.592 ± 0.082	2.225 ± 0.096	1.871 ± 0.182
Substantia nigra	2.156 ± 0.077	2.211 ± 0.060		
Central nucleus of inferior colliculus	2.068 ± 0.112	2.041 ± 0.053	2.025 ± 0.054	1.761 ± 0.115*
Superior colliculus	2.457 ± 0.115	2.367 ± 0.073	2.980 ± 0.175	2.421 ± 0.162*
Deep layer			2.374 ± 0.087	2.151 ± 0.151
Rostroventrolateral medulla	3.765 ± 0.209	3.701 ± 0.074	3.216 ± 0.080	3.107 ± 0.236
Pontine nucleus	1.687 ± 0.054	1.758 ± 0.072	3.895 ± 0.190	3.809 ± 0.390
Parabrachial nucleus	2.218 ± 0.151	2.141 ± 0.094	3.147 ± 0.200	3.154 ± 0.226
Anterior pretectal nucleus			2.505 ± 0.082	2.472 ± 0.196
Nucleus of solitary tract			3.508 ± 0.125	3.499 ± 0.352
Pineal body			1.909 ± 0.016	1.557 ± 0.144*

Brains were sliced in 16 μM sagittal sections from the beginning of the dentate gyrus through the longitudinal fissure. Binding is expressed as nCi bound/mg wet tissue and each value represents mean ± S.E.M. of 6 animals. *Significantly different ($p < 0.05$) from vehicle associated value.

TABLE 3. Comparison of [^{3}H]epibatidine alone or [^{3}H]epibatidine + 150 nM cytisine binding densities in selected brain regions in 16 weeks-old male Wistar rats treated with vehicle (saline) or cotinine (2.0 mg/kg/day) for 14 days

Brain area	Vehicle	Cotinine	Vehicle	Cotinine
	[^{3}H]Epibatidine		[^{3}H]Epibatidine + 150 nM cytisine	
Anterior olfactory nucleus	0.721 ± 0.025	0.732 ± 0.032		
Accumbens nucleus	1.173 ± 0.050	1.187 ± 0.052		
Olfactory tubercle	0.878 ± 0.034	0.864 ± 0.061		
Caudate putamen	1.347 ± 0.027	1.323 ± 0.041	0.319 ± 0.020	0.302 ± 0.012
Lateral globus pallidus	0.537 ± 0.020	0.454 ± 0.016**		
Amygdala	0.570 ± 0.029	0.600 ± 0.017		
Lateral septal nucleus	0.585 ± 0.029	0.560 ± 0.020		
Bed nucleus of stria terminalis	0.675 ± 0.031	0.655 ± 0.017		
Cerebral cortex			0.268 ± 0.015	0.286 ± 0.018
Lamina I, cerebral cortex	0.988 ± 0.032	0.966 ± 0.022		
Lamina II, cerebral cortex	1.399 ± 0.049	1.320 ± 0.031		
Lamina III–VI, cerebral cortex	1.068 ± 0.037	1.029 ± 0.017		
Piriform cortex	0.521 ± 0.026	0.607 ± 0.054		
Prefrontal cortex	1.315 ± 0.026	1.295 ± 0.033		
Hippocampus	0.436 ± 0.024	0.423 ± 0.012		
Presubiculum	2.345 ± 0.064	2.381 ± 0.102	0.633 ± 0.058	0.619 ± 0.036

TABLE 3 (continued)

Brain area	Vehicle	Cotinine	Vehicle	Cotinine
	[^{3}H]Epibatidine		[^{3}H]Epibatidine + 150 nM cytisine	
Subiculum	1.534 ± 0.047	1.547 ± 0.030		
Postsubiculum	2.645 ± 0.112	2.604 ± 0.051	0.584 ± 0.027	0.559 ± 0.020
Entorhinal cortex	1.049 ± 0.028	1.087 ± 0.021		
Habenular nucleus, medial	11.578 ± 0.065	11.613 ± 0.079	10.843 ± 0.124	10.790 ± 0.118
Hypothalamus	0.611 ± 0.022	0.583 ± 0.032		
Zona incerta	0.718 ± 0.028	0.697 ± 0.021		
Thalamus			0.355 ± 0.015	0.343 ± 0.009
Thalamic nucleus, anterior			0.670 ± 0.025	0.635 ± 0.021
Geniculate nucleus, ventral	2.024 ± 0.028	2.156 ± 0.063		
Geniculate nucleus, dorsal	2.798 ± 0.140	2.764 ± 0.068		
Geniculate nucleus, dorsolateral			0.803 ± 0.018	0.846 ± 0.031
Geniculate nucleus, medial	1.901 ± 0.048	1.806 ± 0.026	0.386 ± 0.032	0.386 ± 0.040
Geniculate nucleus, ventrolateral			0.827 ± 0.069	0.784 ± 0.037
Reticular, thalamus	1.630 ± 0.052	1.624 ± 0.060		
Anteroventral, thalamus	4.537 ± 0.500	4.284 ± 0.210		
Anteromedial, thalamus	2.934 ± 0.127	2.920 ± 0.095		
Centromedial, thalamus	2.146 ± 0.083	2.132 ± 0.077		
Lateral posterior, thalamus	2.166 ± 0.106	2.168 ± 0.060		
Laterodorsal, thalamus	2.560 ± 0.092	2.637 ± 0.098		
Mediodorsal, thalamus	2.370 ± 0.072	2.414 ± 0.066		
Paraventricular, thalamus	1.884 ± 0.052	1.950 ± 0.045		
Reuniens, thalamus	2.364 ± 0.056	2.437 ± 0.088		
Posterior, thalamus	2.186 ± 0.111	2.236 ± 0.060		
Ventral posterolateral, thalamus	1.710 ± 0.055	1.723 ± 0.065		
Dorsal tegmental nucleus	1.142 ± 0.059	1.194 ± 0.040		
Periaqueductal gray	0.751 ± 0.037	0.777 ± 0.022		
Interpeduncular nucleus	11.183 ± 0.186	11.499 ± 0.029	9.952 ± 0.217	10.195 ± 0.131
Substantia nigra	1.522 ± 0.088	1.589 ± 0.065	1.234 ± 0.047	1.280 ± 0.285
Pretectal nucleus			1.046 ± 0.097	0.965 ± 0.084
Anterior pretectal nucleus	1.122 ± 0.072	1.070 ± 0.053		
Anterior pretectal nucleus, ventral	0.941 ± 0.058	0.870 ± 0.046		
Anterior pretectal nucleus, dorsal	1.366 ± 0.052	1.234 ± 0.062		
Olivary pretectal nucleus	1.588 ± 0.026	1.622 ± 0.026		
Superior colliculus	3.398 ± 0.086	3.448 ± 0.120	1.464 ± 0.024	1.572 ± 0.063
Superior colliculus deep	1.131 ± 0.054	1.086 ± 0.045		
Central nucleus of inferior colliculus	0.405 ± 0.017	0.323 ± 0.025**		
Medulla	0.517 ± 0.017	0.464 ± 0.018*		
Medial vestibular nucleus	0.999 ± 0.024	1.063 ± 0.043	0.465 ± 0.050	0.400 ± 0.008
Nucleus of the solitary tract	1.888 ± 0.230	1.769 ± 0.135	1.718 ± 0.159	1.873 ± 0.121
Cerebellum			0.197 ± 0.015	0.190 ± 0.008
Cerebellum Purkinje layer	0.910 ± 0.032	0.905 ± 0.015		
Pontine nucleus	0.785 ± 0.023	0.732 ± 0.026		
Fasciculus retroflexus	3.197 ± 0.138	3.527 ± 0.258	2.716 ± 0.258	2.635 ± 0.125
Pineal body	11.436 ± 0.057	11.443 ± 0.102	10.226 ± 0.256	10.662 ± 0.139

Brains were sliced in 16 μM sagittal sections from the beginning of the dentate gyrus through the longitudinal fissure. Binding is expressed as nCi bound/mg wet tissue and each value represents mean ± S.E.M. of 6 animals. *Significantly different ($p < 0.05$) from vehicle associated value; **$p < 0.01$.

TABLE 4. Comparison of [^{125}I]-α-bungarotoxin binding densities in selected brain regions between 16 weeks ols male Wistar rats treated with vehicle (saline) or cotinine (2.0 mg/kg/day) for 14 days

Brain region	Vehicle	Cotinine	Brain region	Vehicle	Cotinine
Anterior amygdaloid area	0.143 ± 0.004	0.131 ± 0.007	Paratenial nucleus	0.166 ± 0.009	0.172 ± 0.004
Posterior cortical amygdala	0.241 ± 0.011	0.232 ± 0.017	Zona incerta	0.082 ± 0.002	0.077 ± 0.002
Dorsal endopiriform nucleus	0.175 ± 0.010	0.166 ± 0.007	Subthalamic nucleus	0.359 ± 0.023	0.365 ± 0.026
Anterior olfactory nucleus	0.104 ± 0.009	0.086 ± 0.004*	Anterior hypothalamic area	0.080 ± 0.010	0.060 ± 0.003*
Dorsoventrolateral olfactory nucleus	0.139 ± 0.009	0.128 ± 0.005	Medial preoptic area	0.140 ± 0.007	0.135 ± 0.003
Posterior olfactory nucleus	0.149 ± 0.009	0.144 ± 0.004	Supraoptic nucleus	0.456 ± 0.037	0.515 ± 0.010
Olfactory bulbs	0.015 ± 0.003	0.014 ± 0.002	Hypothalamic nucleus, ventromedial	0.133 ± 0.003	0.136 ± 0.012
Accessory olfactory bulb	0.605 ± 0.028	0.542 ± 0.033	Mammillary peduncles	0.290 ± 0.010	0.288 ± 0.012
Insular cortex	0.074 ± 0.003	0.068 ± 0.002	Posterior hypothalamic area	0.096 ± 0.005	0.101 ± 0.003
Lamina I–V, cerebral cortex	0.047 ± 0.004	0.043 ± 0.002	Dorsal raphe nucleus	0.614 ± 0.043	0.661 ± 0.027
Lamina VI, cerebral cortex	0.080 ± 0.003	0.078 ± 0.003	Magnocellular nucleus of the PC	0.175 ± 0.010	0.173 ± 0.008
Motor cortex	0.059 ± 0.005	0.053 ± 0.003	Periaqueductal gray	0.095 ± 0.010	0.095 ± 0.005
Orbital cortex	0.084 ± 0.007	0.074 ± 0.005	Dorsal tegmental nucleus	0.562 ± 0.068	0.537 ± 0.032
Parietal association cortex	0.059 ± 0.005	0.049 ± 0.004	Pedunculopontine tegmental nucleus	0.144 ± 0.005	0.149 ± 0.004
Piriform cortex	0.049 ± 0.004	0.043 ± 0.002	Substantia nigra	0.083 ± 0.002	0.073 ± 0.004*
Somatosensory cortex	0.057 ± 0.004	0.054 ± 0.002	Pretectal nucleus	0.146 ± 0.005	0.150 ± 0.006
Visual cortex	0.052 ± 0.004	0.048 ± 0.002	Central inferior colliculus	0.328 ± 0.011	0.324 ± 0.012
Entorhinal cortex	0.088 ± 0.004	0.081 ± 0.007	Inferior colliculus	0.146 ± 0.005	0.147 ± 0.006
CA1 lateral hippocampus	0.093 ± 0.004	0.093 ± 0.008	Superior colliculus	0.350 ± 0.011	0.344 ± 0.012
CA1 medial hippocampus	0.054 ± 0.005	0.037 ± 0.003**	Cochlear nucleus	0.063 ± 0.005	0.068 ± 0.005
CA2/3 lateral hippocampus	0.150 ± 0.006	0.148 ± 0.006	Inferior Olive	0.113 ± 0.003	0.119 ± 0.002
Lateral dentate gyrus	0.497 ± 0.021	0.457 ± 0.023	Medial vestibular nucleus	0.200 ± 0.018	0.246 ± 0.011
Medial dentate gyrus	0.076 ± 0.006	0.064 ± 0.004	Gigantocellular reticular nucleus	0.055 ± 0.003	0.055 ± 0.002
Polymorph layer dentate gyrus	0.231 ± 0.011	0.225 ± 0.016	Lateral lemniscus	0.190 ± 0.015	0.182 ± 0.006
Presubiculum	0.168 ± 0.010	0.150 ± 0.008	Microcellular tegmental nucleus	0.226 ± 0.016	0.220 ± 0.009
Subiculum	0.145 ± 0.008	0.137 ± 0.007	Parabrachial nucleus	0.163 ± 0.005	0.164 ± 0.003
Postsubiculum	0.044 ± 0.002	0.042 ± 0.002	Principal trigeminal sensory nucleus	0.145 ± 0.011	0.150 ± 0.007
Habenular nucleus, lateral	0.078 ± 0.007	0.091 ± 0.010	Spinal trigeminal nucleus	0.100 ± 0.004	0.100 ± 0.004
Geniculate nucleus	0.257 ± 0.016	0.236 ± 0.008	Lateral superior olive	0.226 ± 0.008	0.203 ± 0.006*

Brains were sliced in 16 μM sagittal sections from the beginning of the dentate gyrus through the longitudinal fissure. Binding is expressed as nCi bound/mg wet tissue and each value represents mean ± S.E.M. of 6 animals. * Significantly different ($p < 0.05$) from vehicle associated value; ** $p < 0.01$.

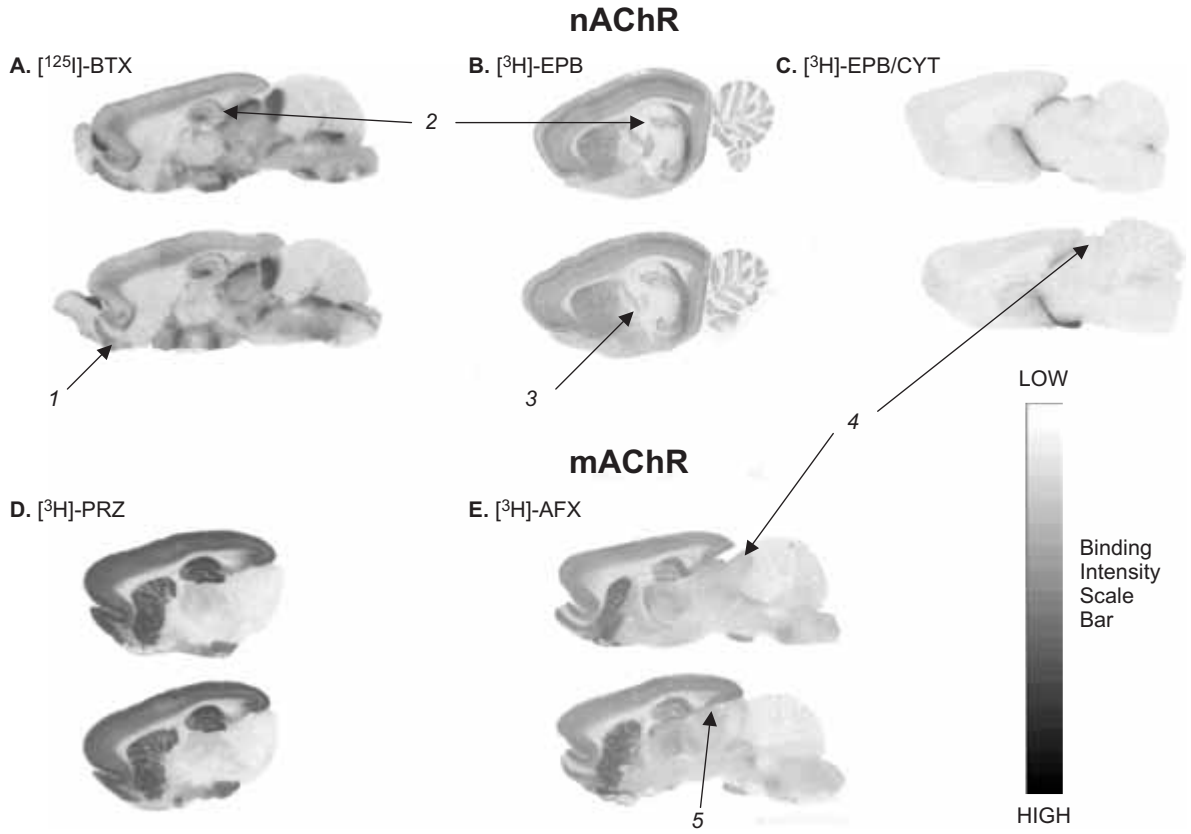

Fig. 9. Representative autoradiograms illustrating nAChR and mAChR subtypes in rats labeled by the following radioligands: (A) [^{125}I]-α-bungarotoxin (α_7), (B) [^{3}H]epibatidine (predominantly $\alpha_4\beta_2$) or (C) [^{3}H]epibatidine + 150 nM cytisine (predominantly $\alpha_3\beta_4$), and mAChR (D) [^{3}H]pirenzipine (M_1) and (E) [^{3}H]AFDX384 (M_2). Experiments were conducted using 16 μM sagittal sections of brains from 16-weeks old male Wistar rats treated with vehicle (saline) or cotinine (2.0 mg/kg/day) for 14 days. Representative brain areas in which significant ($p < 0.05$) treatment-related binding differences were observed are indicated as follows: *1*, anterior olfactory nucleus; *2*, CA1 region of hippocampus; *3*, lateral globus pallidus; *4*, central nucleus of inferior colliculus; *5*, superior colliculus.

Muscarinic Receptor Expression

[^{3}H]PRZ: [^{3}H]PRZ binding was widely distributed in the cortex and hippocampal formation and minimally represented in the thalamus, hypothalamus, and midbrain (Table 2 and Fig. 9). The highest [^{3}H]PRZ binding densities were observed in the telencephalic regions such as CA1 region of the hippocampus, dentate gyrus, nucleus accumbens, basolateral amygdala, neocortex caudate putamen, and anterior olfactory nuclei. There was no consistent pattern of [^{3}H]PRZ binding differences between cotinine and vehicle treated animals.

[^{3}H]AFX: Like [^{3}H]PRZ binding, [^{3}H]AFX binding was widely distributed in the cortex and hippocampal formation. The highest [^{3}H]AFX binding densities were observed in olfactory areas, the caudate putamen, and accumbens nuclei, while moderate binding was found in the cortex, basolateral amygdala, and hippocampal formation (Table 2 and Fig. 9). Of the 40 brain regions measured, significant binding differences ($p < 0.05$) were observed in 4 brain regions, the medial septal nucleus, the pineal body, the central nucleus of the inferior colliculus, and the superior colliculus. In each of these cases binding was lower in the cotinine-treated animals.

Nicotinic Receptor Expression

[^{3}H]EPB: The highest [^{3}H]EPB binding densities were observed in the medial habenular nuclei, interpeduncular nuclei, and pineal gland (Table 3 and Fig. 9). Moderate binding was observed in the anterior thalamus and subicular complex while lower binding densities in the cerebral cortex and individual cortical layers. Out of the 54 areas measured, cotinine-treated animals exhibited a statistically significant difference from vehicle controls in only two areas, the lateral globus pallidus and the medulla. In both cases, cotinine was associated with decreases in [^{3}H]EPB binding.

[^{3}H]EPB + 150 nM cytisine: In order to quantify the density of α_3-containing nAChRs (28), sections were incubated with [^{3}H]epibatidine and 150 nM cytisine. The autoradiographic distributions of [^{3}H]EPB + cytisine binding to central α_3-containing nAChRs were similar between cotinine treated rats and controls (Table 3 and Fig. 9). Similar to [^{3}H]EPB binding, the highest [^{3}H]epibatidine + cytisine binding densities were observed in the medial habenular nuclei, interpeduncular nuclei, and pineal gland. Out of the 20 areas measured, the only significant difference observed between treatment groups was a decrease in binding in the central nucleus of the inferior colliculus.

[^{125}I]BTX: Binding of [^{125}I]BTX was widely distributed across all regions of the brain, with the exception of the striatum and cerebellum (Table 4 and Fig. 9). The highest [^{125}I]BTX binding densities were observed in the accessory olfactory bulb, supraoptic nuclei, mammillary nuclei, dorsal raphe, and medial vestibular nuclei. Moderate binding was observed in the superior colliculus, hippocampus, hypothalamus, and tegmental nuclei. Lower binding densities were observed in the cerebral cortex and amygdala. Out of the 59 areas measured for [^{125}I]BTX, cotinine-treated animals exhibited significantly lower binding densities in five areas: the anterior olfactory nucleus, the CA1 region of the hippocampus (medial region), the anterior hypothalamic area, the substantia nigra, and the lateral superior olive. The decreases in the CA1 region of the hippocampus were striking (e.g., ~30%).

These results are consistent with the ability of chronic administration of cotinine to induce adaptive changes, most notably in α_7 nicotinic receptors. The observation that coti-

nine downregulates α_7 receptors suggests that (unlike nicotine) cotinine does not chronically desensitize the receptors, since chronic desensitization is considered to induce nicotinic receptor upregulation. The reduced ability of cotinine (relative to nicotine) to induce desensitization could be reflected in a prolonged receptor agonist property of the drug. In the least, these data show that one potential site of action of cotinine is the CA1 region of the hippocampus, a region important for memory processes, as well as for the neuroprotective action attributed to nicotinic drugs. This possibility fits well with the results presented in Figs. 2 and 8.

CONCLUSIONS

Until recently cotinine was considered simply an inactive tobacco-derived compound that was used primarily as an indication of smoking cessation compliance. But we now provide compelling evidence that cotinine is indeed a pharmacologically active compound having a number of actions that suggest that it might mediate some of the beneficial effects of nicotine, as well as exhibiting some unique properties.

(1) In the rat, the motor response to acoustic startle can be inhibited by the presentation of a low-level acoustic prepulse presented just in advance of the high-level acoustic pulse; the ability of the CNS to modulate its sensitivity to a sensory stimulus in this way is known as "sensory gating." Drugs like apomorphine, which can induce a schizophreniform response, disrupt sensory gating, and under the baseline conditions, apomorphine suppresses prepulse inhibition of the acoustic startle response. Most drugs with potential antipsychotic action reverse the disruption of acoustic startle by apomorphine. Cotinine significantly reverses the effects of apomorphine on acoustic startle. Cotinine also effectively reversed the disrupting effects of the muscarinic antagonist scopolamine, and the NMDA glutamate receptor agonist MK-801. These findings support the possibility that cotinine possesses the antipsychotic drug-like properties of the more effective "atypical" class of agents.

(2) Cotinine is effective in preventing cytotoxicity associated with the growth factor withdrawal in differentiated PC-12 cells. In this regard, cotinine is nearly as potent as nicotine. Thus, cotinine could prove effective in reducing the progression of neurodegenerative diseases including Alzheimer's disease and Parkinson's disease.

(3) Cotinine produces a dose-dependent increase in accuracy in an automated delayed matching-to-sample (DMTS) task in macaques. Cotinine also significantly reversed distractor-impaired DMTS accuracy in monkeys, indicating the potential of the drug to enhance attention. These properties would be expected to prove effective for the treatment of dementia and in attention deficit disorders. In these studies cotinine was only about 10–100-fold less potent than nicotine (based on effective mg/kg doses) and was able to produce a positive response that is similar in magnitude to that of nicotine (see reference 11). Based on the work of Hatsukami and colleagues (26) the effective doses (i.e., up to 10 mg/kg) of cotinine used in our study would likely be safe in humans, but would be associated with plasma levels that significantly exceed those encountered in human smokers.

(4) Receptor autoradiography was used for measuring nicotinic receptor density in sagittal sections of rat brain. The assay conditions chosen for each receptor subtype provide a reliable and differential distribution of $\alpha_4\beta_2$ and α_7 nicotinic receptors, and for M_1 and M_2 muscarinic receptors in the rat brain. Chronic cotinine treatment decreased the

density of M_2 muscarinic receptors in 4 regions with the greatest effect occurring within the superior colliculus. For nicotinic receptors, the greatest effect of the chronic cotinine treatment was to decrease α_7 receptor density within the CA1 region of the hippocampus. The finding that chronic cotinine treatment decreased nicotinic receptor density in the affected brain regions supports the possibility that the drug may activate nicotinic receptors without inducing significant desensitization. The finding that cotinine also decreased the density of muscarinic receptors may be explained by the ability of the drug to release acetylcholine which in turn acts on muscarinic receptors. Long-term acetylcholine release would be expected to decrease muscarinic receptor density. This again would fit with the possibility of a lack of nicotinic receptor desensitization produced by cotinine, since only chronically released acetylcholine would be expected to reduce muscarinic receptor expression.

These new data suggest the possibility that cotinine is a pharmacologically-active drug capable of activating subtypes of nicotinic receptors with a minimal receptor desensitization. Thus, although cotinine may be a less potent receptor agonist than nicotine, its longer plasma half-life coupled with a reduced ability to desensitize nicotinic receptors may help to explain the prolonged behavioral responses that have been observed with nicotine (i.e., longer than nicotine's presence in the brain or plasma). They also suggest that new cotinine analogs may provide a new approach to the development of drugs for the treatment of neurodegenerative diseases.

Finally, the properties of cotinine as a cognition- and attention-enhancing agent and potential antipsychotic drug having an "atypical" profile combine to provide the basis for a new class of antipsychotic drugs for the treatment of cognitive impairment associated with schizophrenia. Cotinine could also find use in the treatment of Alzheimer's disease, as a memory enhancing drug, and as a neuroprotective and disease modifying agent. Cotinine can also be expected to be useful in the treatment of attention deficit disorders.

ABBREVIATIONS

nAChR, Nicotinic acetylcholine receptor;
mAChR, Muscarinic acetylcholine receptor;
DMTS, Delayed matching-to-sample;
PPI, Prepulse inhibition;
[^{3}H]PRZ, [^{3}H]Pirenzipine;
[^{3}H]AFX, [^{3}H]AFDX 384;
[^{3}H]EPB, [^{3}H]Epibatidine;
[^{125}I]BTX, [^{125}I]α-bungarotoxin.

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