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R-(+) - and S-(-) - Isomers of Cotinine Augment Cholinergic Responses in vitro and in vivo

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Abbreviations:

ACh, acetylcholine
AChE, acetylcholinesterase inhibitor
COT, cotinine
DHβE, dihydro-β-erythroidine
MLA, methyllycaconitine
nAChR, nicotinic acetylcholine receptor
Abstract:

The nicotine metabolite, cotinine (1-methyl-5-[3-pyridynl]-2-pyrrolidinone) like its precursor, has been found to exhibit pro-cognitive and neuroprotective effects in some model systems; however, the mechanism of these effects is unknown. In this study, both the R-(+)- and S-(-)- isomers of cotinine were initially evaluated in an extensive profiling screen and found to be relatively inactive across a wide range of potential pharmacologic targets. Electrophysiological studies on human α4β2 and α7 nicotinic acetylcholine receptors (nAChRs) expressed in Xenopus oocytes confirmed the absence of agonistic activity of cotinine at α4β2 or α7 nAChRs. However, a significant increase of the current evoked by a low concentration of acetylcholine was observed at α7 nAChRs exposed to 1.0 μM R-(+)- or S-(-) cotinine. Based on these results, we employed a spontaneous novel object recognition (NOR) procedure for rodents to test the hypothesis that R-(+)- or S-(-) cotinine might improve recognition memory when administered alone or in combination with the Alzheimer’s disease (AD) therapeutic agent, donepezil. While both isomers enhanced NOR performance when they were co-administered with donepezil, neither isomer was active alone. Moreover, the pro-cognitive effects of the drug combinations were blocked by methyllycaconitine and dihydro-β-erythroidine, indicating that both α7 and α4β2 nAChRs contribute to the response. These results indicate that cotinine may sensitize α7 nAChRs to low levels of acetylcholine (a previously uncharacterized mechanism) and that cotinine could be used as an adjunctive agent to improve the effective dose range of cholinergic compounds (e.g., donepezil) in the treatment of AD and other memory disorders.
Introduction

There is significant evidence from in vitro experiments as well as animal studies to suggest that the nicotine metabolite, cotinine (1-methyl-5-[3-pyridynl]-2-pyrrolidinone), might have potential as a therapeutic agent for some neurologic and psychiatric disorders including Alzheimer’s disease (AD) and schizophrenia (see reviews, Terry et al., 2005; Echeverria and Zeitlin, 2012). For example, in studies relevant to AD, cotinine has been shown to improve the survival of differentiated PC12 cells deprived of nerve growth factor (Buccafusco and Terry, 2003), as well as primary cortical neurons exposed to toxic concentrations of the amyloid beta (Aβ) peptide or glutamate (Burgess et al., 2012; Gao et al., 2014). Cotinine has also been shown to improve working/short term memory performance in monkeys (Terry et al., 2005), to prevent memory loss in transgenic (Tg) 6799 Alzheimer’s disease mice, as well as to stimulate the Akt/GSK3β pathway and reduce Aβ aggregation in their brains (Echeverria et al., 2011; Patel et al., 2014). In animal studies more closely related to schizophrenia, cotinine improved deficits in prepulse inhibition (PPI) of the acoustic startle response in rats in three pharmacologic impairment models (Terry et al., 2005), it attenuated the deficits of sustained attention in rats induced by the NMDA receptor antagonist MK-801 (Terry et al., 2012), and it improved deficits in working/short term memory in monkeys produced by the NMDA antagonist ketamine (Buccafusco and Terry 2009). In recent experiments in mice subjected to prolonged restraint (a chronic stress model) cotinine had antidepressant-like properties and it and reduced cognitive-impairment and synaptic loss in the hippocampus and prefrontal cortex (Grizzell et al., 2014).

As a potential therapeutic agent for humans, cotinine has several advantages over nicotine including a superior safety profile (Hatsukami, et al., 1997), a much longer half-life (Benowitz, 1996) and a lower risk of abuse (Rosecrans 1979). While cotinine is (structurally)
very closely related to nicotine, it is unclear if the positive actions described above are related to pharmacologic effects at nicotinic acetylcholine receptors (nAChRs). The literature available on the effects of cotinine in nAChR binding experiments and in vitro functional assays, suggests that it is a low affinity ligand at heteromeric and homomeric nAChRs with weak agonist effects (Abood et al., 1981; Sloan et al., 1984; Anderson and Arneric, 1994; Briggs and McKenna; 1998; Dwoskin et al., 1999; Vainio and Tuominen, 2001; O’Leary et al., 2008). Recently it has been speculated that cotinine might serve as a positive allosteric modulator of nAChRs (see Grizzell and Echeverria, 2014), however, we are unaware of any publications where this hypothesis has been directly tested.

The purpose of the study described here was, therefore, to further elucidate the pharmacologic effects of cotinine. Both the R-(+)- and S-(-)- isomers of cotinine were first screened across more than 70 neurotransmitter receptors, transporters, ion channels, and enzymes. Subsequent experiments using a well-described in vitro model system were conducted to further investigate the electrophysiological effects of both isomers of cotinine at α4β2 and α7 nAChRs. Based on the results of these experiments where both the R-(+)- and S-(-)- isomers of cotinine enhanced the response to acetylcholine (ACh) at α7 nAChRs (see Results section), we designed a series of experiments in rodents to test the hypothesis that the isomers of cotinine might improve recognition memory when administered alone or that they might amplify the pro-cognitive effects of the commonly prescribed AD-cholinergic agent, donepezil.

Methods

Pharmacological Activity of R-(+)- and S-(-)-Cotinine (in vitro)

The R-(+)- and S-(-)- isomers of cotinine were screened at a single concentration (10 μM) across more than 70 neurotransmitter receptors, transporters, ion channels, and enzymes by
Caliper Life Sciences (Hanover, MD). Binding or activity was determined according to standardized conditioned and validated protocols with reference standards included as an integral part of each assay. Details of each assay condition can be accessed through Caliper's web site at www.caliperls.com.

**Electrophysiological recordings**

Electrophysiological experiments were carried out with human α7 and α4β2 nAChRs expressed in *Xenopus* laevis oocytes. Oocytes were prepared, injected with cDNA encoding α7 nAChR subunits, and recorded using standard procedures (Hogg et al., 2008). Briefly, ovaries were harvested from *X. laevis* females that were deeply anesthetized by cooling at 4 °C and with tricaine mesylate (3-aminobenzoic acid ethyl ester, methane sulfonate salt, 150 mg/l). Small pieces of ovary were isolated in sterile Barth solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 10 mM HEPES, 0.82 mM MgSO4·7H2O, 0.33 mM Ca(NO3)2·4H2O, and 0.41 mM CaCl2·6H2O, pH 7.4) and supplemented with 20 μg/ml kanamycin, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Injections of cDNAs encoding for the receptors were performed in at least one hundred oocytes using an automated injection device (Roboinject, Multi Channel Systems, Reutlingen, Germany); and receptor expression was examined at least two days later. Oocytes were impaled with two electrodes filled with 3 M KCl, and their membrane potentials were maintained at −80 mV throughout the experiment. All recordings were performed at 18 °C and cells were superfused with OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1.8 mM CaCl2·2H2O, and 1.8 mM MgCl2·6H2O, pH 7.4). Currents were recorded using an automated process equipped with standard two-electrode voltage-clamp configuration (HiClamp, Multi Channel Systems). The principle of this system differs from standard electrophysiology because instead of applying the compound in the perfusion, the oocyte is moved into a well from a 96
wells microtiter plate containing the desired solution. Data were captured and analyzed using Matlab (Mathworks, Inc., Natick, MA) or Excel (Microsoft, Redmond, WA) software. ACh and the isomers of cotinine were prepared as concentrated stock solutions in water and then diluted in the recording medium to obtain the desired test concentrations. All experiments were carried out using three or more cells.

**Animal Care**

All animal procedures employed during this study were reviewed and approved by the Institutional Animal Care and Use Committee and are consistent with AAALAC guidelines. Measures were taken to minimize pain or discomfort in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. Significant efforts were also made to minimize the total number of animals used while maintaining statistically valid group numbers.

**Study Subjects**- Male albino Wistar rats (Harlan Sprague-Dawley, Inc.) approximately two months old were housed in pairs in a temperature controlled room (25°C), maintained on a standard 12-hour light/dark cycle with free access to food (Teklad Rodent Diet 8604 pellets, Harlan, Madison, WI) and water.

**Behavioral testing**

**Spontaneous Novel Object Recognition (NOR) Task**

The NOR task was adapted from Ennaceur and Delacour (1988) as we have published previously (Callahan et al, 2014). Briefly, test subjects were acclimated to laboratory conditions (i.e., tail marking, daily handling, and weighing) for at least 3 days prior to experimentation. During experimentation, the animals were transported to the laboratory and acclimated for 30 min prior
to initiating the experimental phase; the animals remained in the laboratory for 15 min following study completion.

**Habituation** - The animals were acclimated, weighed, and individually placed in a dimly lit (10 lux) training/testing environment (an opaque plastic chamber, 78.7 cm × 39.4 cm × 31.7 cm with bedding on the floor) for 10 min of chamber exploration. The NOR chamber was placed on a table positioned along the short wall of the laboratory. HVAC ventilation provided masking noise to reduce any extraneous background noise, and there were no room orienting cues or wall-mounted visual cues (except for the small B/W camera positioned above the NOR chamber). At the beginning of each series of NOR experiments, fresh bedding material was placed in the chamber prior to habituation and allowed to become saturated with animal odors. Animal droppings were removed between experimental sessions, however, the same bedding remained in the chamber for remainder of the each study (i.e., during training and testing), thus preventing any specific olfactory cues over the course of experimentation.

**Training trial** - Twenty-four hours after the habituation session, the animals were acclimated, weighed, and injected with test compound (drug or vehicle) and after the appropriate pretreatment interval, placed in the chamber with their nose facing the center of a long wall and allowed to explore two identical objects for 10 min. The animal’s behavior was observed and recorded on videotape via a camera located 69 cm above the chamber; the investigator sat quietly 10–15 ft. away from the NOR chamber.

**Test trial** – Initially, retention (delay) intervals ranging from 1-48 hr were evaluated for effects on the recognition of a novel object (see Fig 4). Subsequently a delay interval that reliably produced complete forgetting (48 hr) was used throughout the rest of the behavioral studies. In the NOR task, two objects, one object identical to training (familiar) and a novel
object were placed in the chamber, and the animal was allowed to explore the objects for 5 min. Experimental objects to be discriminated were a plastic multicolored Duplo-Lego block configured tower (12 cm in height, 6 cm in width) paired with a ceramic conical-shaped green Christmas tree salt/pepper shaker (12 cm in height, 5 cm in diameter); all objects existed in duplicate. The objects were placed 19.3 cm from the sides of the two short walls and 19.3 cm from the sides of the long walls of the chamber; distance between the two objects was approximately 40 cm. The role of familiar and novel object as well as chamber position of object was randomly assigned across subjects and treatments, and objects were cleaned between sessions with a dilute 50 % ETOH solution to eliminate olfactory cues. Object exploration occurred when the animal directed its nose to the object at a distance of $\leq 2$ cm and/or touching it with its nose, rearing up against the object to investigate if the object was also considered exploration, whereas physically climbing on the object, using the object to support itself while rearing to investigate the chamber arena or digging at the base of the objects, was not considered appropriate object exploratory behavior. The primary behavioral measure was time (s) spent investigating each object. A discrimination index ($d_2$) was calculated on each test trial and was defined as the difference in time spent exploring the novel and familiar objects divided by the total exploration time for both objects: $d_2$ index = (novel – familiar)/(novel + familiar). This measure is considered as an index of recognition memory and takes into account individual differences in the total amount of exploration time. For data inclusion, the rat had to explore each individual object at least 4 sec and spend a minimum of 12 sec of total object exploration. Experimental groups contained 6-9 rats per treatment (or testing) condition which provided sufficient sample size to observe statistical significance. Animals were tested only once, and
object exploration time was scored live under blind testing methods (i.e., the investigator was
unaware of treatment assignment).

**Drug administration**

All compounds were prepared in physiological saline (0.9 % NaCl) and administered by
intraperitoneal (i.p) injection in a volume of 1 ml/kg. Doses refer to the weight of the salt,
except where noted. The drugs used and suppliers were: R-(+)-cotinine base (Toronto Research
Chemicals Inc, North York, ON Canada), S-(-)-cotinine (Toronto Research Chemicals Inc, North
York, ON Canada), dihydro-β-erythroidine Hydrobromide (Santa Cruz Biotechnology, Dallas,
TX), donepezil HCl (Memory Pharmaceutical Corporation, Montvale, NJ) and
methyllycaconitine citrate (Tocris Bioscience, Ellisville MO). The pretreatment interval for
donepezil and cotinine was 30 min prior to the NOR training trial. In the studies where
donepezil and cotinine were combined, donepezil was administered first followed immediately
by the specific test dose of cotinine. For the antagonist studies where MLA and DHBE were
evaluated for their ability to reverse the behavioral effects of the combination of donepezil and
cotinine, the antagonist was administered first (60 min before the NOR training trial) followed
30 min later (i.e., 30 min before the NOR training trial) with the combination of donepezil and
cotinine.

**Statistical Analyses**

Statistical analysis was performed using SigmaPlot 11.2 and statistical significance was
assessed using an alpha level of 0.05. For one and two factor comparisons, analysis of variance
(with repeated measures when indicated) was used followed by the Student Newman Keuls or
Dunnett’s method (for comparisons with vehicle controls only) for post hoc analysis. All results
are expressed as the mean (±SEM).
Results

R-(+)- and S-(-) - isomers of cotinine lack significant activity across a wide range of pharmacological targets.

Pharmacological Screen

The results of the initial pharmacological screen for the R-(+)- and S-(-) - isomers of cotinine across more than 70 neurotransmitter receptors, transporters, ion channels and enzymes are provided in Table 1. As per the standard protocols used by Caliper Life Sciences (Hanover, MD), significant activity at any of these sites was defined as the inhibition of ligand binding (or enzyme activity) by $\geq 50\%$. Using this criterion neither the R-(+)- nor the S-(-) - isomer of cotinine demonstrated significant activity at any of the targets that were evaluated, although the R-(+)- and S-(-) isomers were associated with $\sim 36\%$ and $\sim 47\%$ inhibition of ligand binding at D2 receptors, respectively.

R-(+)- and S-(-) - isomers of cotinine display no agonistic activity at human $\alpha_4\beta_2$ or $\alpha_7$ nAChRs

To evaluate the possible agonistic activity of R-(+)- or S-(-) -cotinine at human $\alpha_4\beta_2$ and $\alpha_7$ receptors, a protocol of 30 sec exposure to 3 concentrations of the compounds was designed. In addition, to test any putative antagonistic activity of cotinine on the nAChRs compound exposure was immediately followed without wash by a brief ACh test pulse (see arrows) at a concentration near the receptor EC$_{50}$. Typical currents recorded at human $\alpha_4\beta_2$ and average data obtained in 7 cells are illustrated in the upper panels of Fig 1. These data illustrate that exposure to cotinine at 1, 10 or 100 $\mu$M (see horizontal bars) evokes no detectable current and causes no significant inhibition of the subsequent ACh response. Results obtained using the same experimental protocol in cells expressing human $\alpha_7$ nAChRs are shown in the lower panel of Fig 1. Typical
currents recorded during drug exposure show that the isomers of cotinine cause no detectable activation of α7 receptors. Average amplitude of currents was obtained in 9 cells and illustrates that R-(+)- or S-(-)-cotinine causes no inhibition of the ACh responses.

**Sustained exposure to the S-(-) - isomer of cotinine antagonizes the ACh-evoked current in human α4β2, but not α7 nAChRs.**

To probe a possible longer term effect of cotinine, cells expressing α4β2 receptors were incubated for 48 hours in presence of 10 μM S-(-)-cotinine and the amplitude of the current evoked by 1 mM ACh was subsequently tested. The average amplitude of current cells exposed to 10 μM cotinine was 13.3 ± 2.4 μA (n = 18) whereas the average amplitude recorded in sibling oocytes recorded at the same time but not exposed to cotinine was 25.51 ± 4.2 μA (n = 13). These data suggest that sustained exposure to cotinine reduces the amplitude of the ACh-evoked response and that cotinine might interact with the α4β2 receptors although on a slow time course. Experiments conducted in cells expressing the α7 nAChR with the S-(-) isomer of cotinine using the 48 hour incubation protocol in the presence of 10 μM of compound revealed no significant difference attributable to cotinine. Namely, cells incubated for 48 hr with 10 μM S-(-) cotinine showed on average a current of 5.85 ± 0.28 μA (n = 9) when exposed to 1 mM ACh whereas control cells responded with 6.28 ± 1.37 (n = 7). These data indicate that sustained exposure to S-(-) cotinine causes no significant modification of the ACh-evoked currents in human α7 nAChRs.

**R-(+) - and S-(-) - isomers of cotinine enhance the ACh-evoked current in human α7 nAChRs.**

As has been shown previously, in some conditions, low concentrations of agonist can yield an unusual potentiation of ACh-evoked currents at α7 nAChRs (Wallace et al., 2010; Prickaerts...
2012). Thus, we tested if this protocol of irregular stimulation of the receptor would allow us to
detect a possible interaction of the cotinine isomers with nAChRs. This protocol is designed to
mimic the fact that neurons do not discharge in a sustained and continuous manner, but rather in
bursts of different durations. Cells are exposed first at regular intervals to brief ACh test pulses
to assess the stability of their responses to the ACh test pulse. After this first phase, cells are
exposed to a sustained concentration of cotinine and the ACh-evoked currents are measured for 6
minutes. Stimulation by ACh is then suspended for 8 minutes to mimic a period of silence in
neuronal activity. The effect of cotinine on the ACh-evoked current is then examined by a brief
ACh test pulse. The process is repeated a second time before returning to control conditions. As
shown in Fig 2A, only minor variations in the amplitude of the ACh-evoked current was
observed in cells exposed to control conditions which indicates that brief exposure to 40 μM
ACh causes no major desensitization of the receptors that could be revealed by a longer time
interval between applications. Strikingly different results were observed when testing the effects
of R-(+)- or S-(−)-cotinine with a significant increase of the ACh-evoked current (Fig 2B and
2C). Typical currents evoked by 40 μM ACh at human α7 receptors using this protocol are
illustrated in Fig 2. Average results obtained using this same experimental protocol obtained for
R-(+)- or S-(−)-cotinine at α4β2 and α7 nAChRs are shown in Fig 3. Surprisingly, exposure to
1 μM R-(+)- or S-(−)-cotinine caused a significant enhancement of the ACh-evoked current at
the α7 receptor whereas the same experimental condition yielded no significant modification of
the α4β2 response. To examine the concentration dependency of the α7 potentiation cells were
exposed either to 0.1 or 10 μM R-(+)- or S-(−)-cotinine using the same experimental paradigm
and are summarized (with the response to 1.0 μM concentrations of the isomers) in the histogram
in Fig 4. Collectively, these data suggest that both the R-(+)- and S-(−) isomers of cotinine can
modulate the ACh-evoked current at the α7 nAChRs with a maximal efficacy at about 1 μM and should yield functional differences in vivo.

**Performance of a rodent task of recognition memory is delay dependent.**

The effects of different delay intervals in the NOR task (A/B retention sessions) are provided in Fig 5. The individual exploration times of the novel and familiar objects are illustrated in Fig 5A with the calculated discrimination (d2) ratio’s illustrated as scatter plots and histograms in Figs 5B and 5C, respectively. As shown, there was a delay-dependent decrease in preference for the novel object with subjects displaying complete forgetting of the familiar object at the 24 and 48 hr time point with discrimination (d2) ratios near zero. Statistical analysis of exploration times revealed the following: main effect of delay F(4,35)=0.86, p=0.50, object type (F(1,35)= 171.57, p<0.001), delay by object type interaction (F(4,35)= 30.59, p<0.001. Post hoc analysis indicated that there was a significant preference for the novel object (i.e., versus the familiar object, p<0.001) at the 1, 3, and 6 hr delays, but that this preference was lost at the longer delays. This (delay-related) effect on preference for the novel object was also evident when d2 ratios were analyzed, F(4,35)=41.40, p<0.001 (see Fig 5 for the significant differences between individual delays).

**Donepezil is associated with dose-dependent improvements in recognition memory**

The effects of the AD treatment, donepezil in the NOR task (A/B retention sessions) after a 48 hr retention interval are provided in Fig 6. The individual exploration times of the novel and familiar objects are illustrated in the main figure with the calculated discrimination (d2) ratio’s illustrated in the inset. As shown, donepezil was associated with a dose-dependent increase in preference for the novel object, main effect of dose F(3,29)=4.0, p=0.017, object type (F(1,29)= 74.9, p<0.001), dose by object type interaction (F(3,29)= 18.2, p<0.001. Post hoc analysis
indicated that the 1.0 and 2.5 mg/kg doses of donepezil were associated with a significant preference for the novel object (p<0.001 versus familiar). This (dose-related) effect of donepezil was also observed when the d2 ratios were analyzed, F(3,29)=19.0, p<0.001 (see Fig 6 for the significant dose-related difference in D2 ratios).

R-(+) - and S-(-) - isomers of cotinine (administered alone) do not affect performance of the NOR task.

The effects of different doses (0.1-10.0 mg/kg) of R-(+)-cotinine (A) and S-(-)-cotinine (B) in the NOR task (A/B retention sessions) after a 48 hr retention interval are provided in Fig 7. The individual exploration times of the novel and familiar objects are illustrated in the main Figs with the calculated discrimination (d2) ratio’s illustrated in the Fig insets. There were no significant effects of either isomer of cotinine at any of the doses that were evaluated (main effects of dose and the dose by object type interactions, p>0.05).

R-(+) - isomer of cotinine enhances the effects of a subthreshold dose of donepezil on NOR performance.

In these experiments, several doses of R-(+)-cotinine were combined with a subthreshold dose of donepezil (i.e., 0.5 mg/kg) operationally defined as a dose that did not significantly affect NOR performance in prior studies. The combined drug effects on the individual exploration times of the novel and familiar objects are illustrated in the main part of Fig 8 with the calculated discrimination (d2) ratio’s illustrated in the inset. Statistical analysis of object exploration revealed the following, main effect of treatment F(5,42)=2.0, p=0.11, object type (F(1,42)= 33.6, p<0.001), treatment by object type interaction (F(5,42)= 3.76, p=0.007. Post hoc analysis indicated that the combinations of R-(+)-cotinine 3.0 and 10.0 mg/kg with donepezil 0.5 mg/kg were associated a significant preference for the novel object (p<0.01 versus familiar). This effect...
was also observed in the statistical analysis of d2 ratios, main effect of treatment, F(5,42)=5.25, p<0.001 (see Fig 8 for the significant treatment-related difference in D2 ratios).

**Selective nicotinic antagonists block the positive effects of R-(+)- cotinine plus donepezil on NOR performance.**

In these experiments (Fig 9), test subjects were pretreated with either the α7-selective nAChR antagonist methyllycaconitine (MLA) (A) or the α4β2 selective nAChR antagonist dihydro-β-erythroidine (DHβE) (B) before administering an active dose combination of R-(+)-cotinine and donepezil (identified in the previous set of studies). The individual exploration times of the novel and familiar objects are illustrated in the main portion of the figures with the calculated discrimination (d2) ratio’s illustrated in the insets. For the MLA experiments, the following statistical results were obtained, main effect of treatment F(2,21)=0.08, p=0.92, object type (F(1,21)= 18.7, p<0.001, treatment by object type interaction (F(2,21)= 7.10, p=0.004. Post hoc analysis indicated that the combination of R-(+)- cotinine 10.0 mg/kg with donepezil 0.5 mg/kg was associated with a significant preference for the novel object (p<0.001 versus familiar), and that MLA 3.0 mg/kg blocked this effect. The same effect was observed when the d2 ratios were analyzed, main effect of treatment F(2,21)=9.21, p=0.001 (see Fig 9 for the significant treatment-related difference in D2 ratios). For the DHβE experiments, the following statistical results were obtained, main effect of treatment F(2,21)=0.91, p=0.42, object type (F(1,21)= 45.17, p<0.001, treatment by object type interaction (F(2,21)= 24.15, p<0.001. Post hoc analysis indicated (again) that the combination of R-(+)- cotinine 10.0 mg/kg with donepezil 0.5 mg/kg was associated with a significant preference for the novel object (p<0.001 versus familiar), and that DHβE 3.0 mg/kg blocked this effect. The same effect was observed when the d2 ratios were
analyzed, main effect of treatment $F(2,21)=16.46$, $p<0.001$ (see Fig 9 for the significant treatment-related difference in D2 ratios).

S-(-)- isomer of cotinine enhances the effects of a subthreshold dose of donepezil on NOR performance.

In the next set of experiments, several doses of S(-)-cotinine were combined with a subthreshold dose of donepezil. The combined drug effects on the individual exploration times of the novel and familiar objects are illustrated in the main part of Fig 10 with the calculated discrimination (d2) ratio’s illustrated in the inset. Statistical analysis of object exploration revealed the following, main effect of treatment $F(5,42)=2.5$, $p=0.045$, object type ($F(1,42)=66.81$, $p<0.001$, treatment by object type interaction ($F(5,42)=9.44$, $p<0.001$. Post hoc analysis indicated that the combinations of S(-)- cotinine 1.0, 3.0 and 10.0 mg/kg with donepezil 0.5 mg/kg were associated with a significant preference for the novel object ($p<0.001$ versus familiar). This effect was also observed in the statistical analysis of d2 ratios, main effect of treatment, $F(5,42)=5.20$, $p<0.001$ (see Fig 10 for the significant treatment-related difference in D2 ratios).

Selective nicotinic antagonists block the positive effects of S-(-)- cotinine and donepezil on NOR performance.

As in the case of the R-(+) isomer of cotinine, in subsequent experiments (Fig 11), we pretreated test subjects with either MLA (A) or DHβE (B) before administering an active dose combination of R-(−)-cotinine and donepezil. The individual exploration times of the novel and familiar objects are illustrated in the main portion of the figures with the calculated discrimination (d2) ratios illustrated in the inset. For the MLA experiments, the following statistical results were obtained, main effect of treatment $F(2,21)=0.74$, $p=0.49$, object type ($F(1,21)=24.0$, $p<0.001$, treatment by object type interaction ($F(2,21)=6.84$, $p=0.005$. Post hoc analysis indicated that the
Combination of S-(-) -cotinine 10.0 mg/kg with donepezil 0.5 mg/kg was associated with a significant preference for the novel object (p<0.001 versus vehicle), and that MLA 3.0 mg/kg blocked this effect. The same effect was observed when the d2 ratios were analyzed, main effect of treatment F(2,21)=7.27, p=0.004 (see Fig 11 for the significant treatment-related difference in D2 ratios). For the DHβE experiments, the following statistical results were obtained, main effect of treatment F(2,21)=0.86, p=0.44, object type (F(1,21)= 89.76, p<0.001), treatment by object type interaction (F(2,21)= 41.90, p<0.001. Post hoc analysis indicated that the combination of S-(-) -cotinine 10.0 mg/kg with donepezil 0.5 mg/kg was associated with a significant preference for the novel object (p<0.001 versus vehicle), and that DHβE 3.0 mg/kg blocked this effect. The same effect was observed when the d2 ratios were analyzed, main effect of treatment F(2,21)=41.15, p<0.001 (see Fig 11 for the significant treatment-related difference in D2 ratios).

Discussion

The most notable results of this study can be summarized as follows: 1) the R-(+)- and the S-(-) isomer of cotinine appear to be relatively inactive across a wide range of potential pharmacologic targets including those that might have relevance to neuropsychiatric disorders or be associated with adverse drug reactions, 2) however, in electrophysiological studies, both isomers of cotinine, significantly increased responses evoked by low concentrations of acetylcholine in oocytes expressing the human α7 nAChR, 3) in the behavioral (NOR) studies, both isomers of cotinine enhanced NOR performance when co-administered with a subthreshold dose of donezepil, while neither isomer was active alone, 4) the positive effects of the combinations of the isomers of cotinine and donepezil on NOR performance were blocked by
MLA and DHβE indicating that both α7 and α4β2 nAChRs contribute to this specific behavioral response.

The electrophysiological studies conducted in human receptors expressed in *Xenopus* oocytes indicate without ambiguity that the R-(+)- and the S-(-) isomers of cotinine (when administered in brief pulses of 30 sec) do not evoke inward currents at either the α4β2 or α7 receptor, nor do they cause any significant inhibition of these receptors. These data are in agreement with previously reported studies (Briggs, 1998). This suggests that in vivo at concentrations relevant to those commonly observed in smokers’ blood (e.g., 250-300 ng/ml or 1.4-1.7 μM, see Hukkanen et al., 2005), cotinine does not act as an agonist or antagonist at α4β2 or α7 receptors. We did, however, observe that sustained (48 hr) exposure to S-(-) cotinine reduced the amplitude of the ACh-evoked response in α4β2, but not α7 receptors, thus indicating that cotinine might interact with α4β2 receptors although on a slow time course. As the reduction of the amplitude of current can occur through different mechanisms it would be too speculative to interpret these data further at this point.

Additional electrophysiological experiments were conducted with a low concentration of ACh applied at irregular intervals, using a protocol that unveiled the co-agonist activity of RG 3487 (Wallace et al., 2010) and EVP-6124 at α7 nAChRs (Prickaerts et al., 2012). Experiments conducted with this protocol at human α4β2 receptors showed no significant effect of cotinine (see Fig. 2), whereas in the same experimental conditions, a major effect was observed at α7 nAChRs. These data clearly indicate that exposure to 1 μM R-(+)- or S-(-)- cotinine enhances the response of α7 receptors to 40 μM ACh, an effect that could explain the pro-cognitive effects observed with cotinine treatment in NOR experiments. These results are suggestive of an interaction of cotinine with the α7 binding site which is supported by the observation that
cotinine acts, albeit at high concentration, as an agonist at the L9'T mutant of α7 (Briggs et al., 1999).

While there was some evidence in both the electrophysiological experiments with α7 nAChRs (at the 1 μM concentration, see Fig 4) and in the NOR experiments where cotinine was combined with donepezil (see Figs 8 and 10, respectively) that the responses to the R-(-) isomer might be somewhat lower in magnitude when compared with the S-(-) isomer, there was little evidence of clear (i.e., logarithmic) differences in stereospecificity detected in this study. While these observations are bit difficult to interpret, the behavioral effects might imply that the compounds have subtle effects across multiple targets (i.e., at different nAChR subtypes or at other receptors not yet identified). The fact that both an α7 nAChR antagonist (MLA) and an α4β2 antagonist (DHβE) blocked the effects of the drug combination does imply that both of these nAChR subtypes contribute to the capacity of the cotinine isomers to amplify the effects of donepezil on synaptic acetylcholine levels. Interestingly, in a sensory inhibition paradigm in DBA/2 mice, blockade of α4β2 nAChRs with DHβE or α7 nAChRs with α-bungarotoxin blocked the increase in the conditioning amplitude and sensory gating improvements (respectively) induced by cotinine (Robb et al., 2013; Wildeboer-Andrud et al., 2014), indicating that both nAChR subtypes contribute to these in vivo responses as well.

While it is always a challenge to reconcile data obtained in vitro with results obtained in vivo using very different approaches, results obtained from brain slice experiments are shining a new light on our understanding of the role of nAChRs in brain circuits (Arroyo 2014; Bloem 2014). These studies clearly show that α7 and α4β2 nAChRs are expressed in different layers of the cortex, but that some interneurons express both types of receptors as shown by MLA and DHβE inhibition (Bennett 2012). Similar observations were also reported for recordings in the
hippocampus (Alkondon 1993; Alkondon 2001; Christophe 2002). Moreover, in this work it was shown that treatment with an acetylcholinesterase inhibitor (ambenonium dichloride) caused a marked slowing down of the slow phase of the response time course of synaptic potentials evoked by basal forebrain stimulations. These results were interpreted as reflecting the slowing down of degradation of ACh which caused a more prolonged response at \( \alpha_4\beta_2 \) receptors that was sensitive to DH\( \beta E \) (Bennett 2012). These dual phases in the synaptic-evoked currents with \( \alpha_7 \) and \( \alpha_4\beta_2 \) receptors suggest that both the transient and phasic responses might contribute to the regulation of brain function and it was shown that \( \alpha_4\beta_2 \) responses of interneurons can cause a prolonged disynaptic inhibition of cortical neurons in the mouse forebrain (Arroyo 2012). The complexity of receptor expression with the phasic and tonic responses evoked in the same cell might reconcile the puzzling observation (noted above) that the pro-cognitive effects of cotinine were inhibited by both MLA and DH\( \beta E \). Namely, as pro-cognitive effects of cotinine are observed in the presence of a low concentration of acetylcholinesterase inhibitor, the subsequent exposure to DH\( \beta E \) might reduce the \( \alpha_4\beta_2 \) component of the response that is otherwise indispensable to unveil the enhancement of \( \alpha_7 \) activity caused by cotinine.

The ability of the isomers of cotinine to effectively increase the response to ACh at \( \alpha_7 \) nAChRs may have several implications. The \( \alpha_7 \)-nAChR has long been considered a therapeutic target in disorders like AD and schizophrenia given the deficits in \( \alpha_7 \)-nAChR protein that have been observed in the brains of patients who suffered from these disorders (Freedman et al., 1995; Burghaus et al., 2000; Guan et al., 2000). Moreover, \( \alpha_7 \) nAChRs are abundant in the hippocampus and prefrontal cortex (important structures for cognition and AD, reviewed, Gotti et al., 2007) and they modulate several calcium-dependent events in neurons including neurotransmitter release (McGehee et al., 1995; Gray et al., 1996), postsynaptic signaling (Chang
and Berg, 1999; Hefft et al., 1999) and neuronal survival (Messi et al., 1997; Berger et al., 1998). In addition, agonists of α7 nAChRs have been shown to increase the phosphorylation of ERK and CREB (signaling pathways linked long term potentiation and memory formation) in the rodent brain (Bitner et al., 2007, 2010) and to improve performance in a variety of learning and memory-related tasks in animals (for review, see Kem, 2000).

The ability of the isomers of cotinine to improve the pro-cognitive dose range of donepezil could also have important clinical implications and there is significant interest in the AD field in any strategy that might enhance the efficacy of the currently available treatments (see Riordan et al., 2011). One limitation to donepezil (and other AChEIs) is the variety of dose-limiting side effects that may prevent the administration of doses that are high enough for optimal effects on cognition. While both muscarinic acetylcholine receptors (mAChRs) and nAChRs are considered important therapeutic targets in AD, doses of AChEIs high enough to significantly improve nAChR signaling (via the increase in synaptic acetylcholine) are often accompanied by adverse reactions (e.g., nausea, vomiting, and diarrhea) that likely the result from muscarinic overstimulation (see Maelicke and Albuquerque, 2000). Accordingly, an alternative (nAChR-based) treatment strategy that would theoretically be less susceptible to adverse reactions would be to selectively activate or “sensitize” nAChRs to acetylcholine, thus allowing for lower doses of the AChEI to be used. Recently we tested an adjunctive treatment strategy which included the nicotinic “positive allosteric modulator (PAM), PNU-120596 and subthreshold doses of donepezil and found it to be effective in both aged rats and aged monkeys (Callahan et al., 2013). The results of the current study indicate that the isomers of cotinine might also serve as an important part of a similar adjunctive strategy.
The translational significance of the rodent behavioral experiments described in this report is also an important subject of discussion. Object recognition memory is one of the domains of cognition that is often impaired in aged (non-demented) individuals as well as in patients with AD (Flicker et al., 1987; Purdy et al., 2002; Schiavetto et al., 2002). The rodent NOR task has been described as a model of (non-spatial) recognition memory (Ennaceur and Delacour 1988). This form of memory is believed to consist of a recollective (episodic) and a familiarity component (Squire et al., 2004), i.e., behaviors that are demonstrated in the NOR task when subjects explore a novel object more than a familiar one. Moreover, there is considerable (albeit debated) evidence that the hippocampus (an important structure in the neuropathology of AD) is actively involved in object recognition memory in both rodents (Myhrer, 1988; Rampon et al., 2000; Broadbent et al., 2004) and humans (Reed and Squire, 1997; Squire, 1992).

In conclusion, the results of this study indicate that cotinine may sensitize α7 nAChRs to low levels of acetylcholine (a previously uncharacterized mechanism) and that both the R-(+) and S (-) isomer of cotinine could be used as part of an adjunctive treatment strategy to improve the effective dose range of cholinergic compounds (e.g., donepezil) used in AD. Our behavioral data in young rats also support the premise that neither the pro-cognitive effects of donepezil nor the donepezil-cotinine combination requires innate cholinergic deficits (as are normally associated with old age or AD), suggesting potential applications across a wide range of cognitive disorders.
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Authorship Contribution

Participated in research design: AVT, PMC, DB

Conducted experiments: PMC, DB

Performed data analysis: AVT, PMC, DB

Wrote or contributed to the writing of the manuscript: AVT, PMC, DB
References


Cholinergic Modulation of the Medial Prefrontal Cortex: The Role of Nicotinic Receptors in Attention and Regulation of Neuronal Activity.


Footnotes:

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Legends for Figures

**Fig 1.** Putative agonistic activity of cotinine at human α4β2 and α7 nAChRs. To evaluate the possible agonistic activity of R-(+)- or S-(-)-cotinine, cells expressing either the human α4β2 or α7 receptor were exposed to brief pulses of the compounds (30 sec) at three different concentrations (indicated by the horizontal bars). Current evoked by a brief ACh test pulse (30 μM for α4β2 and 200 μM for α7, indicated by the arrows) was applied immediately after cotinine exposure to evaluate a possible antagonistic activity of this compound. Typical currents obtained in a cell expressing the α4β2 are shown in the upper panel. Average results, normalized versus the ACh response recorded in control condition are shown by the histogram (n = 7). Results obtained at α7 nAChRs are shown in the lower panel and average responses were obtained on n= 9.

**Fig 2.** Sustained exposure to R-(+)- and S-(-)-cotinine at human α7 nAChRs. Probing the effects of sustained exposure to 1 μM cotinine on currents evoked by 40 μM ACh was conducted using the irregular stimulation paradigm described in Prickaerts et al., 2012. ACh-evoked currents were recorded first in control using a two minute interval between brief ACh-test pulse (40 μM, 5 s, green traces). Exposure to 1 μM cotinine was then applied (indicated by the horizontal bar above the traces) and currents evoked by 40 μM was tested at irregular intervals (red traces). Note the enhancement of the response observed after the 8 minute time period (middle trace) in the α7 nAChRs treated with both the R-(+)-isomer (A) and -S-(-)- isomer (B) of cotinine. Recovery from cotinine exposure was determined by applying the same ACh test pulses at regular intervals upon return in control (green traces). A control trace obtained without cotinine is presented in (A).
Fig 3. Average effects of sustained exposure to 1 μM R-(+) – or S-(−) –cotinine at the human α4β2 and α7 nAChRs. Experiments were conducted using the same experimental protocol shown in Figure 2. Currents were normalized to the average response recorded in control and represented in the form of histograms. Bars indicate the standard error for n=9 for S-(−) –cotinine at α4β2, and n=5 for α7 and n=6 for R-(+) –cotinine at α4β2 and n=5 for α7. Green bars indicate the ACh-evoked responses recorded in control and grey or red bars indicate the response recorded during cotinine exposure. Note the difference in scale used for the α4β2 and α7 graphs.

Fig 4. Concentration-dependent effects of sustained exposure to R-(+) – or S-(−) –cotinine at the human α7 nAChRs. Experiments were conducted using the same experimental protocol shown in Figure 2. Currents were normalized to the average response recorded in control and represented in the form of histograms. Bars indicate the standard error for n=5-6 for each isomer at α7 nAChRs.

Fig 5. Delay-dependent decrease in performance (recognition memory) by young Wistar rats in a spontaneous novel object recognition (NOR) procedure. (A) Mean (± S.E.M) exploration times of the familiar and novel objects (A/B retention sessions). (B). Scatter plots of discrimination (d2) ratios by individual rats. (C). Mean (± S.E.M) discrimination (d2) ratios illustrated as histograms. d2 ratio = (novel - familiar)/(novel + familiar). Bars represent the mean (± S.E.M.), N=8 rats per delay condition. +++p<0.001 novel vs familiar object; *** = p<0.001 sig different from the 1 hr time point; #### = p<0.001 sig different from the 3 hr time point; □□□□ = p<0.001 sig different from the 6 hr time point.
Fig. 6. Dose-related effects of donepezil by young Wistar rats on performance of a spontaneous novel object recognition task. In these experiments, donepezil (or vehicle) was administered by i.p injection 30 min before the training trial. Mean (± S.E.M) exploration times of the familiar and novel objects after 48 hr delays (A/B retention sessions) are illustrated in the main Fig. The inset illustrates the mean (± S.E.M) discrimination (d2) ratios. d2 ratio = (novel - familiar)/(novel + familiar). +++p<0.001 novel vs familiar object; *p<0.05 vs VEH. N=8-9 for each group.

Fig 7. Dose-related effects of R-(+)-cotinine (A) and S(-)-cotinine (B) in the NOR task by young Wistar rats on performance of a spontaneous novel object recognition task. In these experiments, cotinine (or vehicle) was administered by i.p injection 30 min before the training trial. Mean (± S.E.M) exploration times of the familiar and novel objects after 48 hr delays (A/B retention sessions) are illustrated in the main Figs. Insets illustrate the mean (± S.E.M) discrimination (d2) ratios. d2 ratio = (novel - familiar)/(novel + familiar). N=6 for each group.

Fig 8. Effects of co-administration of a subthreshold dose of donepezil (0.5 mg/kg, ip) plus R-(+)-cotinine (1.0-10 mg/kg, ip) on performance of a spontaneous novel object recognition task. In these experiments, donepezil (or vehicle) was administered 30 min before the training trial followed immediately by the specific test dose of cotinine. Mean (± S.E.M) exploration times of the familiar and novel objects after 48 hr delays (A/B retention sessions) are illustrated in the main Fig. Inset illustrates the mean (± S.E.M) discrimination (d2) ratios. d2 ratio = (novel - familiar)/(novel + familiar). ++ = p<0.01; +++p<0.001 novel vs familiar object * = p<0.05; ** =
Fig 9. Effects of co-administration of a subthreshold dose of donepezil (0.5 mg/kg), an active dose of R (+)-cotinine (10 mg/kg) and the nicotinic antagonists methyllycaconitine (A) and dihydro-β-erythroidine (B) on performance of a spontaneous novel object recognition task. In these experiments the antagonist was administered first (60 min before the NOR training trial) followed 30 min later (i.e., 30 min before the NOR training trial) with the combination of donepezil and cotinine. All compounds were administered by i.p. injection. Mean (± S.E.M) exploration times of the familiar and novel objects after 48 hr delays (A/B retention sessions) are illustrated in the main Figs. Insets illustrate the mean (± S.E.M) discrimination (d2) ratios. d2 ratio = (novel - familiar)/(novel + familiar). +++p<0.001 novel vs familiar object; *** = p<0.001 sig different from vehicle response. N=8 for each group. COT = cotinine; DON = donepezil; DHβE = dihydro-β-erythroidine; MLA= methyllycaconitine.

Fig 10. Effects of co-administration of a subthreshold dose of donepezil (0.5 mg/kg, i.p.) plus S(-)-cotinine (1.0-10 mg/kg, i.p.) on performance of a spontaneous novel object recognition task. In these experiments, donepezil (or vehicle) was administered 30 min before the training trial followed immediately by the specific test dose of cotinine. Mean (± S.E.M) exploration times of the familiar and novel objects after 48 hr delays (A/B retention sessions) are illustrated in the main Fig. Inset illustrates the mean (± S.E.M) discrimination (d2) ratios. d2 ratio = (novel - familiar)/(novel + familiar). +++p<0.001 novel vs familiar object * = p<0.05; ** = p<0.01 sig
different from vehicle response. \# = p<0.05 sig different versus DON alone response. N=8 for each group. COT = cotinine; DON = donepezil.

**Fig 11.** Effects of co-administration of a subthreshold dose of donepezil (0.5 mg/kg), an active dose of S (-)- cotinine (10 mg/kg) and the nicotinic antagonists methyllycaconitine (A) and dihydro-\(\beta\)-erythroidine (B) on performance of a spontaneous novel object recognition task. In these experiments the antagonist was administered first (60 min before the NOR training trial) followed 30 min later (i.e., 30 min before the NOR training trial) with the combination of donepezil and cotinine. All compounds were administered by i.p. injection. Mean (± S.E.M) exploration times of the familiar and novel objects after 48 hr delays (A/B retention sessions) are illustrated in the main Figs. Insets illustrate the mean (± S.E.M) discrimination (d2) ratios. d2 ratio = (novel - familiar)/(novel + familiar). +++p<0.001 novel vs familiar object; *** = p<0.001 sig different from vehicle response. COT = cotinine; DON = donepezil; DH\(\beta\)E = dihydro-\(\beta\)-erythroidine; MLA= methyllycaconitine. N=8 for each group.
Table 1. In Vitro Binding/Activity Profile of R-(+)- and S-(-)- Cotinine

<table>
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<tr>
<th>Target</th>
<th>% Inhibition at 10 μM</th>
<th>R-(+)-Cotinine</th>
<th>S-(-) Cotinine</th>
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<tr>
<td>Adenosine Transporter (h)</td>
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<tr>
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**Ion Channels**

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<td>(Dihydropyridine Site)</td>
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</table>

**Second Messengers**

| Nitric Oxide, NOS (Neuronal-Binding) | -7.02% | 3.35% |

**Prostaglandins**

| Leukotriene, LTB4 (BLT) | 6.08% | 6.06% |
| Leukotriene, LTD4 (CysLT1) | 1.87% | 5.42% |
| Thromboxane A2 (h) | -16.05% | -4.82% |

**Brain/Gut Peptides**

| Angiotensin II, AT1 (h) | 8.14% | 6.73% |
| Bradykinin, BK2 | -0.84% | -1.20% |
| Endothelin, ET-A (h) | -3.19% | 5.19% |
| Neurokinin, NK1 | 6.48% | 17.49% |
| Neuropeptide, NPY2 (h) | -14.58% | -2.44% |

**Enzymes**

| Esterase, Acetylcholine | -5.12% | -1.22% |
| Phosphodiesterase, PDE3A1A (h) | 8.72% | 3.36% |
| Phosphodiesterase, PDE5A1 (h) | 5.21% | 3.41% |

**Enzymes, Kinases**

| Kinase, Protein, PKA (h) | 13.42% | 13.63% |
| Kinase, Protein, PKCa (h) | -12.42% | -6.87% |

Values are expressed as the percent inhibition of specific binding or activity and represent the average of replicate tubes at each of the concentrations tested.