Inhibition and Inactivation of Presynaptic Cholinergic Markers Using Redox-Reactive Choline Analogs

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Inhibition and inactivation of two presynaptic cholinergic "markers", choline acetyltransferase and high affinity choline transporter, has been investigated using inhibitors designed with a redox-reactive catechol tethered to a quaternary ammonium group. Two quaternary ammonium alkyl-substituted catechols, 3[(trimethylammonio)methyl]catechol (TMC, 1) and *N*,*N*-dimethylepinephrine (catecholine, 2) were shown to bind weakly and noncompetitively to bovine choline acetyltransferase yet inactivated the enzyme in a time course consistent with the involvement of early intermediates in the spontaneous oxidation of these catechols. Both agents also inhibited high-affinity choline uptake. The time course of TMC and catecholine spontaneous oxidation-dependent inactivation of high affinity choline uptake sites was slower than, if it occurred at all, the spontaneous degradation of measurable choline transport in synaptosomes. When compared with inhibition of uptake of other neurotransmitters, it was shown that catecholine demonstrated more selectivity than TMC toward inhibition of choline transport. K_m (μM) and V_{max} (pmol/min per mg of protein) were measured for high affinity transport of choline, dopamine, and serotonin and were observed to be $K_m = 2.04 \pm 0.31$, $V_{max} = 22 \pm 1$; $K_m = 1.4$, $V_{max} = 53$; and $K_m = 0.15$, $V_{max} = 23$, respectively, in good agreement with published literature values. K_i's (mM) for catecholine and TMC, calculated from experimentally determined IC_{50}'s, were for catecholine 0.13 ± 0.06, 0.53 ± 0.09, and 0.39 ± 0.10, and for TMC 0.06 ± 0.03, 0.09 ± 0.03, and 0.09 ± 0.08, for choline, dopamine, and serotonin transport, respectively. *In vivo* studies using catecholine suggest that this compound impairs learning ability associated with long-term memory. Thus, catecholine represents a lead compound in a potential series of redox-reactive choline analogs, which may become useful irreversible antagonists of the critical cholinergic macromolecular targets underlying cholinergic hypofunction in disorders such as Alzheimer's disease.

Introduction

Selective cholinergic neuronal inactivation is useful in the dissection of cholinergic contributions in neurobiologically complex systems *in vitro* and in the study of biochemical mechanisms underlying consciousness and cognition. In particular, selective inactivators of macromolecules, which are key to the function of the presynaptic cholinergic neurons, would be useful as tools for identifying and manipulating neurotransmitter pathways and for studying the mechanisms involved in cholinergic disorders. Such selective cholinergic toxins can be used to develop animal models for disorders associated with cholinergic deficit, such as Alzheimer's disease.[1,2]

Alzheimer's disease is characterized behaviorally by severe impairment in cognitive function and neuropathologically by the appearance of neuritic plaques and neurofibrillary tangles.[3] It has been shown that in Alzheimer's patients there is a severe reduction of functional neurons in the nucleus basalis of Meynert, the primary source of cholinergic input into the cortex.[4] In the cerebral cortex and hippocampus of patients with Alzheimer's disease, there is a dramatic reduction of acetylcholine levels, acetylcholinesterase, choline acetyltransferase, and high-affinity choline uptake.[5-9] These "markers" characterize the pathology of Alzheimer's disease and primarily implicate selective destruction of the acetylcholine generating presynaptic neurons in the disease process. Among the cholinergic markers cited, the two most appropriate macromolecules to target in studies on selective inactivation are the acetylcholine synthesizing enzyme, choline acetyltransferase (ChAcT), and high-affinity choline uptake (HAChU) transporter system.

Choline acetyltransferase is known to be concentrated in the cytoplasm of the presynaptic neuron.[10] Agents designed to inhibit this enzyme need to penetrate the neuron before they can interact with the enzyme. If these agents were actively transported through the presynaptic high affinity choline uptake system, they would be accumulated in the presynaptic neurons. The ChAcT inhibitors that are effectively bound to the HAChU protein but inefficiently transported by such a mechanism are, nevertheless, very likely to inhibit choline uptake and affect the functional activity of the neuron. Thus, inhibition and/or inactivation of either or both ChAcT or HAChU will result in a decrease in acetylcholine levels in the presynaptic neuron and consequent functional inactivation of the synapse. The inhibition of ChAcT would result in depressed synthesis of acetylcholine while inhibition of HAChU would have the effect by preventing the reac-

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quisition of choline from the synaptic cleft where released acetylcholine is rapidly hydrolyzed to choline and acetic acid.

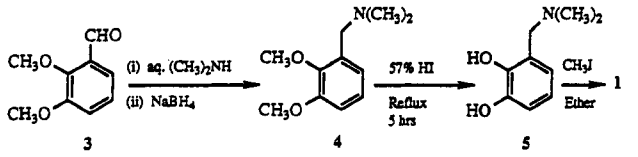
Therefore, it may be possible to design effective cholinotoxic agents which are (1) nontransportable inhibitors of HAChU, which functionally inactivate presynaptic neurons without directly inhibiting ChAcT; (2) selective ChAcT inhibitors concentrating in presynaptic cholinergic neurons through facilitated cotransport at the HAChU transport site without measurably inhibiting choline transport; or (3) slowly transportable agents which both inhibit choline uptake and directly inhibit ChAcT.

Some of the earlier efforts directed toward developing specific cholinotoxins have identified the nitrogen mustard choline analog, AF64A, as an experimentally useful compound with selectivity for presynaptic cholinergic elements.[11,12] Since then, several structural analogs of AF64A have been designed and tested.[13] Recently, halomethyl analogs of choline with high-affinity choline transport inhibitory activity have been reported.[14] Nevertheless, the search for new and more selective cholinergic inactivators is a continuing process.

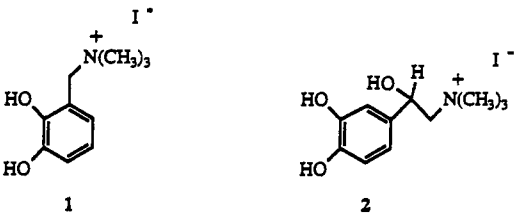
The inhibitors investigated here were quaternary ammonium alkyl-substituted catechols. These are redox-reactive agents possessing affinity for choline-binding macromolecules. One of the reagents tested has been previously shown to irreversibly inactivate neurotoxin (acetylcholine) binding sites in the nicotinic acetylcholine receptor, and both have been reported preliminarily to be inhibitors of ChAcT.[15,20] Here we have investigated their potential for selectively blocking active transport of choline and the synthesis of acetylcholine. Selective transport of quaternary ammonium alkyl-substituted catechols into the presynaptic cholinergic cells would be expected to compromise the cholinergic neuronal function. In cholinergic neurons, there are no known protective mechanisms against catechol toxicity. On the other hand, the catecholaminergic neurons are well protected against such catechols due to the presence of intraneuronal catecholamine storage granules which are believed to maintain a reducing environment (low pH, high ascorbate concentration).[16] In addition, catecholaminergic synapses possess catechol-*O*-methyl transferase, a relatively nonspecific enzyme that catalyzes the transfer of methyl groups from *S*-adenosylmethionine to the *m*- and *p*-hydroxy group of catecholamines and various other catechols.[17]

Two quaternary ammonium alkyl-substituted catechols, 3-[(trimethylammonio)methyl]catechol (TMC, 1) and *N*,*N*-dimethylepinephrine (catecholine, 2), were examined. In theory, the quaternary ammonium portion of these molecules will provide affinity for cholinergic macromolecules whereas the redox-reactive part will cause inactivation of the macromolecular sites to which these molecules are bound. The catechol fragment undergoes spontaneous oxidation leading to the formation of reactive, electrophilic quinones and reduced molecular oxygen products, and there is ample evidence that both classes of products are toxic to cells.[16] Oxidized catecholamines form indolequinones through intramolecular reactions of the amino functions, contributing significantly to the overall rate of polymerization and indiscriminant reactivity of the intermediates through which polymerization also occurs. However, the quaternary ammonium catechols, studied here, possess no capacity to react intramolecularly and polymerize through the intermediacy of indolequinones.[18]

Scheme I



Thus, the class of agents represented by 1 and 2 may be generally more selective on chemical grounds than other hydroxylated catecholamines.



This paper describes aspects of the synthesis and *in vitro* biological evaluation of two redox reactive choline analogs, 1 and 2. In addition to the inhibition and inactivation of ChAcT, catecholine (2) is shown to be a selective *in vitro* inhibitor of high-affinity choline uptake. We also report an *in vivo* study using 2, demonstrating that these agents can affect learning of a representational memory task.

Chemistry

Compound 1 was synthesized using the method reported by Nickoloff *et al.*[15] with minor modifications. As shown in Scheme I compound 5 was obtained by reductive amination of 2,3-dimethoxybenzaldehyde (3) followed by cleavage of methyl ethers with hydriodic acid. This tertiary amine was not isolated and was directly subjected to methylation with iodomethane, resulting in small pale yellow crystals with a sharp melting point which was identical to the melting point reported by Nickoloff et al.[15] The yield of recrystallized product 1 was 40% based on 2,3-dimethoxybenzaldehyde. Structure was confirmed earlier by elemental analysis.[15] Spectroscopic properties (FT-NMR and FTIR) were all consistent with the assigned structure.

Compound 2 was synthesized based on the scheme reported by LaManna et al. with minor modifications as reported in an earlier preliminary communication.[19,20] The three-step procedure, from the parent chlorodihydroxyacetophenone (6), is shown in Scheme II. The final product was not stereochemically resolved. Spectroscopic and chemical analyses were consistent with the assigned structure.

Synthesis of both these compounds was achieved with reasonably high yields. However, the steps which involved the workup procedure under basic conditions were challenging. As expected, under basic conditions the catechols undergo spontaneous oxidation followed by rapid polymerization. Thus, the extractions of the secondary amine derivatives of these catechols into ether were carried out very rapidly. The crystalline products (1, 2) were stable and could be stored at −20 °C under an argon atmosphere. Since these precautions were taken during synthesis, the agents obtained were sufficiently pure to be used for biochemical experiments.

Biological Results

(A) Choline Actyltransferase Activity. ChAcT activity was assayed using the method described by

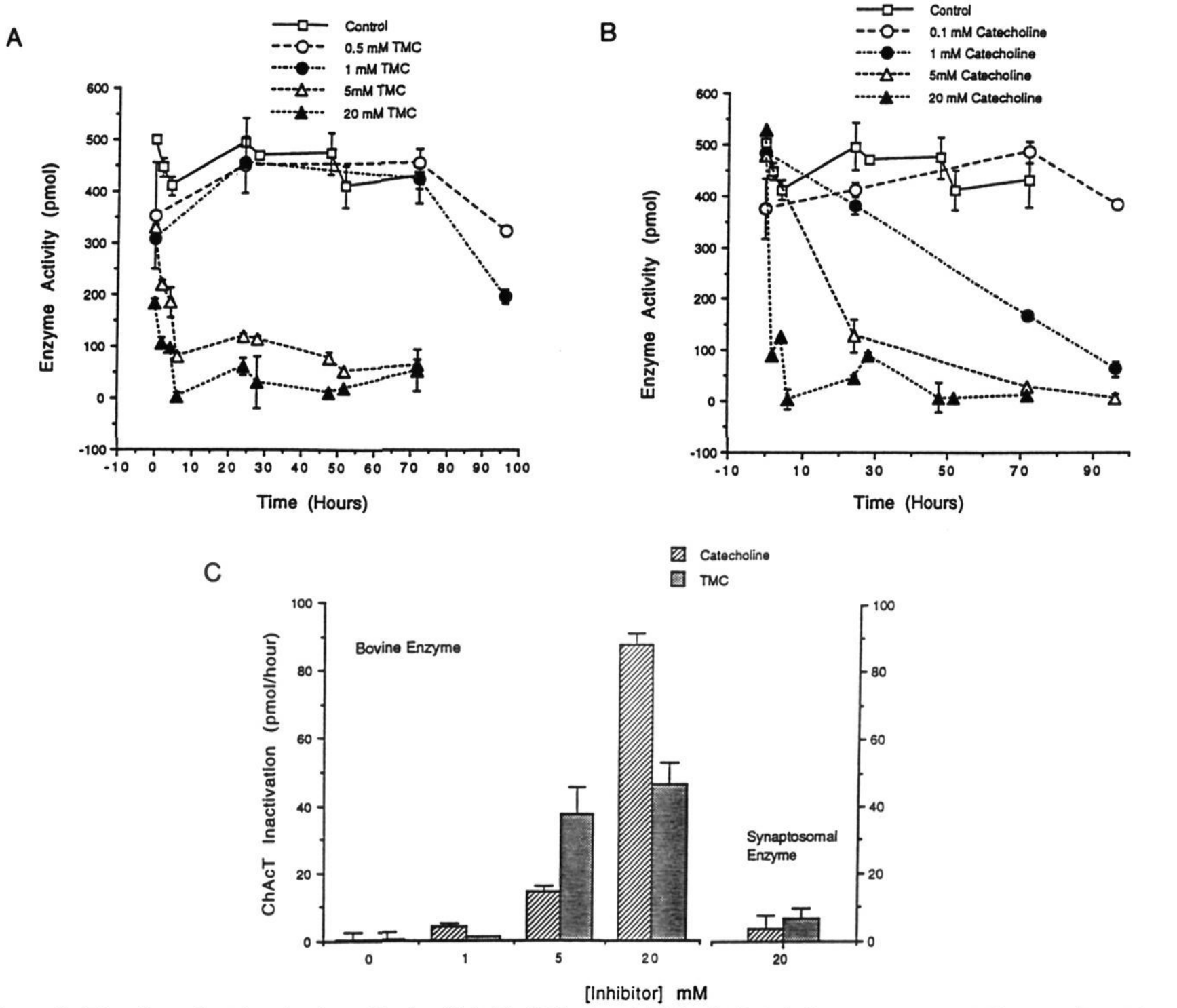
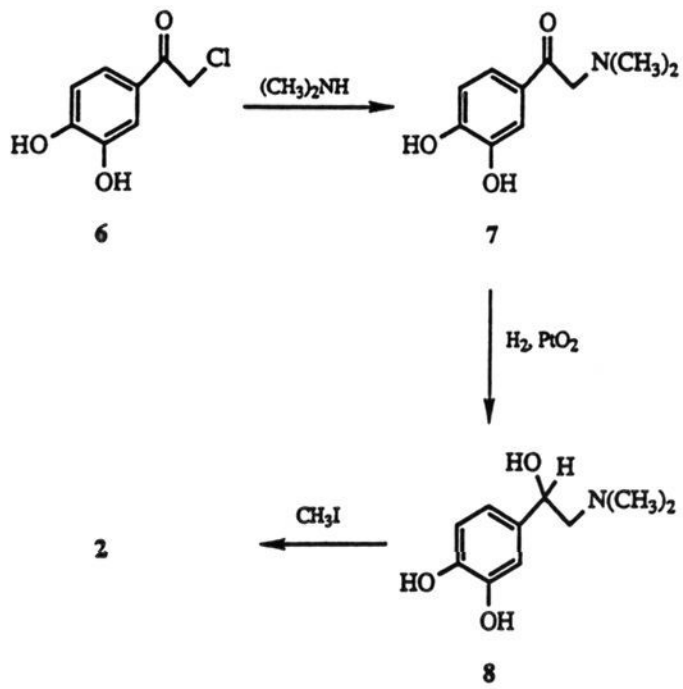


Figure 1. Time-dependent inactivation of bovine ChAcT: (A) by 1 and (B) by 2. Data is from one representative experiment (n = 2), and the values represent mean ± sem of duplicate determinations. (C) Rate of inactivation of bovine ChAcT expressed in picomoles of enzyme inactivated per hour versus inhibitor concentration for 1 and 2. At 20 mM inhibitor concentration, the inactivation rate of bovine enzyme is compared with that of rat brain synaptosomal enzyme. Values represent the mean ± sem of duplicate determinations.

Kozlowski and Arbogast.[21] Formation of [^{14}C]acetylcholine was determined by separation of product formed from [^{14}C]acetyl coenzyme A ([^{14}C]AcCoA, substrate) on Dowex 1 × 8 chloride. Physostigmine was routinely employed in ChAcT preparations suspected of acetylcholinesterase (AcChE) contamination. We previously demonstrated that purified bovine choline acetyltransferase (ChAcT) was inhibited by both 1 and 2.[20] Both inhibitors bound to enzyme sites noncompetitively with choline. The calculated K_i values for 1 and 2, respectively, were 15 ± 6 and 25 ± 4 mM, as reported earlier.

The purified enzyme was slowly inactivated at the high inhibitor concentration in a concentration-dependent manner for both the inhibitors (Figure 1). Compound 1, apparently because of relatively higher affinity for the enzyme, inactivated the enzyme more rapidly at lower concentrations (5 mM) than did catecholine. In spite of the higher inactivation rate at a low concentration, the rate of inactivation by 1 did not increase markedly when its concentration was increased. In contrast, 2 inactivated the enzyme more slowly at low concentrations, but at higher concentrations (20 mM) reached a higher rate of inactivation than that seen in 1.

Scheme II



Both 1 and 2 inactivated the rat brain synaptosomal enzyme. The rate of inactivation of the rat brain synaptosomal enzyme by 1 and 2 was found to be much slower than that of bovine ChAcT (Figure 1C). In the rat brain

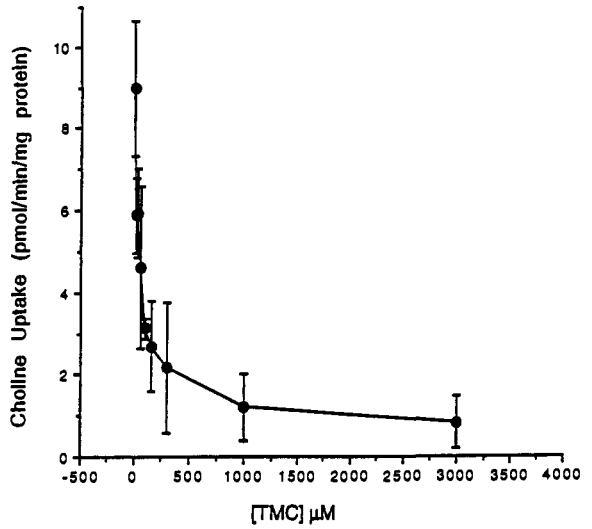


Figure 2. Measurement of high-affinity choline uptake in the presence of various concentrations of **1**. The concentration of [^{3}H]choline used was 1 μM. The values represent means ± sem for four experiments, where each determination was in duplicate.

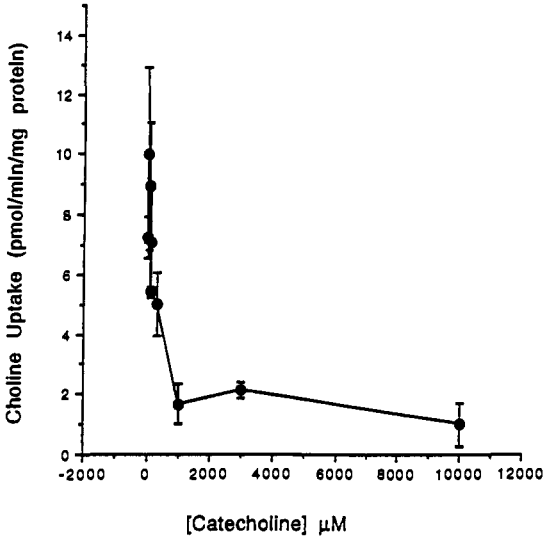


Figure 3. Measurement of high-affinity choline uptake in the presence of various concentrations of **2**. The concentration of [^{3}H]choline used was 1 μM. The values represent means ± sem for three experiments, where each determination was in duplicate.

synaptosomes, most of the enzyme is trapped intrasynaptosomally, and inhibitors were required to enter the synaptosomes before they reached the enzyme. It was likely that these charged compounds penetrate the synaptosomal membrane using the same active transport mechanism as used by the natural substrate choline. However, this process was slow and could not be accurately measured in this *in vitro* preparation. That is, transport rates were of the same order of magnitude or less than the rate of loss of viability of synaptosomal membranes. If the inhibitors are taken into the synaptosomes by sodium-dependent high-affinity choline uptake (HAChU), then they would also compete with choline for the high-affinity uptake sites, resulting in the inhibition of the intrinsically more rapid high-affinity choline uptake. Based on this argument, studies on inhibition of high-affinity choline uptake were undertaken.

(B) High-Affinity Choline Uptake. From the dependence of choline concentration on the rate of [^{3}H]-choline uptake, K_m for choline transport was estimated to be 2.04 ± 0.31 μM, which was consistent with the range of values (1.3–3.1 μM) reported in literature[22-24] for the high-affinity component of choline transport system. V_{max} for choline transport was found to be 22 ± 1 pmol/min per mg of protein. Rate of transport of [^{3}H]choline (0.5 or 1 μM) by HAChU system was measured in the presence and absence of **1** or **2** and was observed to be inhibited by both **1** and **2** in a concentration-dependent manner (Figures 2 and 3). IC_{50} values for each inhibitor were calculated by nonlinear regression analysis of inhibition curves containing 5–10 individual drug concentrations and are shown in Table I. K_i's shown in Table II were calculated from IC_{50} using the Cheng–Prusoff equation.[25]

To measure the selectivity of inhibition of high-affinity choline uptake relative to other neurotransmitter transport systems, inhibition of uptake of [^{3}H]dopamine and [^{3}H]-serotonin were studied. Inhibition of norepinephrine was not investigated as both dopamine and norepinephrine are catecholamines, and reports have suggested a common uptake transporter for both neurotransmitters.[26]

The dependence of concentration on the dopamine uptake was measured, and K_m and V_{max} for transport of dopamine were estimated to be 1.4 μM and 53 pmol/min per mg of protein, respectively. The observed K_m was consistent with the K_m of 1.4 μM estimated by Snyder and Coyle.[27] Inhibition of [^{3}H]dopamine uptake by various concentrations of 1 and 2 were measured and IC_{50}'s for each inhibitor were calculated by nonlinear regression analysis of inhibition curves containing 5–10 individual drug concentrations (Table I). K_i's shown in Table II were calculated from IC_{50} and the K_m for dopamine uptake, using the Cheng–Prusoff equation.[25]

When velocities of [^{3}H]serotonin accumulation at varying serotonin concentrations were measured and analyzed, the presence of two sites was suggested. This was consistent with the findings reported in literature.[28] The K_m and V_{max} for the high-affinity component of serotonin transport were estimated from the Eadie–

Table I. IC_{50}'s for Inhibition of High Affinity Choline Uptake, Dopamine Uptake, and Serotonin Uptake

	IC_{50}[a] (mM) ± SD		
inhibitor	choline uptake	dopamine uptake	5-HT uptake
catecholine	0.221 ± 0.089	0.949 ± 0.203	3.6 ± 0.2
TMC	0.083 ± 0.048	0.196	0.213

[a] IC_{50}'s shown here are from inhibition studies using a 1 μM concentration of respective neurotransmitter.

Table II. Comparison of Inhibition Constants (K_i) for the Inhibition of High-Affinity Choline Uptake, Dopamine Uptake, and 5-HT Uptake

	K_i (mM) ± SD		
inhibitor	choline uptake	dopamine uptake	5-HT uptake
catecholine (**2**)[e]	0.13 ± 0.06	0.53 ± 0.09[f]	0.39 ± 0.1[f]
TMC (**1**)[a,d]	0.06 ± 0.03[c]	0.09 ± 0.03[b]	0.09 ± 0.08[b]

[a] Two-way factorial ANOVA indicated that, overall, TMC (in comparison with catecholine) had a significantly higher affinity ($p < 0.001$) for all neurotransmitter uptake systems taken together. [b] Specifically, TMC had higher affinity than catecholine for dopamine and serotonin uptake systems ($p < 0.02$), [c]but not for the choline uptake system ($p > 0.02$). [d] TMC had comparable affinity at each neurotransmitter uptake sites ($p > 0.025$), [e]while catecholine discriminated between the three neurotransmitter uptake systems ($p < 0.025$). [f] Tukey test indicated that the affinity of catecholine for choline uptake system was significantly different than that for dopamine uptake system ($p < 0.01$) and serotonin uptake system ($p < 0.05$) and that catecholine does not discriminate between dopamine and serotonin uptake systems ($p > 0.05$).

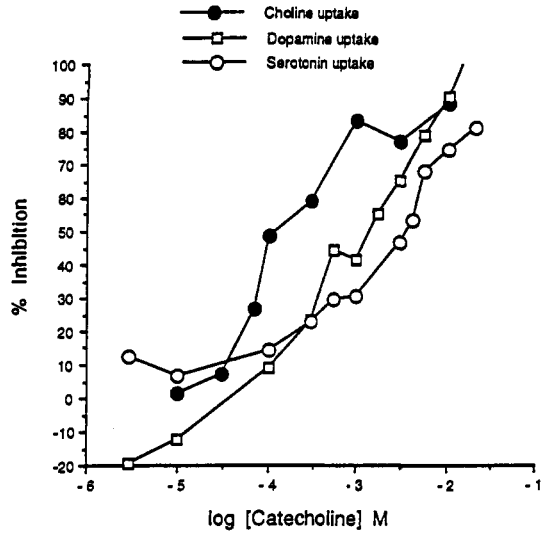


Figure 4. Comparison of the effect of 2 on high-affinity choline uptake, dopamine uptake, and serotonin uptake. These studies were performed using a 1 μM concentration of the respective neurotransmitter.

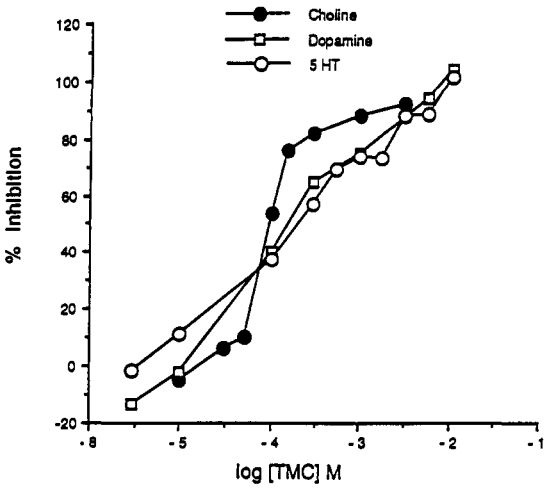


Figure 5. Comparison of the effect of TMC on high-affinity choline uptake, dopamine uptake, and serotonin uptake. These studies were performed using a 1 μM concentration of the respective neurotransmitter.

Hofstee plot (data not shown) and were found to be 0.15 μM and 23 pmol/min per mg of protein, respectively. For the low-affinity component, the K_m and V_{max} were estimated to be 7.05 μM and 197 pmol/min per mg of protein, respectively (data not shown). These kinetic constants are in agreement with the K_m values reported by Shaskan and Snyder (high-affinity K_m = 0.14–0.17 μM; low-affinity K_m = 8 μM).[28] Inhibition of serotonin uptake by 1 and 2 was measured and IC_{50}'s for each inhibitor were calculated by nonlinear regression analysis of inhibition curves containing 5–10 individual drug concentrations (Table I). Since the concentration of serotonin (1 μM) used for inhibition studies is close to the cross-over point for the low-affinity component (K_m = 7.05 μM) and the high-affinity component (K_m = 0.153 μM), the measured IC_{50}'s reflect combined contributions of two uptake sites. Calculation of apparent K_i's was done by the method of Cheng and Prusoff[25] using these apparent IC_{50} values and the K_m for high-affinity component of serotonin uptake (Table II). The use of K_m for the high-affinity component of serotonin uptake gave a more conservative estimate for apparent K_i for demonstrating selectivity of these agents for cholinergic sites over serotonergic sites.

Both 1 and 2 inhibit the high-affinity choline uptake at micromolar concentrations. Graphical comparison suggested that compound 2 demonstrated some level of selectivity (Figure 4) while 1 inhibited the uptake of all three neurotransmitters without much discrimination (Figure 5). Comparison of the inhibition constants (K_i) for the inhibition of choline uptake with those for inhibition of uptake of dopamine and serotonin are summarized in Table II. Two-way factorial analysis of variance (ANOVA) indicated that, overall, TMC (in comparison with catecholine) had a significantly higher affinity ($p < 0.001$) for all neurotransmitters. In the same analysis, the statistical interaction term reflecting the discrimination of the compounds for the neurotransmitter uptake systems was significant ($p < 0.0001$). Thus, TMC had a higher affinity than catecholine for dopamine and serotonin uptake systems ($p < 0.02$), but not for the choline uptake system ($p > 0.02$). Further, TMC had comparable affinity at each neurotransmitter uptake site ($p > 0.025$), while catecholine discriminated between the three neurotransmitter uptake systems ($p < 0.025$). A Tukey test was conducted to examine how catecholine discriminated between the neurotransmitter uptake systems. The affinity of catecholine for the choline uptake system was significantly different than that for dopamine uptake system ($p < 0.01$) and serotonin uptake system ($p < 0.05$). The Tukey test also indicated that catecholine does not discriminate between dopamine and serotonin uptake systems ($p > 0.05$). Collectively, these results indicated that catecholine was, indeed, more selective than TMC for choline uptake inhibition when compared with inhibition of other neurotransmitter uptake systems.

(C) *In Vivo* Behavioral Studies with Catecholine (2). Young male rats were trained to perform a representational memory task as described previously.[29,30] Animals learned the paired-run alternation (win-shift) task rapidly and were responding above 95% correct as a group by the seventh session. Rats were implanted with cannulae for hippocampal injections when they had achieved criterion of 100% correct for 3 consecutive days. Following a 1-week recovery period from surgery, rats were divided into three groups for bilateral injections of either saline or two doses of catecholine. Response times were not affected by either control or drug injections as mean response times remained under 4 for information runs. Injection sites were verified histologically following removal of the brain and were located within the dorsal hippocampus (data not shown).

Animals were not impaired directly following intrahippocampal (ih) injections of 2 (data not shown). Over a 1-month period, each group of animals performed significantly above chance levels ($p < 0.05$, by binomial expansion). Following the injection schedule, animals were retrained to perform a win-stay task.[31] As can be seen from Figure 6, all animals learned the task, although animals that had received the lower dose of 2 were modestly impaired in learning the new task. The animal receiving the highest dose of catecholine was much slower in learning the win-stay task. The data indicated that a 1-month exposure to 2 did not directly impair behavioral function but did impair subsequent learning of a new task.

Discussion

The quaternary ammonium alkyl-substituted catechols reported here are proposed to be the first generation of

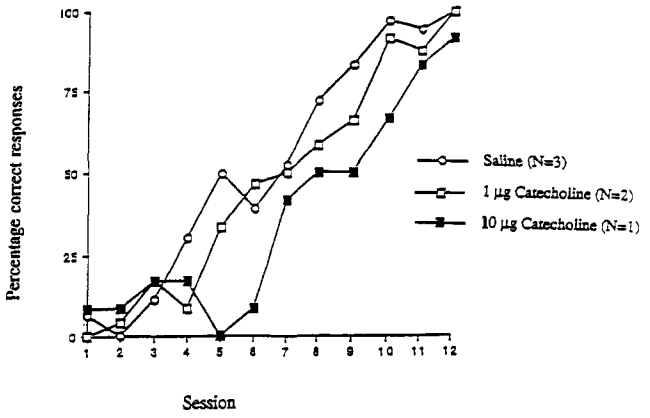


Figure 6. Learning of a win-stay representational memory task in a t-maze, following 1 month of injections of either saline, 1 μg of catecholine, or 10 μg of catecholine. Each group of animals learned the task over time ($p < 0.05$), although catecholine significantly slowed learning ($p < 0.05$).

a novel class of selective redox reactive cholinotoxic agent analogs. Two molecules, TMC (1) and catecholine (2), were examined in some detail. The hydroxy intermediates formed during oxidation of 1 have been spectroscopically characterized.[15] Such hydroxy derivatives of both 1 and 2, if isolated, could prove to be highly reactive choline analogs. The synthesis of these proposed intermediates in redox inactivation has mechanistic significance and may yield more reactive though still selective agents.

Due to the presence of choline-like side chain, 2 matched the description of redox-reactive choline analog to a greater extent than did 1. Both cholinomimetic analogs, 1 and 2, have been shown to inhibit choline acetyltransferase and high-affinity choline uptake. The enzyme inhibitor dissociation constants for both these inhibitors were in the millimolar range (K_i's for TMC and catecholine, respectively, are 15 ± 6 and 25 ± 4 mM). This was expected as these compounds are analogs of the low-affinity substrate, choline (K_m for choline is 0.4–1.0 mM). Both compounds inhibited the enzyme noncompetitively. Since these compounds were structural analogs of choline, noncompetitive inhibition was not expected. Such inhibition can be rationalized if the enzyme has a noncompetitive binding site with affinity for these catechols, partially overlapping the choline recognition center. It was also shown that the enzyme was subject to slow irreversible inactivation but was somewhat more sensitive to 2 than it was to 1. A reduced oxygen species or a covalent mechanism may mediate inactivation as had been proposed earlier.[15,20]

The rate of inactivation of the rat brain synaptosomal enzyme was found to be slower than that of the partially purified bovine enzyme. This was consistent with the requirement for the inhibitors to penetrate the synaptosomes before they could reach the enzyme. There was a possibility of transport of these inhibitors via the high-affinity choline uptake system. Competition with choline for uptake sites, and thereby inhibition of choline transport at micromolar levels was consistent with an inefficient though potentially selective transport of 1 and 2 through the high-affinity choline uptake system. Preliminary experiments using radioactive [^{3}H]catecholine suggested a very slow transport rate which was difficult to measure accurately due to the relatively short-term stability of the synaptosomal preparation.

Both 1 and 2 inhibited the high-affinity choline uptake. They displayed higher affinity for uptake sites than for ChAcT. Selectivity of each inhibitor for cholinergic sites was investigated by comparing the inhibition of choline uptake with that of dopamine or serotonin uptake. Uptake of 1 μM concentration of each neurotransmitter was compared (Table I), and the IC_{50}'s obtained for the inhibition of 1 μM choline uptake were compared with those for the inhibition of 1 μM dopamine uptake or 1 μM serotonin uptake. As each neurotransmitter under comparison has a different K_m for transport, inhibition constants (K_i) were calculated and utilized as the more suitable parameter for direct comparison. The relationship between the inhibition constant (K_i) and IC_{50} has been discussed by Cheng and Prusoff.[25]

Calculated K_i's are summarized in Table II. Catecholine (2) was about 5-fold and 3-fold more selective for inhibition of choline uptake in comparison to that of dopamine and serotonin uptake, respectively. The level of selectivity was statistically significant. Also, it was clear that TMC (1) did not show any preference for inhibition of cholinergic sites over dopaminergic or serotonergic sites. Thus, catecholine (2) shows more selectivity than TMC (1). This was expected on structural grounds as catecholine possesses a choline like side chain, whereas TMC represented a choline homologue only when employing the phenolic hydroxy on the ring carbon γ to the quaternary nitrogen. The behavioral studies, which are interesting but preliminary, indicated impaired learning of a representational memory task occurs following long-term administration of catecholine. Further studies are necessary to determine the time course of impairment and to document *in vivo* inhibition of choline uptake following intracerebral injection of catecholine and its analogs.

On the basis of these studies, catecholine (2) represented a good lead compound for a class of choline analogs capable of causing selective cholinergic destruction through a novel redox-reactive mechanism. It appeared to be slowly transported into the presynaptic neuron through the high-affinity choline transport system, for which it possessed a higher binding affinity, and was capable of slowly and irreversibly inactivating ChAcT which it also inhibited.

The approach used here has taken advantage of the electrophilic reactivity of quinone intermediates formed by oxidation of catechol. Involvement of such quinone intermediates has been suggested to explain the neurotoxicity of 6-hydroxydopamine.[16] The intracellular formation of this metabolite evidently was normally blocked in catecholaminergic neurons by mechanisms that were not clearly understood.[16] It has also been suggested that superoxide and hydroxy radical was the actual cytotoxic species.[32,33] The mechanisms involved are still not clear. However, involvement of superoxide, hydroxy radical, semiquinone radical, and singlet oxygen all have been implicated.[34–36]

The concept of site-directed oxidative inactivation is well-known, although it has not been examined extensively in the context of reactions mediated by catechol oxidation. Site directed inactivation of the nicotinic acetylcholine receptors (nAChR) by TMC has been reported.[15] TMC was spontaneously oxidized to reactive quinone intermediates which were proposed to mediate the demonstrated covalent labeling of the neurotoxin binding sites of nAChR.[15] It was suggested that TMC, in the presence of molecular oxygen, was oxidized to *o*-quinone which was subsequently converted to hydroxycatechol intermediates by addition of water molecules. These hydroxylated

oxidation intermediates mediated a site-dependent reduction of the easily reduced disulfide present at the receptor binding site. The corresponding *p*-quinone thus produced reacted with one of the free thiols to form a covalent linkage.[15] Such covalent labeling by choline analogs of catechol also inactivated other cholinergic sites, such as high-affinity choline uptake sites and the cholinergic enzyme choline acetyltransferase.

Conclusions

The inhibition and inactivation of macromolecular sites important in cholinergic neurotransmission have been demonstrated using catecholine, a redox-reactive quaternary ammonium agent. Catecholine showed promise as a selective pharmacological tool for manipulating cholinergic pathways. Catecholine may slowly penetrate presynaptic sites at cholinergic synapses through the choline high affinity; however, no firm evidence of this is presented. Both catecholine and TMC competitively inhibit high-affinity transport of choline which is consistent with but does not require slow transport. When compared with inhibition of uptake of other neurotransmitters, it was shown that catecholine demonstrated more selectivity than TMC toward inhibition of choline transport. K_m (μM) and V_{max} (pmol/min per mg of protein) were measured for high-affinity transport of choline, dopamine, and serotonin and were observed to be $K_m = 2.04 \pm 0.31$, $V_{max} = 22 \pm 1$; $K_m = 1.4$, $V_{max} = 53$; and $K_m = 0.15$, $V_{max} = 23$, respectively, and in good agreement with published literature values. K_i's (mM) for catecholine and TMC, calculated from experimentally determined IC_{50}'s, were for catecholine 0.13 ± 0.06, 0.53 ± 0.09, and 0.39 ± 0.10, and for TMC 0.06 ± 0.03, 0.09 ± 0.03, and 0.09 ± 0.08 for choline, dopamine and serotonin transport, respectively. These data suggest that suitable concentrations of catecholine may selectively compromise cholinergic function of presynaptic sites by significantly blocking choline reuptake—even in the absence of a sizable uptake of catecholine by these neurons. Thus, catecholine and potentially other derivatives based on catecholine may selectively reduce cholinergic function by two mechanisms: (1) blockade of choline uptake attendant with diminished synthesis of acetylcholine and (2) inhibition and inactivation of choline acetyltransferase subsequent to catecholine uptake and attendance with reduced acetylcholine synthesizing capacity. Simultaneous function of these two mechanisms would be expected to provide the best conditions for the establishment of selective cholinergic deficit. *In vivo* studies using catecholine suggest that this compound impairs learning ability associated with representational memory. Based on behavioral experiments, catecholine, when administered into the hippocampus, impaired learning ability in a representational memory task. While the exact sites and degree of selectivity of action have not been determined, catecholine appears to be capable of introducing cholinergic deficit *in vivo*. Catecholine thus represents a beginning point in the development of a series of redox-reactive choline analogs in the study of selective cholinotoxicity.

Thus, a lead compound in a series of novel selective cholinergic toxins has been established. The results reported here support the rationale for the design of such reagents and suggest that this approach toward site-directed cholinergic inactivation is very promising.

Experimental Section

Chemistry. Reagents, starting materials, and solvents were purchased from commercial suppliers. Melting points were determined on a Thomas-Hoover Unimelt Capillary melting point apparatus and were uncorrected. Routine absorption measurements were performed on a Varian DMS-200 UV/visible spectrophotometer. Infrared (IR) spectra were obtained using Perkin-Elmer 1600 series FTIR. NMR spectra were recorded either on a Varian VXR-400 (400 MHz) or on a Bruker AC-F 300 (300 MHz) spectrometer. ^{13}C NMR samples made in D_2O were referenced using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard. Chemical shifts are expressed in δ (ppm) units; coupling constants (J) are reported in hertz (Hz), and spin multiplicities are described as s (singlet), d (doublet), dd (doublet of doublets), bs (broad singlet), and m (multiplet). Elemental microanalysis was performed on final products by Galbraith Laboratories, Knoxville, TN.

3-[(Trimethylammonio)methyl]catechol Iodide (1). This compound was prepared in three steps from 2,3-dimethoxybenzaldehyde following the detailed procedures given by Nickoloff et al.;[15] mp 175–176 °C [lit.[37] mp 181 °C (crystallized from different solvents)]. The following spectroscopic data, which were not cited in the original reference, were determined. IR (KBr): ν_{max} 3436, 3178, 1472, 1313, 1261, 873 cm^{-1}. ^1H NMR (D_2O): δ 2.93 (s, 9 H), 4.32 (s, 2 H), 6.74 (t, 1 H, J = 7.72 Hz), 6.80 (dd, 1 H, J = 7.78, 1.62 Hz), 6.90 (dd, 1 H, J = 7.84, 1.64 Hz). ^{13}C NMR (D_2O): δ 55.3 (9 H), 66.7 (2 H), 118.2 (0 H), 120.9 (1 H), 123.2 (1 H), 128.3 (1 H), 147.5 (0 H), 148.2 (0 H).

(*R,S*)-[(Dimethylamino)methyl](3,4-dihydroxyphenyl)carbinol Methiodide (Catecholine, 2). Catecholine was synthesized in a three-step procedure essentially as described earlier by Patel et al.[20] This procedure was adapted from an earlier synthesis by LaManna et al.[19] and proceeds from 2-chloro-3′,4′-dihydroxyacetophenone (6) through α-(dimethylamino)-3′,4′-dihydroxyacetophenone hydrochloride (7) and [(dimethylamino)-methyl](3,4-dihydroxyphenyl)carbinol (8) to give catecholine. Chemical analyses, FTIR, and NMR data given in Patel et al.[20] were consistent with the assignment of structure for catecholine as well as for the synthetic intermediates. However, we report mp 225–227 °C dec (*cf.* lit.[20] mp 206–208 °C) for intermediate 7. We have no explanation for this discrepancy with the melting point reported earlier. Compound 8 was routinely isolated as a precipitate from ethyl acetate and was quite suitable for direct conversion to catecholine in 75 ± 5% yield. As isolated this precipitate has mp 133–135 °C (and can vary 2–5 °C about the midpoint depending on the sample). While this intermediate shows some tendency to decompose on handling, it may be purified further by recrystallization from ethyl acetate: mp 139–141 °C (*cf.* lit.[19] mp 142–143 °C). The purification of this intermediate does not improve the yield of catecholine obtained in the final step of the synthesis. Catecholine (2), mp 181–183 °C (lit.[19] mp 180–182 °C). ^{13}C NMR (D_2O): δ 56.9 (9 H), 70.7 (1 H), 73.3 (2 H), 116.6 (1 H), 119.0 (1 H), 121.3 (1 H), 135.3 (0 H), 146.9 (0 H), 147.0 (0 H).

Biological Evaluation. Male Long–Evans rats were purchased from Harlan Sprague–Dawley. Bovine brain choline acetyltransferase was purchased from Sigma (Lot 98F-9625). Radiolabeled compounds were purchased from Amersham. All other reagents were purchased from Sigma or Mallinckrodt.

(A) Choline Acetyltransferase Assay. The general procedure used for the enzyme assay was the same as reported in an earlier communication.[20]

Enzyme Preparations. Bovine choline acetyltransferase (2 mg/mL) was prepared in 40 mM phosphate buffer (pH 7.4) and used immediately.

Synaptosomal Preparations Used for Enzyme Assay. Long–Evans rats (Sprague–Dawley) were sacrificed via cervical dislocation. The brain was removed, without the cerebellum and olfactory tubules, placed in 10 volumes of phosphate buffer (40 mM, pH 7.4), and thoroughly homogenized using a Brinkman polytron (all glass) on setting 4. The homogenate was centrifuged at 1000*g* for 10 min. The supernatant was recentrifuged at 18500*g* for an additional 30 min. The pellet obtained was then resuspended in phosphate buffer to protein concentrations between 2.4 and 5.0 mg/mL. These preparations could be stored

for several weeks at −82 °C without loss of enzyme activity. Membrane protein was determined in every case by the method of Lowry.[38]

Determination of Enzyme Activity. [^{14}C]AcCoA solutions (10 μCi/mL) were prepared by diluting the solid commercial preparation (0.16 mg, 10 μCi) in 1 mL of sodium acetate buffer (0.01 M, pH 6.0). Routinely the above [^{14}C]AcCoA (240 μL) was added to unlabeled AcCoA (20 mg) and diluted with 80 mL of an unbuffered solution of sodium chloride (150 mM) and physostigmine (0.15 mM) to give a final concentration of 30 nCi/mL [^{14}C]AcCoA and 0.25 mg/mL AcCoA. This solution was distributed, usually in 10-mL aliquots, into tubes containing varying amounts of choline chloride (0.1–30 mM). Rates of acetylation in the presence or absence of inhibitor were estimated using the procedures of Kozlowski and Arbogast.[21]

Determination of Rates of Enzyme Inactivation by 1 and 2. At inhibiting and lower concentrations of **1** and **2**, both the bovine enzyme and rat brain synapatosomal enzyme inactivation was measured by incubation in the presence or absence of specific concentration of the inactivator at 4 °C. Control inactivation of both ChAcT preparations allowed measurements of inhibitor stimulated inactivation over a 72–96-h period. The enzyme activity was determined at 20 mM choline versus a 0 mM control.

(B) High-Affinity Choline Uptake Assay. HAChU was carried out with some modifications to the procedure described by Yamamura and Snyder.[22]

Synaptosomes. Synaptosomes were prepared essentially by the method of Gray and Whittaker.[39] Forebrains of adult male Long–Evans Sprague–Dawley rats (125–150 g), sacrificed by cervical dislocation, were removed, chilled, rinsed in cold 0.32 M sucrose (pH 7.4, adjusted with Tris buffer), and homogenized by hand in a loose-fitting all-glass Dounce homogenizer in 10 volumes of 0.32 M sucrose. The homogenate was centrifuged for 10 min at 1000*g* and washed once to remove large tissue fragments and cellular debris. The combined supernatants were centrifuged at 17500*g* for 20 min to give the crude synaptosomal–mitochondrial pellet. This particulate fraction, containing predominantly synaptosomes, mitochondria, and myelin fragments, was washed by resuspension in 0.32 M sucrose and recentrifugation (17500*g* for 20 min). The final pellet was resuspended on iced Krebs-Ringer phosphate buffer (KRB) aerated with 95% O_2–5% CO_2. The composition of this buffer was (in mM): NaCl, 124; KCl, 5; Na_2HPO_4, 20; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $CaCl_2$, 0.75; glucose, 10; pH 7.4. The crude synaptosome suspension contained about 1.25 mg of protein/mL of suspension as measured by the method of Bradford.[40]

Determination of K_m for Choline Transport. The uptake of [^{3}H]choline was measured for a range of choline concentrations from 10 nM to 10 μM. Aliquots (200 μL, 0.27–0.3 mg of protein) of the synaptosome suspension were equilibrated to 30 °C, and then 75 μL of [^{3}H]choline (208.5 nCi) was added to give the desired choline concentration in a final volume of 275 μL. Incubations were carried out at 30 °C for a fixed period of 3 min during which the choline transport was found to be linear with time. Parallel incubations were run at 0 °C to correct for nonspecific binding and diffusion. After 3 min, incubations were terminated by rapidly cooling in ice, the samples were centrifuged for 7 min at 800*g* at 4 °C, and the crude synaptosome pellets were washed once with 1.5 mL of ice-cold KRB. Radioactivity was counted in the pellets after solubilizing in 2 × 0.5 mL of Triton X-100 (1% v/v in water). Counts per minute were converted into picomoles of [^{3}H]choline transported. Uptake of [^{3}H]choline was determined as a measure of the difference between transport at 30 °C and 0 °C. The Michaelis constant K_m for transport of choline by the high affinity carrier was estimated using the method of Lineweaver and Burk.[41]

Determination of Inhibitor Constants (K_i) for 1 and 2 for Inhibition of [^{3}H]Choline Transport. For inhibition studies, tritiated choline concentrations of 0.5 or 1 μM were used, which were close to value of K_m (2.04 ± 0.31 μM) for the high-affinity component of choline accumulation. These concentrations were well below the apparent K_m for low-affinity uptake of choline (44–93 μM).[22,23] Choline transport was measured at 30 °C, for 3 min, as described above using a solution of [^{3}H]choline containing a range of concentrations of the added inhibitor (10 μM to 10 mM). The degree of inhibition of choline uptake was determined by comparing the uptake rate at each inhibitor concentration with that in the absence of inhibitor. IC_{50}'s for each inhibitor was calculated from the Hill plots, which were subsequently corrected for K_m for choline transport to yield K_i values, using the following equation derived by Cheng and Prusoff:[25]

$$K_i = \frac{IC_{50}}{1 + [S]/K_m}$$

Determination of K_m for Dopamine and Serotonin Transport. Transport of various concentrations (10 nM to 10 mM) of [^{3}H]dopamine or [^{3}H]serotonin was measured for 3 min, at 30 °C, by the procedure described for choline. K_m for dopamine transport was determined by Lineweaver–Burk analysis. For serotonin transport Eadie–Hofstee plots were used to calculate K_m.

Determination of Inhibitor Constants (K_i) for 1 and 2 for Inhibition of [^{3}H]Dopamine and [^{3}H]Serotonin Transport. For inhibition studies, tritiated dopamine or serotonin concentrations of 0.5 or 1 μM were used. Uptake of [^{3}H]dopamine (0.72 Ci/mmol) or [^{3}H]serotonin (0.727 Ci/mmol) was measured in the presence and absence of inhibitors as described for the inhibition of choline uptake, and IC_{50} values were calculated from the Hill plots and subsequently used with K_m for respective neurotransmitter transport to determine K_i values, according to the method of Cheng and Prusoff.[25]

Statistical Analysis. All data are given as mean ± standard deviation (SD). Two-way analysis of variance (ANOVA) was used to calculate statistical significance between groups when differences between different data sets were compared. A follow-up test for simple effects and a Tukey test were utilized to determine which differences were significant.

(C) *In Vivo* Studies. Young male Long–Evans rats (weighing 200–250 g) were allowed to feed *ad lib* for 1 week and were weighed daily. At the end of the week, animals were placed on a restricted diet of wet mash sufficient to lower and then maintain weight at 85% of *ad lib* levels. The animals then were introduced to a standard T-maze and trained to enter the arms for food (wet mash). Food was supplemented to maintain body weights at 85% of *ad lib* levels throughout the study. Once animals responded by moving rapidly through the maze for food, they were trained to perform the memory task. Rats were rewarded with food if they entered the arm opposite the one entered on the previous information run. A 30-s delay was imposed between runs, while 10–15 min separated each trial. Trials were given according to a random (but fixed) schedule.

Performance on the win-shift task was measured by recording both the percentage of correct choices and the response times for each run following daily injections of either saline or the appropriate drug (in saline). The effects of drug (or control injections) were measured on single sessions consisting of 12 paired-run trials. Once animals achieved criterion (100% correct choices for 3 consecutive days), they were implanted with cannulae via stereotactic surgery.

Surgical Procedures. The animals were cannulated for ih injection at least 1 week before the drug testing began. Rats were anesthetized by intraperitoneal (ip) injection of Chloropent (20 mg/kg; pentobarbital and chlorohydrate) for guide cannulae implantation by means of stereotactic technique. With bregma and lambda at the same vertical coordinate, the guide cannulae were inserted at AP-4.8 mm behind bregma, + 3.7 mm (re midline), and 3.0 mm below the skull surface for hippocampal injections according to the atlas of Paxinos and Watson. The injection cannula extend 1.0 mm beyond the guide cannula to deliver the drug into the appropriate area (4.0 mm below the surface of the skull). Guide cannulae were secured to the skull with stainless steel anchoring screws and cranioplastic cement.

Injection and Behavioral Schedules. On the first day of ih injections, animals received 0.5-mL injections of either saline (as a control) or catecholine (1 or 10 μg) over a 1-min period. Pressure injections were given bilaterally 10–30 min prior to behavioral testing. Animals were hand-held loosely for injections and did not appear distressed by the injection procedure. Animals received injections, followed by behavioral testing each day for a 1-month period.

At the end of the 1-month period, animals were retrained to perform a win-stay task. Animals were required on the choice run to enter the same goal box entered on the previous information run. Animals were not rewarded for choosing incorrectly, but were allowed to correct their choice. The data were analyzed by two factors analysis of variance with replication, followed by ANOVA for simple effects.

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