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Galanthamine, an Acetylcholinesterase Inhibitor: A Time Course of the Effects on Performance and Neurochemical Parameters in Mice

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SWEENEY, J E , P S PUTTFARCKEN AND J T COYLE *Galanthamine, an acetylcholinesterase inhibitor A time course of the effects on performance and neurochemical parameters in mice* PHARMACOL BIOCHEM BEHAV **34**(1) 129–137, 1989 —The time course of the effects of the long-acting acetylcholinesterase (AChE) inhibitor, galanthamine, on a spatial navigation task and on AChE and acetylcholine (ACh) levels were investigated in mice Mice received either saline or ibotenic acid injections into the nucleus basalis magnocellularis (nBM) The control and nBM group were then trained to perform a modified Morris swim task and the time to find the hidden platform was recorded The nBM group took significantly longer to find the platform than the control group in the reversal phase of testing Galanthamine attenuated the performance deficit in the nBM-lesioned group in a time-dependent manner, with peak performance at four hours after injection of 5 0 mg/kg galanthamine IP This dose impaired performance of the task in control mice, with the most severe deficits observed at two hours after injections when motor activity was severely reduced Galanthamine (5 0 mg/kg IP) significantly decreased cortical AChE activity and significantly increased cortical ACh content in control mice in a time-dependent manner The time courses of the neurochemical effects, however, did not correlate precisely with the behavioral time course Galanthamine concentrations up to 1×10^{-5} M did not affect choline acetyltransferase (ChAT) activity, [^{3}H]hemicholinium-3 (HCh-3) binding to the choline carrier, [^{3}H]quinuclidinylbenzilate (QNB) binding to muscarinic receptors, or [^{3}H]acetylcholine binding to nicotinic receptors in cortical homogenates AChE activity was inhibited by galanthamine in cortical homogenates with an IC_{50} of $4\ 1 \times 10^{-7}$ M Galanthamine's ability to reverse cognitive deficits induced by nBM lesions, its relatively long half-life and its specificity of effects suggest that this drug may be effective in treating the central cholinergic deficits in Alzheimer's disease and related disorders

Spatial navigation Galanthamine ACh content AChE inhibitor nBM lesion
[^{3}H]ACh binding HCh-3 binding QNB binding ChAT Mice

AN intact cholinergic system appears to be critical for normal mammalian learning and memory (1,7) Several pharmacological and lesion strategies have revealed the importance of this system in cognitive function Blockade of muscarinic cholinergic receptors with selective receptor antagonists induces a transient disruption of memory in both rodents (23) and primates (2), including humans (8) In experimental animals, lesions of the nucleus basalis magnocellularis (nBM), the major cholinergic projection to the cerebral cortex in rodents, produce profound deficits on a number of learning and memory tasks (22,29) In humans, the loss of cells in the nucleus basalis of Meynert and reductions in central cholinergic markers correlate with cognitive impairments in Alzheimer's disease (4,6)

Assuming that a cholinergic deficit may be responsible, in part, for some of the Alzheimer's disease-induced cognitive deficits, replacement strategies in nBM-lesioned animals may serve as a useful model to evaluate potential pharmacologic interventions. Galanthamine is a centrally active acetylcholinesterase (AChE) inhibitor that attenuates spatial memory and passive avoidance deficits in nBM-lesioned BALB/C mice in a dose-dependent fashion (30,31) The time course of the behavioral effects in nBM-lesioned mice, however, has not been documented, an

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important factor in evaluating its potential clinical utility Physostigmine, another AChE inhibitor, is quite effective at improving performance in nBM-lesioned animals (13,21). However, its relatively short half-life (20–30 minutes) is one factor that limits its clinical usefulness

The effects of galanthamine and its metabolites on AChE in the periphery have been documented in the blood, serum, and urine (18, 19, 27, 33, 35) There is a paucity of information, however, on the effects of galanthamine on neurochemical parameters in the central nervous system (CNS) Before clinical application can be considered, it is essential that galanthamine's effects on central cholinergic neurochemical parameters be investigated

The purpose of the present study was to examine the in vivo time course of galanthamine's effects on a spatial navigation task, AChE activity, and endogenous acetylcholine and choline content in BALB/C mice An additional goal of this study was to examine the specificity of the effects of galanthamine on AChE, in vitro The essential neurochemical parameters involved in synthesis, degradation and action of acetylcholine include the following 1) the high-affinity choline uptake system, the rate-limiting step in synthesis, 2) choline acetyltransferase (ChAT), the synthetic enzyme, 3) muscarinic receptors, 4) nicotinic receptors, and 5) acetylcholinesterase (AChE), the catabolic enzyme Frequently, drugs chosen to intervene at one particular step in this pathway may exert action at more than one site involved in cholinergic functioning (36)

To examine the effects of galanthamine on the sodium-dependent high-affinity choline uptake (SDHACU), hemicholinium-3 (HCh-3), a potent antagonist of SDHACU, was chosen As mentioned previously, blockade of muscarinic receptors impairs performance of a number of behavioral tasks in rodents and primates Therefore, the effects of galanthamine on muscarinic binding were measured using [^{3}H]quinuclidinylbenzilate (QNB), a selective antagonist at the muscarinic acetylcholine receptor Effects of galanthamine on nicotinic binding were measured using [^{3}H]acetylcholine (ACh) Finally, the effects of galanthamine on cortical ChAT activity were measured

METHOD

Subjects

Male BALB/cByJ mice (Jackson Laboratories) were used in all of the following experiments. At the beginning of the studies, the mice were 6–8 weeks old and weighed 26–32 grams The mice were housed either individually, or in pairs, in a temperature- and light-controlled environment with food and water available ad lib except for the brief periods in which behavioral testing was conducted Mice were maintained on a 13-hour light/11-hour dark cycle, with light starting at 0700

Experimental Design

Experiment 1 Mice received injections of either saline (controls) or ibotenic acid (nBM group) into the nucleus basalis magnocellularis (nBM) region Seven days after the lesion, motor activity of each mouse was monitored for 12 hours after an IP injection of saline and on the following day after an injection of 5 0 mg/kg IP galanthamine Two days later, each mouse was trained to perform a spatial navigation task When the mouse reached a criterion level of performance on the training phase (with the platform in one position), the mouse began reversal testing where the position of the platform was changed daily Galanthamine (5.0 mg/kg, IP) was administered at various times before the beginning of reversal testing Forty-eight hours after behavioral testing, mice were killed and their brains processed for ChAT activity and histology to assess the efficacy of the lesions

Experiment 2 Mice were injected with 5 0 mg/kg galanthamine and killed by cervical dislocation at various times after the injections for determination of AChE levels in cortical tissue

Experiment 3 Mice were injected with saline or 5 0 mg/kg galanthamine and killed by focused microwave at various times after the injections for determination of ACh levels in cortical tissue

Experiment 4 Mice were killed by cervical dislocation and fronto-parietal cortical samples were collected The effects of various concentrations of galanthamine, in vitro, on AChE activity, ChAT activity, [^{3}H]QNB binding, and [^{3}H]HCh-3 binding, were determined

Surgery

Mice were anesthetized with 3% halothane (Ayerst Laboratory Inc) at a rate of 5–8 liters per minute The stereotaxic procedure is described elsewhere (14) and summarized below Each mouse was placed in a stereotaxic instrument, and an incision was made into the scalp Holes were drilled anterior to the fronto-nasal suture on both sides of the central suture The nBM region was approached by lowering an injection needle through the olfactory bulb and moving it in an anterior to posterior and medial to lateral direction Thus, neither the hippocampus nor cortex was directly damaged by the injection route The lesion coordinates were 2 0 mm anterior to the frontal-nasal suture and 1 5 mm lateral to the midline The needle was lowered 8 0 mm below the skull surface and then retracted to 7 0 mm Three injections of 0 2 μl of saline (control group) or 10 μg/μl ibotenic acid (nBM group) was made at 7.0 mm, 6 5 and 6 0 mm below the surface of the skull During the same surgical procedure, three injections were also made on the contralateral side of the brain

Behavioral Testing

Motor activity The mice were placed individually into 40 × 40 × 30 cm Digiscan activity monitors (Omnitech Model RX-YCM) Movement was detected by infrared light beam sensors located around the perimeter of the instrument Total distance travelled in 1 hour by each mouse was measured and data were analyzed by a Digiscan Analyser (Omnitech Model DCM) and stored on an Apple II Plus computer Food and water were available without restriction during the testing period between 0900 and 2100 Temperature and light in the testing room were regulated to conform to conditions in which the mice were ordinarily housed

Spatial Navigation (Swim) Task

Training This procedure is described elsewhere (30) and is summarized here Each mouse was trained to swim to a platform that was submerged 1 cm below the surface of 24–26°C water made opaque by the addition of milk The swim tank was 72 cm in diameter and contained different black and white patterns in each quadrant for visual orientation The 5 × 5 cm platform was placed 10 cm from the wall of the tank and in the middle of the quadrant

At the start of the trial, the mouse was placed on the platform for 20 seconds The mouse was then placed into different quadrants of the tank (not containing the platform) and allowed to swim back to the concealed platform The time to find the platform was recorded If a mouse did not find the platform in 120 seconds, it was placed back on the platform Each mouse received one training session per day which consisted of three trials, one from

each of the quadrants not containing the platform. The scores from the 3 trials were totaled

Testing took place between 0900 and 1100 daily. The platform remained in the same position for each day during the training phase. Criterion level performance was ≤60 seconds/session (3 trials), for two consecutive days

Reversal trials Once a mouse reached criterion during the training phase, testing in the reversal phase began During this phase, the position of the platform was changed daily in a quasi-random order At the beginning of testing, the mouse was placed onto the platform in the new position for 20 seconds for orientation Next, the mouse was placed into the middle of one of three quadrants (which did not contain the platform) and allowed to swim back to the platform in the new position The time to find the platform was recorded—a total of 3 trials The mice were tested one day on the reversal task following saline injections. The next day, the mice tested on the reversal task following injections of 5 mg/kg galanthamine IP that was administered either 12, 6, 4, 2 or 1 hour before testing

Preparation of Tissues for Biochemical Analyses

For all biochemical assays except ACh, each mouse was killed by cervical dislocation followed immediately by decapitation The brain was removed onto an ice-cooled metal plate Tissue samples (approximately 17 mg/hemisphere) were taken from fronto-parietal cortex, excluding the cingulate area Tissue samples for ChAT activity and QNB binding were stored at −70°C until the time of assay Tissue samples for AChE, [^{3}H]HCh-3, [^{3}H]ACh measurements were maintained on ice and used fresh All assays were performed in triplicate and protein values determined (14)

For measurement of endogenous ACh levels, each mouse was killed by microwave irradiation focused on the skull (1300 W, 2450 MHz, for 1 5 sec) in a Menumaster Microwave oven adapted by Medical Engineering Consultants (Lexington, MA) The brain was rapidly removed and fronto-parietal cortical samples were collected, as described previously, and stored at −70°C until the time of assay

Cortical AChE

AChE activity was determined on cortical homogenates (30 mg/ml) from mice in Experiments 2 and 4 according to the acetylthiocholine method of Ellman (9) To determine the effects of galanthamine on AChE activity, in vitro (Experiment 4), homogenized tissue samples were first incubated with buffers containing various concentrations of galanthamine for 30 minutes at room temperature before addition of the substrate

Cortical ChAT Activity

ChAT was measured by a modified method of Fonnum (10) for the mice in both Experiments 1 and 4. Briefly, [^{14}C] acetyl coenzyme (Co) A (New England Nuclear, 57 2 mCi/mmol) was used as the substrate (500 μM). The [^{14}C]acetylcholine product was separated from the acetyl CoA substrate via an organic (acetonitrile) inorganic (AcCoA into buffer) separation.

Tissue from the mice in Experiment 1 were assayed without any drug to determine the efficacy of each nBM lesion To determine the effects of galanthamine, in vitro, on ChAT activity (Experiment 4), tissue homogenates were sonicated in 100 volumes (wt./vol.) buffer that contained various concentrations of galanthamine and BW813U, a ChAT inhibitor

Endogenous Cortical ACh and Ch

ACh and Ch content were assayed in tissue from mice in Experiment 3 using HPLC with electrochemical detection, based on a method by Potter (24). A Bioanalytical Systems (BAS) LC-4B electrochemical detector with a platinum electrode (detector setting +0 50 volts, sensitivity· 5 nA) was connected to a Waters M-45 pump (flow rate 0.8 ml/min) The mobile phase was 40 mM sodium phosphate buffer, pH 8 5 (Sigma Chemical Co) made up in HPLC-grade water (J. T. Baker, Inc) A polymeric analytical column (BAS) separated acetylcholine (ACh) and choline (Ch) from unretained peaks. A second reactor column (BAS) containing covalently-bound AChE and choline oxidase enzymes converted the ACh and Ch to H_2O_2 and betaine The H_2O_2 product was detected

Tissue samples were sonicated in 10 volumes (wt /vol) of 0 1 M perchloric acid (PCA) and centrifuged at 15,000 × g for 10 minutes in centrifugal filter tubes (Ranin Instrument Co) The PCA extract samples (20 μl) were then injected directly into the HPLC

Binding Experiments

Termination procedures Filters were presoaked in a solution containing 0.2% ([^{3}H]HCh-3 binding assay) or 0.05% ([^{3}H]ACh binding assay) polyethyleneimmine to reduce nonspecific binding to the filter paper. The incubation for all binding assays was terminated by addition of 4 ml ice-cold buffer and rapid filtration through Whatman GF/B glass fiber filter paper using a Brandel Cell Harvester (Gaithersburg, MD). The filters were washed twice more, transferred to scintillation vials containing 5 ml of Beckman EP Ready Solv and counted for 2 minutes. Specific [^{3}H]HCh-3 binding was determined by subtracting total from nonspecific binding in the absence of added membranes. This value was then subtracted from specific binding obtained in the presence of added membranes. Specific [^{3}H]ACh and [^{3}H]QNB binding was determined by subtracting total from nonspecific binding in the presence of added membranes

Competition studies employed 16 unlabeled ligand concentrations between 0.1 nM and 10 μM. All unlabeled ligands were dissolved in the appropriate buffer and added immediately before addition of the membrane suspension.

QNB binding. The [^{3}H]QNB binding assay was a modification of the procedure reported by Boggan *et al* (3) Briefly, tissue from mice in Experiment 4 was weighed (2 mg/ml final protein concentration in assay), homogenized in 100 volumes (wt /vol) of sodium-potassium phosphate buffer (Na-K-PO4, pH 7.4), and centrifuged at 48,000 × g for 10 minutes. The pellet was resuspended in fresh buffer and centrifuged again at the same speed and time.

Receptor binding was carried out by incubating membrane suspensions in 0 5 ml final reaction volume containing 0.025 M Na-K-PO4 and 2 nM [^{3}H]QNB. The reaction was terminated after 60 minutes. Nonspecific binding was determined in the presence of 10 μM atropine

Hemicholinium-3 binding [^{3}H]Hemicholinium binding was assayed as described previously (25, 26, 37) and summarized here. Fresh mouse cortex (from Experiment 4) was weighed and sonicated in 20 volumes (wt./vol.) of ice-cold 50 mM glycylglycine buffer, pH 7 4 containing 200 mM NaCl. The homogenate was centrifuged at 20,000 × g for 20 minutes at 4°C. The supernatant fluid was discarded and the pellet was resonicated and resuspended in glycylglycine buffer to yield 200–600 μg protein/ml.

Receptor binding was initiated by incubating membrane suspensions (120 μg protein/tube) in a 200 μl final reaction volume containing 10 nM [^{3}H]HCh-3 at 25°C for 30 minutes. Nonspecific binding was determined in the presence of 1 μM unlabeled HCh-3.

TABLE 1

TIME COURSE OF THE EFFECTS OF GALANTHAMINE ON MOTOR ACTIVITY

Group		Total Distance Travelled at Various Times After Injections*				
		1 Hr	2 Hr	4 Hr	6 Hr	12 Hr
Control Group (n=6)	+Sal	38 2 ± 4	25 9 ± 3	24 5 ± 3	22 3 ± 3	12 9 ± 4
	+Gal	0 5 ± 0 2	3 9 ± 0 6	25 9 ± 6	20 6 ± 4	14 7 ± 7
nBM Group (n=7)	+Sal	60 6 ± 2	45 4 ± 3	37 9 ± 6	23 7 ± 3	28 1 ± 6
	+Gal	9 1 ± 4	11 8 ± 4	34 9 ± 7	27 4 ± 9	22 8 ± 9

*Meters in 1 hour travelled ± S E M following injections of either 0 1 cc saline (Sal) or 5 0 mg/kg galanthamine (Gal) on consecutive days

Nicotinic receptor binding [^{3}H]ACh binding was assayed as previously described (28) Briefly, mouse cortex (from Experiment 4) was weighed and homogenized within 50 mM Tris-HCl buffer containing 1 5 μM atropine, 1 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, and 2 mM $CaCl_2$ (pH 7 4) The homogenate was centrifuged twice at 49,000 × g for 10 minutes and the supernatant discarded after each spin The final pellet was resuspended in fresh buffer containing 100 μM diisopropylfluorophosphate (DFP)

The binding assay was initiated with the addition of the membrane suspension (650 μg) to a 500 μl final reaction volume containing 10 nM [^{3}H]ACh at 0°C for 40 minutes Nonspecific binding was determined in the presence of 100 μM nicotine

Histology

After removing samples for ChAT activity, the remaining brain tissue from mice in Experiment 1 was fixed by submersion in a 4% phosphate-buffered formalin, pH 7 4 and 20% sucrose solution (w/v) Brains were sectioned frozen on a sliding microtome into 50 μm coronal sections Sections through the lesion site were mounted and stained for Nissl substance

Analysis of Competition Studies

All radioligand binding experiments were analyzed by the computer program LIGAND (20), although the actual data curves were drawn by hand In analyzing the competition studies, curves were modeled for the existence of one or more multiple affinity states If the estimates for a two site did not result in a significant improvement in fit over a one site fit, the simpler model is reported

Statistics

Motor activity data were analyzed using a split-plot repeated measures analysis of variance (ANOVA) control mice were analyzed in one plot and nBM-lesioned mice in another Spatial navigation, AChE, ACh and Ch data were analyzed using a one-way ANOVA Differences between means were assessed, post hoc, using Scheffe's tests Biochemical data were analyzed using unpaired Student's *t*-tests

RESULTS

The nBM-lesioned mice that did not have at least a 20% reduction in ChAT activity in fronto-parietal cortex, as compared to controls, were excluded from behavioral analysis (1 mouse) In addition, mice not reaching criterion after 6 days of training on the spatial navigation task were eliminated from the study (1 control and 1 nBM-lesioned mouse)

Motor Activity

Motor activity (as assessed by total distance travelled) decreased steadily with time after the saline injection in both the control and nBM groups (Table 1) Although activity in both groups decreased with time, the nBM group was significantly more active as compared to the control group This led to a significant (control × nBM) interaction effect, $F(4,44) = 5\ 71$, $p<0\ 01$

Galanthamine injections (5 0 mg/kg, IP) decreased motor activity in both the control and nBM groups by 98% and 85%, respectively, one hour after injections At 2 hours, activity was decreased by 85% in the control group and 74% in the nBM group Four hours after galanthamine injections, however, motor activity in both the control and nBM group was similar to that on the saline-injection day (24 5 ± 3 vs 25 9 ± 3 in the control group and 34 9 ± 2 vs 37 9 ± 5 in the nBM group) Different levels of activity at the different pretreatment times lead to a highly significant effect, $F(4,44) = 14\ 7$, $p<<0\ 01$

Spatial Navigation

Both the control and nBM groups acquired the swim task at similar rates in the training phase, when the platform remained in one position, as previously reported (30) (data not shown) The mean time to find the platform on the first day of training was 160 ± 38 sec for controls and 167 ± 33 for the nBM group The time to find the platform decreased steadily for both groups, similarly to previously reported acquisition rates on this task (30,31) On day 6 of training, both groups had reached criterion levels, the control and nBM groups took a mean of 36 ± 3 sec and 40 ± 4 sec, respectively, to find the hidden platform (a total of three trials) On the reversal phase, when the position of the platform changed, the saline-injected nBM group took 360% longer than controls to find the hidden platform—162 ± 15 as compared to 45 ± 5 sec, respectively

Galanthamine (5 0 mg/kg, IP) impaired the performance of control mice in a time-dependent fashion (Fig 1A) Many of the

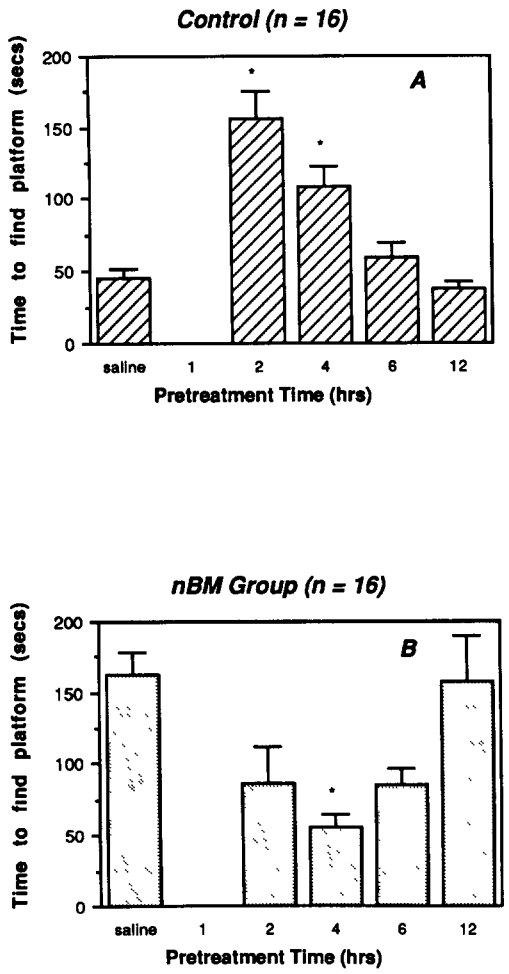

FIG 1 Time course of the effects of galanthamine on the spatial navigation reversals in the control (A) and nBM group (B) The times to find the platform (± SEM) are shown for mice injected with saline (0 1 cc, IP), or galanthamine (5 0 mg/kg, IP) 2, 4, 6, or 12 hours before testing

mice showed peripheral side effects immediately following galanthamine injections, such as reduced motor activity. None of the mice injected with 5.0 mg/kg were able to perform the swim task one hour later At later time points, however, performance varied significantly with pretreatment time, $F(4,39)=20$, $p<0$ 01. The control group took significantly longer to find the platform at 2 and 4 hours after galanthamine injections (156 ± 19 sec and 108 ± 15 sec, respectively) (Scheffe's $F=13$ 4, and 5.1, respectively, $p<0$ 05) The 6- and 12-hour pretreatment time points, however, did not differ significantly from saline-injected levels for the controls

Galanthamine attenuated the performance deficits in the nBM group in a time-dependent fashion, $F(4,39)=5$ 3, $p<0$ 05 (Fig 1B) Similar to the control group, the nBM group was unable to perform the swim task one hour after injections of 5 0 mg/kg galanthamine At four hours after injections, however, the nBM group took significantly less time to find the platform (56 ± 8 sec) than they did with saline injections (162 ± 15 sec) (Scheffe's $F=3$ 3, $p<0$ 05). Performance was improved at both 2 and 6 hours pretreatment (86 ± 27 and 85 ± 12 sec, respectively) over saline-injected levels; however, these values did not reach statistical significance At the 12-hour pretreatment time, the time to find the platform was similar in the nBM group to that on the saline-injection day

Lesion Confirmation

ChAT activity in the fronto-parietal cortex (for animals in Experiment 1) was decreased significantly by 21%, from $86\ 9\pm2$ nmol ACh/mg protein/hr in the control group to $69\ 4\pm4$ nmol ACh/mg protein/hr in the nBM group ($t=3$ 7, $p<0.01$) Endogenous ACh levels, but not Ch levels, were decreased by 37% in nBM-lesioned mice as compared to controls (Experiment 2) In addition, in the nBM group, the surgical needle tract could be followed from substantia innominata to the ventral medial globus pallidus area Gliosis around the needle tract and loss of magnocellular neurons indicated destruction of the nBM region.

Effects of Galanthamine on AChE Activity and ACh and Ch Content Following Injections

A time course of the effects of galanthamine on the AChE, ACh and Ch content in control mice are shown in Fig. 2 Cortical AChE varied significantly with pretreatment time, $F(5,35)=5$ 5, $p<0.01$ One hour after galanthamine injections (5 0 mg/kg), cortical AChE was significantly inhibited by 23% in control mice (Scheffe's $F=4$ 4, $p<0$ 05) In the Ellman assay, the concentration of galanthamine was diluted more than 900-fold Therefore, AChE inhibition by galanthamine in this assay was an underestimate of AChE inhibition in vivo In vivo values were estimated by extrapolation from the in vitro concentration-response curve (see Table 2)

Cortical ACh varied significantly with galanthamine pretreatment times in control mice, $F(5,35)=3$ 7, $p<0.05$. Cortical ACh was significantly increased at 1 and 2 hour pretreatment times (37% and 20%, respectively) (Scheffe's $F=3$ 9, $p<0$ 05). ACh levels were increased by 13% and 6% at 4 and 12 hours respectively These values, however, did not reach statistical significance At the 6-hour pretreatment time, ACh levels were decreased by 10%

Cortical Ch did not vary significantly with pretreatment time, $F(5,35)=0$ 21, $p>>0$ 05 For all of the pretreatment time points, Ch levels were increased between 3% and 6% over saline-injected levels

Effects of Galanthamine, In Vitro, on AChE and ChAT Activity, HCh-3 and QNB Binding

Galanthamine inhibited cortical AChE levels in a concentration-dependent manner (Fig 3). The estimated IC_{50} for AChE inhibition was $4\ 1\times10^{-7}$ M. Galanthamine at concentrations up to 1×10^{-5} M did not affect ChAT activity (Fig 4A). BW813U, a specific inhibitor of ChAT, reduced ChAT activity with an estimated IC_{50} of $3\ 7\times10^{-7}$ M Neither galanthamine, nor physostigmine, produced inhibition of 10 nM [^{3}H]HCh-3 binding at concentrations up to 1×10^{-5} M (Fig. 4B) Mouse cortical membranes, however, did show high-affinity displaceable binding of 10 nM [^{3}H]HCh-3 by unlabeled hemicholinium, with a K_i of 2.6×10^{-8} M Neither galanthamine, nor muscarinic agonists, produced significant inhibition of 10 nM [^{3}H]ACh binding at concentrations up to 1×10^{-5} M (Fig. 4C) Binding of 10 nM [^{3}H]ACh in mouse cortical membranes was completely inhibited by nicotine with a K_i of $6\ 3\times10^{-10}$ M In addition, neither galanthamine, nor the nicotinic agonist nicotine, produced significant inhibition of [^{3}H]QNB binding at concentrations up to

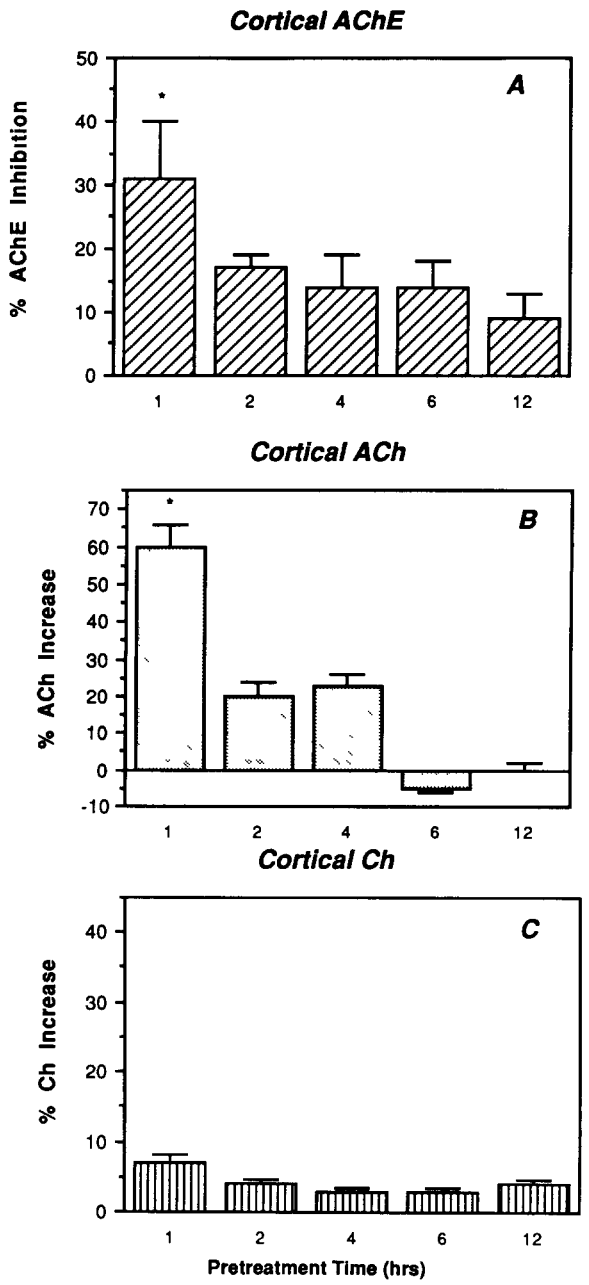

FIG 2 Time course of the effects of galanthamine in control mice on AChE activity (A), ACh levels (B), and Ch levels (C) The percent changes (±SEM), with respect to saline-injected levels, are shown following injections of galanthamine (5 0 mg/kg, IP) 2, 4, 6, or 12 hours before the time of killing

1×10^{-5} M (Fig 4D) Binding of 2 nM [^{3}H]QNB by mouse cortical membranes was completely inhibited by atropine, a selective muscarinic antagonist, with a K_i of $1\ 57 \times 10^{-9}$ M Oxotemorine, a muscarinic agonist, also inhibited [^{3}H]QNB binding with a K_i of $1\ 7 \times 10^{-8}$ M These data are summarized in Table 3

TABLE 2

ESTIMATED* % AChE INHIBITION

Time After Injection	% In Vitro Inhibition	% Estimated In Vivo Inhibition
1	31	91
2	19	83
4	15	78
6	15	78
12	9	63

*Estimate based on extrapolation of concentration-response curve (Fig 3) because of a 965 × dilution of galanthamine concentration in the Ellman assay

DISCUSSION

In the present study, galanthamine significantly improved performance of nBM-lesioned mice on a spatial navigation task 4 hours after injections of 5 0 mg/kg IP At 6 hours, the time to find the platform was reduced from 167 ± 15 sec in saline-injected mice to 85 ± 12 sec in mice injected with galanthamine 6 hours before testing, however, this decrease was not statistically significant These data indicate that the time course of the effects of galanthamine on performance is, therefore, considerably longer than those described for other AChE inhibitors, such as physostigmine, where peak performance occurs 30–60 minutes after injections (17,21) In a study using unlesioned C57BL/10 mice, galanthamine (0 1 mg/kg, IP) improved performance of a Morris water maze task with a peak effect occurring at 2 hours (34) While a time-dependent improvement in performance was noted in both the studies, the precise time courses are not directly comparable because of the two different strains of mice used [BALB/c in the present study and C57BL/10 in the Vincent study (34)] and the different doses of galanthamine used [5 0 mg/kg in nBM-lesioned mice in the present study and 0 1 mg/kg in control mice in the Vincent study (34)] Furthermore, C57BL/10 mice acquire a swim maze task more slowly than other strains of mice and are more sensitive to the facilitating effects of physostigmine (32)

Galanthamine did significantly affect motor activity within the first two hours of treatment This decreased motor activity is the most likely explanation for why neither control nor nBM-lesioned

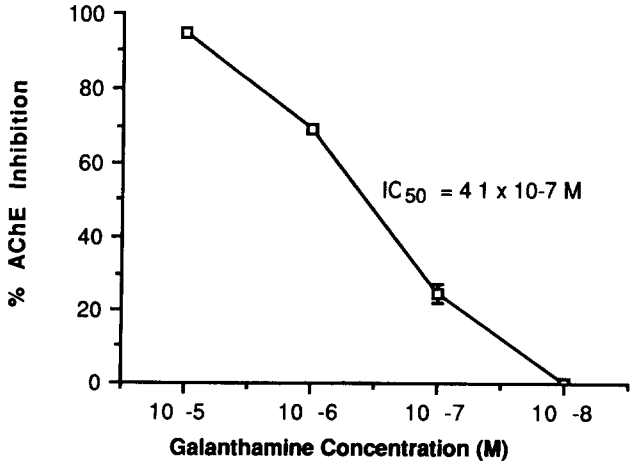

FIG 3 The effects of increasing concentrations of galanthamine, in vitro, on AChE activity in cortical homogenates Each value represents a mean of triplicates from four different experiments

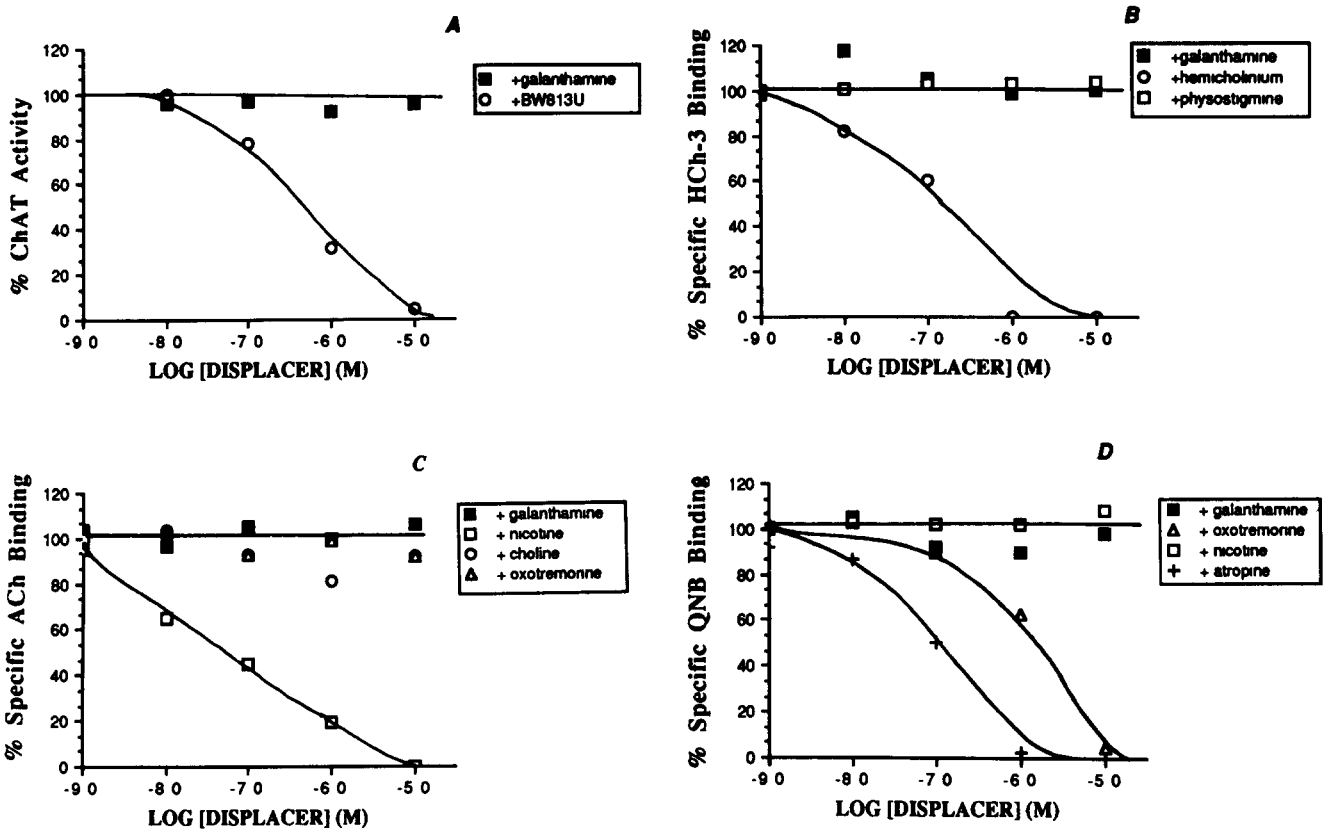

FIG 4 The effects of increasing concentrations of galanthamine, in vitro, on ChAT activity (A), HCh-3 binding (B), QNB binding (C), and ACh binding (D) in cortical homogenates (A) ChAT activity measured in the presence of increasing concentrations of galanthamine and BW813U (B) Inhibition of specific [^{3}H]HCh-3 binding, 10 nM, by increasing concentrations of galanthamine, hemicholinium, and physostigmine (C) Inhibition of specific [^{3}H]ACh binding, in the presence of 1 5 μM atropine (to block muscarinic binding), by increasing concentrations of galanthamine, nicotine, choline, and oxotremorine (D) Inhibition of specific [^{3}H]QNB binding,2 nM, by increasing concentrations of galanthamine, oxotremorine, nicotine, and atropine Each point is a mean value of triplicate determinations from three experiments Each point is a mean value of triplicate determinations from three experiments % specific binding refers to % specifically bound with respect to control values

mice were able to perform the swim task one hour after injections of 5 0 mg/kg galanthamine. At 2 hours, the time to find the platform was not improved significantly in nBM-lesioned mice, but was impaired significantly in the control group The lack of a significant improvement in the nBM group and the impairment in the control group at this time point was probably also due, at least in part, to the decreased motor activity in the mice At four hours when motor inhibition was no longer evident, the effect of galanthamine on memory became apparent The performance of nBM-lesioned mice was improved significantly and the performance of control mice was still impaired significantly

Administration of a single dose of galanthamine produced a time-dependent decrease in AChE levels and a time-dependent increase in ACh levels in control mice. The magnitude of the changes in neurochemical parameters was similar to that seen with physostigmine in the rat (11,12). In the Hallak and Giachobini study (11), the peak inhibition of AChE, however, occurred 5 minutes after injections of physostigmine and ACh levels in the cortex peaked 20 minutes after injection, and by 60 minutes after injections, AChE activity and ACh levels were indistinguishable from control levels. In the present study, the peak of AChE inhibition and ACh increase occurred 1 hour after injection, however, values were indistinguishable from controls 6 hours after injections Thus, galanthamine has a substantially longer duration of effect on cortical cholinergic parameters than physostigmine

Although the performance, AChE activity and ACh levels exhibited time-dependent changes following galanthamine treatment, the time courses of neurochemical parameters and performance alterations did not occur contemporaneously The effects of galanthamine on AChE inhibition were longer-lasting than the effects of ACh levels A dissociation between AChE inhibition and ACh levels has also been noted following administration of physostigmine and tacrine (12) Furthermore, at four hours after injection, control mice were significantly impaired on the spatial navigation task, however, neither AChE activity nor ACh levels were significantly different from saline-injected levels There are a number of possible explanations for this disparity, including· 1) changes in cholinergic parameters trigger a change in another parameter (which has a longer time course) that results in changes in behavior, 2) the assay method was not sensitive enough to detect small biologically relevant changes, 3) ACh levels in a different area of the brain, such as the hippocampus, may correlate more directly with performance, 4) partial AChE inhibition could prolong the duration of action of ACh without resulting in a detectable buildup of the transmitter in the tissue, or 5) galanthamine may affect other noncholinergic parameters that correlate better with performance Furthermore, the lack of correlation does not necessarily imply that there is no association between cholinergic transmission and performance of the task For example, the efficacy of synaptic conduction following a given dose of an

TABLE 3

BINDING IN MOUSE CORTICAL MEMBRANES

Radioligand	Unlabeled Ligand	Receptor/ Carrier	K_i (nM)	B_{max} (fmol/mg protein)
QNB	atropine	muscarinic	1 57 ± 0 036	55 0
QNB	oxotremorine	muscarinic	17 40 ± 6 8	35 0
QNB	galanthamine	muscarinic	>10,000	—
QNB	nicotine	muscarinic	>10,000	—
ACh	nicotine	nicotinic	0 625 ± 0 49	0 76
ACh	oxotermorine	nicotinic	>10,000	—
ACh	galanthamine	nicotinic	>10,000	—
HCh-3	HCh-3	choline carrier	26 0 ± 9 3	0 33
HCh-3	galanthamine	choline carrier	>10,000	—
HCh-3	physostigmine	choline carrier	>10,000	—
HCh-3	choline	choline carrier	>10,000	—

Results from two independent replicate experiments for each radioligand are reported below Results were analyzed by a nonlinear curve-fitting algorithm The tabulated values are the estimates of K_i or B_{max} (± standard error of the parameter estimate) from a combined analysis of the results from two independent experiments The concentration of [^{3}H]QNB was 2 nM, of [^{3}H]ACh and [^{3}H]HCh-3 was 10 nM Dashed lines indicate the unlabeled ligand was unable to interact with the labeled site, thus making it impossible to obtain a B_{max} estimate

AChE inhibitor depends on the rate of ACh release during transmission and on the sensitivity of the postsynaptic membrane (7) Therefore, to understand the effects that galanthamine has on cholinergic neurotransmission, which may correlate better with performance measurements, ACh turnover and receptor sensitivity would have to be measured in addition to ACh levels

The IC_{50} for galanthamine is $4\ 1 \times 10^{-7}$ M in cortical homogenates This value is similar to other reported values in the periphery, however, it is one order of magnitude lower than the IC_{50} for physostigmine (5,19) While galanthamine is not as potent an inhibitor of AChE as physostigmine, the longer half-life and considerably lower LD_{50} suggest that former might be of more clinical utility Galanthamine did not affect the other cholinergic parameters measured [^{3}H]HCh-3 binding, ChAT activity, or [^{3}H]QNB or [^{3}H]ACh binding These data indicate specificity for AChE inhibition, however, the effects of galanthamine on non-cholinergic systems still need to be explored

In conclusion, these data indicate that galanthamine reverses behavioral deficits resulting after nBM lesions in a time-dependent manner Galanthamine's effects generally correlate with the inhibition of cortical AChE and the subsequent increase in endogenous ACh levels in control mice Clearly, destruction of enough nBM cholinergic neurons will limit the ability of any AChE inhibitor to augment cortical ACh levels produced by surviving neurons Nonetheless, its relatively long half-life and specificity of action on cholinergic parameters suggest that galanthamine may have therapeutic utility in attenuating the cholinergic deficits in the early stages of Alzheimer's disease

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