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Neurochemical and Behavioral Effects of N-Ethyl-Acetylcholine Aziridinium Chloride in Mice

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POPE, C. N., B. T. HO AND A. A. WRIGHT. *Neurochemical and behavioral effects of N-ethyl-acetylcholine aziridinium chloride in mice.* PHARMACOL BIOCHEM BEHAV 26(2) 365–371, 1987.—N-ethyl-choline aziridinium (ECA) and N-ethyl-acetylcholine aziridinium (EAA) were shown to be inhibitors of high affinity choline uptake *in vitro* (IC_{50}=0.4 μM and 1.5 μM, respectively), and intraventricular administration showed that EAA was more selective in its inhibition of hippocampal choline uptake *in vivo*. EAA significantly reduced the activity of choline acetyltransferase in the hippocampus 3 to 28 days following intraventricular infusion, but not in the striatum or parahippocampal cortex. Neither muscarinic receptor binding nor glutamic acid decarboxylase activity were affected in any of the three brain regions. EAA (12 or 16 nanomoles, intraventricular) significantly impaired memory performance of mice in a radial arm maze when tested two weeks after treatment. A subgroup analysis implicated long-term reference memory as the mechanism disrupted.

Hippocampus Radial arm maze Mice Working memory Reference memory Cholinotoxins

THE central cholinergic system has been implicated in the acquisition, storage or retrieval of information in man and other species [2, 8, 33, 41, 43, 44]. Compounds which inhibit central cholinergic neurotransmission, e.g., atropine, have long been known to produce retrograde amnesia in humans [23]. Compounds which facilitate central cholinergic transmission, i.e., cholinomimetics, have been shown to enhance learning and memory in man and other animals [2, 7, 12, 13, 40]. High doses of cholinomimetics can begin to inhibit learning and memory [7, 9, 12, 13], thus producing an inverted U-shaped function between performance and dose. This type of dose-response curve suggests a regulatory role for cholinergic neurons. In addition, it appears that a central cholinergic deficit may be the most significant neurochemical manifestation of Alzheimer's disease (AD), a progressive disorder of cognitive and mnemonic function affecting approximately 2 million persons in the United States alone [3,6]. In short, there is a great deal of evidence that the central cholinergic system plays a vital role in learning and memory.

An animal model of impaired central cholinergic function would be of value both for the study of underlying processes in cognition and memory and for the design of therapeutic agents for the treatment of AD and related disorders. The toxic choline analog, N-ethyl-choline aziridinium chloride (ECA, AF64A), has been proposed to be a cholinergic-specific neurotoxin, capable of inducing long-term impairment in central cholinergic function after central administration [11,25]. ECA is a rather selective and irreversible inhibitor of high affinity choline uptake [25,37], a regulatory and possible rate-limiting step of acetylcholine synthesis [1,39]. Several groups of investigators have reported selective long-term reductions in presynaptic cholinergic parameters (e.g., high affinity choline uptake, choline acetyltransferase) following central administration of ECA [11, 35, 38, 42]. However, there have been reports of non-cholinergic toxicity following treatment with ECA. While Walsh *et al.* [42] observed no signs of degeneration in septum, fimbria-fornix, caudate or hippocampus after as much as 30 nanomoles ECA (intracerebroventricular) in rats, Jarrard *et al.* [20] reported that intraventricular ECA in rats resulted in nonselective necrosis in brain areas surrounding the injection site (e.g., fimbria-fornix). Signs of non-cholinergic toxicity have also been reported following injection of ECA into the substantia nigra in rats [22].

ECA is a close structural analog of choline, and associates *in vivo* with cellular sites which exhibit affinity for choline (e.g., the site for high affinity choline uptake). Choline is essential for all cellular membranes in addition to being a precursor for the neurotransmitter acetylcholine, thus all types of neurons would be expected to have some affinity for toxic choline analogs including the toxin ECA.

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Thus, there is a mechanism whereby toxic choline analogs could produce nonspecific toxicity as has been reported [20,22]. Acetylcholine, unlike choline, is not essential for all cells and is highly regulated within the central nervous system. There is, thus, less reason to expect general toxicity from a toxic analog of acetylcholine than choline, and the toxicity can be better confined to the target site. Several studies [4, 11, 36] have suggested that N-ethyl-acetylcholine aziridinium (EAA) can be deacetylated *in vivo* to the active choline analog (ECA) by physostigmine-sensitive enzymes. Such enzymes (i.e., acetylcholinesterase) are concentrated in brain areas receiving cholinergic innervation. Thus, it was hypothesized in this study that EAA would be a more selective cholinergic neurotoxin than ECA, on the basis that the deacetylated active toxin (ECA) would be produced in areas of high cholinesterase activity (i.e., through target-site biotransformation).

We report here neurochemical and behavioral effects of intraventricular administration of N-ethyl-acetylcholine aziridinium chloride in mice. The relative inhibitory potencies of ECA and EAA on high affinity choline uptake *in vitro* and *in vivo* were examined. The cholinergic markers choline acetyltransferase (CAT) and muscarinic receptor (QNB) binding were determined as indices of presynaptic and postsynaptic function, respectively. In addition, glutamic acid decarboxylase activity, a neurochemical marker for GABAergic neurons, was measured as an index of nonspecific toxicity. The effect of EAA treatment on memory was assessed by radial arm maze behavior.

METHOD

Chemicals

Synthesis of N-ethyl-acetylcholine mustard HCl (the precursor for both ECA and EAA) was by a modification of the method of Jackson and Hirst [18] for acetylcholine mustard. ECA and EAA were prepared fresh by dissolving the precursor in double-distilled water to make a 20 mM solution. This solution was allowed to stand at room temperature for 60 minutes for formation of the aziridinium species [36]. ECA was prepared by adjusting the pH of the precursor solution to 11.5 with 1 N NaOH and maintaining it there for 20 minutes. This solution was then adjusted to approximately pH 7.0 at 25° with 0.1 N HCl and diluted to the appropriate concentration with phosphate-buffered saline (PBS, pH 7.4). EAA was obtained by adjusting the pH of the precursor solution (prepared as for ECA) to approximately 7.0 with 0.1 N NaOH, followed by dilution to the appropriate concentration with PBS.

Acetyl Coenzyme A, eserine sulphate, l-glutamic acid, pyridoxal phosphate, and bovine serum albumin were purchased from Sigma Chemical Company. Tetraphenylboron was purchased from Aldrich Chemical Company. Acetyl Coenzyme A (acetyl-1-^{14}C), specific activity 30–50 mCi/mmol, was purchased from ICN Chemical and Radioisotope Division. Choline chloride (methyl-^{3}H), specific activity 60–85 Ci/mmol, l-glutamic acid (1-^{14}C) specific activity 50–60 mCi/mmol, and 1-quinuclidinyl[*phenyl*-4-^{3}H] benzilate were purchased from Amersham. All other chemicals were reagent grade.

Animals and Treatments

Male, outbred albino mice (CD-1, Charles River Breeding Laboratories, Wilmington, MA) weighing 25–35 grams were used throughout. Seventy-two mice were used for neurochemical analyses and an additional sixty mice were used for behavioral studies. Mice were anethetized with sodium pentobarbital (85 mg/kg, IP) and positioned in a stereotaxic apparatus for treatment. A parasagittal incision was made in the scalp and fascia lightly scraped from the surface of the skull to expose the reference point bregma. Bilateral burr holes were drilled in the skull 1.1 mm on either side of the midline, lateral to bregma. Bilateral cannulae (27 gauge) were lowered to a depth of 2.7 mm below the dura and a syringe pump was used to deliver 1.0 μl into each lateral ventricle simultaneously (0.4 μl/minute). The cannulae were left in place for an additional two minutes to allow for diffusion away from the injection site.

TABLE 1

EFFECTS OF EAA (12 nmoles, ICV) ON HIPPOCAMPAL NEUROCHEMICAL MARKERS, SEVEN DAYS AFTER TREATMENT

Treatment*	Lethality	CAT†	QNB‡	GAD§
Vehicle	0/8	10.2 ± 0.3	298 ± 15	13.1 ± 0.8
EAA				
4 nmoles	0/8	8.3 ± 0.9¶	283 ± 17	12.2 ± 1.0
8 nmoles	0/8	6.4 ± 1.3¶	288 ± 15	12.7 ± 0.9
12 nmoles	2/8	5.0 ± 1.7#	270 ± 28	13.0 ± 0.6

*All treatments made by bilateral infusion into the lateral ventricles under Nembutal anesthesia as described in the Method section.

†Choline acetyltransferase activity expressed as μmole/hr/g tissue ± standard error.

‡QNB binding expressed as pmole/mg protein ± standard error.

§Glutamic acid decarboxylase activity expressed as μmole/hr/g ± standard error.

¶$p<0.05$, Student's t-test; $df=14$.

#$p<0.05$, Student's t-test; $df=12$.

Tissue Preparation and Biochemical Assays

Animals were sacrificed by decapitation. Brains were rapidly removed and placed into ice-cold 50 mM sodium phosphate buffer (pH 7.4) for determination of CAT, GAD and QNB binding *or* were dissected at room temperature for choline uptake studies. Brain regions were dissected on ice essentially as described by Glowinski and Iversen [15] for rat brain. "Parahippocampal cortex" was defined as that directly overlying the hippocampal formation.

High affinity choline uptake was measured in crude synaptosomes prepared essentially as described by Gray and Whittaker [16]. Briefly, tissue was homogenized with Potter-Elvehjem glass homogenizers (0.25 mm clearance) in 29 volumes 0.32 M sucrose. These suspensions were centrifuged for 10 minutes at 1,000 × g, after which resultant supernatants were recentrifuged for 20 minutes at 17,000×g. The pellet (referred to as P2) was resuspended in the original homogenate volume with ice-cold, oxygenated medium (40 mM Tris-(hydroxymethyl)aminomethane buffer, pH 7.5 (at 25°), 4.2 mM $MgSO_4$, 9.6 mM KCl, 10 mM dextrose, 2.4 mM $CaCl_2$ and 125 mM NaCl).

High affinity choline uptake was measured by a modification of the method by Nordberg [29]. Aliquots (50 μl) of P2 were incubated with 0.43 ml of above Tris-buffered medium at 37° for five minutes, after which 20 μl of [^{3}H]choline (0.5 μCi) in medium was added (final concentration of choline

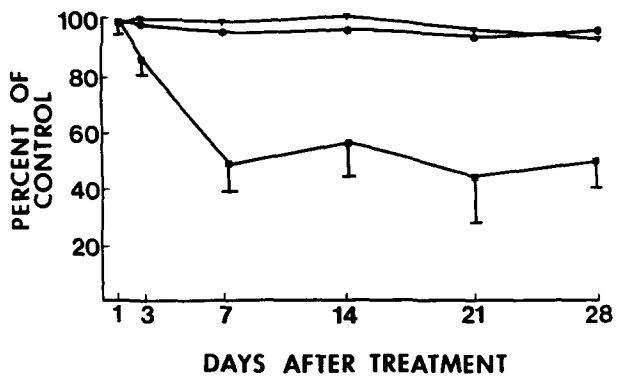

FIG. 1. Choline acetyltransferase activity in cortex, striatum and hippocampus at various times after 12 nanomoles EAA (ICV infusion). Mice were anesthetized with Nembutal (85 mg/kg) and positioned in a stereotaxic apparatus. Twelve nanomoles EAA was infused bilaterally into the lateral ventricles (6 nanomoles/side) and mice sacrificed between one and 28 days after treatment. Choline acetyltransferase activity was measured in brain regions as in the Method section. Each point represents the mean (±s.e.m.) of six animals. ▼ Striatum; ● cortex; ■ hippocampus.

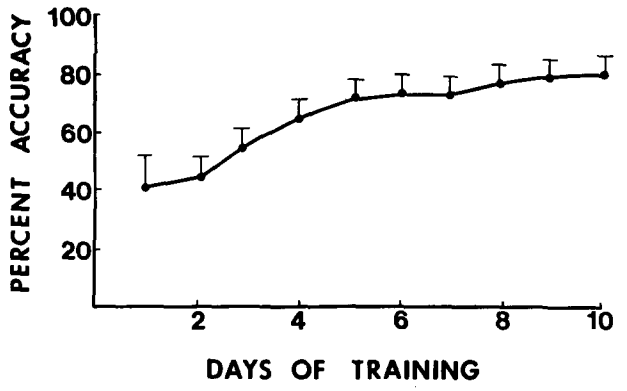

FIG. 2. Acquisition of radial arm maze behavior. Fifty μl of isotonic sucrose was placed in wells at the end of each arm before a trial. A trial began by placing a mouse into the central area and allowing the mouse to choose freely until all eight arms had been entered. Mice were given two trials per day. Performance was corrected for chance probability as described in the Method section. Each point represents the mean (±s.e.m.) of two daily trials with sixty mice.

adjusted to 0.04 to 4.0 μM choline with cold choline chloride). Incubation was continued for another four minutes. Paired samples were incubated at 0° for blanks. Incubation was stopped by addition of 3 ml ice-cold 1 mM isotonic choline chloride. Tissue was collected onto glass fiber filters (GF/C, Whatman, Inc.) by gentle vacuum filtration and the filters washed three times with 3 ml isotonic choline chloride. The filters were then placed in glass scintillation vials containing 5 ml Instagel counting scintillant (United Packard International). The vials were shaken for 30 minutes and counted at 43% efficiency. High affinity uptake was determined as the difference in tissue radioactivity between paired samples incubated at 37° and those incubated at 0°.

Choline acetyltransferase was assayed in frozen (−70°C)/thawed tissue samples by a modification of the method of Fonnum [14]. Thawed samples were homogenized (Polytron, setting 6 for 15 seconds) in 39 volumes 50 mM sodium phosphate buffer (pH 7.4). The homogenate (180 μl) was added to 20 μl of 10 mM disodium EDTA (pH 7.4) containing 15% Triton X-100 and vortexed vigorously for 10 seconds. Ten μl of this suspension was added to 25 μl of the reaction mixture (300 mM NaCl, 41 mM sodium phosphate pH 7.4, 0.1 mM physostigmine sulphate, 10 mM EDTA pH 7.4, 10 mM choline chloride and 0.4 mM [^{14}C]acetyl coenzyme A (about 100,000 dpm/reaction) and incubated at 37° for 15 minutes. The reaction was stopped by addition of 3 ml ice-cold 10 mM sodium phosphate buffer pH 7.4. One ml of 3-heptanone containing 15 mg sodium tetraphenylboron was added to each tube and vortexed lightly ten times (up/down). The tubes were then centrifuged for ten minutes at 700 × g. A 0.5 ml sample of the organic phase was added to 1 ml acetonitrile and 4 ml organic counting scintillant (OCS, Amersham) and counted at 73% efficiency.

Muscarinic cholinergic receptor binding was determined by the method of Yamamura and Snyder [49] using [^{3}H]quinuclidinyl benzilate (QNB) as ligand. Specific binding was determined by the difference in binding between samples incubated in the presence or absence of 100 nanomolar atropine. Glutamic acid decarboxylase (GAD) activity was determined by the method of Wilson *et al.* [46] as modified by Coyle and Enna [5]. Analysis of protein content in tissue samples was by the method of Lowry *et al.* [24] using bovine serum albumin as standard.

Behavioral Methods

An eight-arm maze similar to that described by Pick and Yanai [34] was constructed of clear Plexiglas. Each arm was 5 cm in height × 34 cm in length × 5 cm in width. Arms radiated outward at equal angles from a central area, 12 cm in diameter. The maze was covered with a clear Plexiglas top which had a small hole (5 cm diameter) in the center to allow mouse entry. The maze was elevated 70 cm from the floor in a room enriched with various cues, e.g., glassware shelves, pictures, lights and a radio. In addition, four objects (fluorescent light, ring stand, small cart and oxygen tank) were positioned adjacent to the maze at approximately equivalent distances from each other. The experimenter was positioned above and directly to the side of the maze. Mice were water-deprived (i.e., ad lib water was available for only 15 minutes each day sometime between 1600 and 1800 hours) for seven days before training. During training and retention testing, supplemental water was available only on non-testing days (i.e., on weekends). Isotonic sucrose (50 μl) was placed in wells at the ends of each arm for reinforcer.

A trial began by placing a water-deprived mouse through the hole in the maze top. The mouse was allowed to choose arms freely until all eight arms had been entered. An entry was recorded by the experimenter when the mouse crossed a point 10 cm from the perimeter of the central area.

Optimal performance in this task was entry into eight different arms without repeating earlier choices. Accuracy was thus measured as number of different (correct) arms entered out of the first eight attempts. Assuming completely random choices (i.e., no memory for prior visits) in the eight-arm maze task, the number of correct choices expected out of eight attempts is 5.3 [30]. Therefore, accuracy was corrected for chance performance by subtracting 5.3 from the observed number of different arms chosen out of the first eight at-

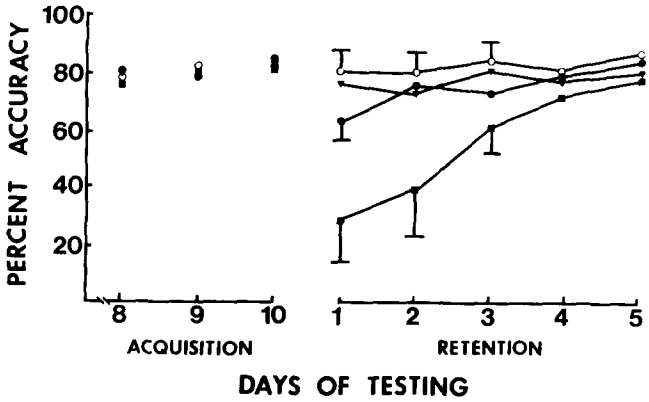

FIG. 3. Effects of EAA on radial arm maze behavior. Sixty mice were given twenty acquisition trials in the maze. Four performance-matched groups (n=15) were established on the basis of the last six acquisition trials. Mice were then treated with either vehicle or 8, 12, or 16 nanomoles EAA by intraventricular infusion. Retention testing (two trials per day for five days) was begun thirteen days after treatment. ○ Controls; ▼ 8 nanomoles EAA; ● 12 nanomoles EAA; ■ 16 nanomoles EAA.

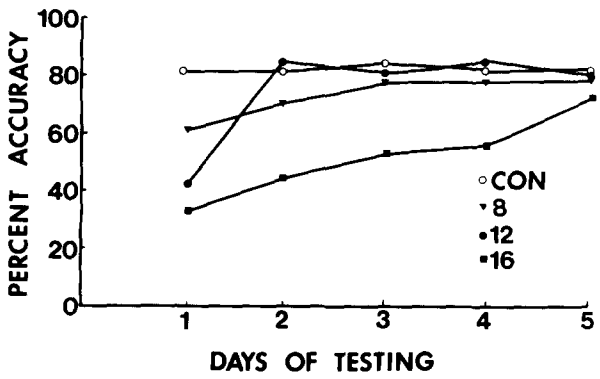

FIG. 4. Effects of EAA on radial arm maze performance in pattern response group. Mice were classified as "pattern" responders based on performance during last six trials of acquisition training, as described in the Method section. ○ Controls; ▼ 8 nanomoles EAA; ● 12 nanomoles EAA; ■ 16 nanomoles EAA.

tempts. This corrected score was then normalized by dividing by 2.7 [the difference between perfect performance (8.0) and random performance (5.3)] and multiplying by 100 to give corrected percent accuracy.

Sixty mice were trained in the eight-arm radial maze. Pretreatment acquisition training lasted for two weeks (20 trials). Four performance-matched groups of 15 mice each were established. Mice were then treated with either vehicle or 8, 12 or 16 nanomoles EAA by intraventricular infusion as previously described. Treated animals were maintained on food and water ad lib for seven days, after which water deprivation was begun as before. Retention testing (two trials per day) was initiated two weeks after treatment and one week after beginning water deprivation.

RESULTS

Neurochemical Effects of EAA

The K_T or transport constant (i.e., concentration of choline resulting in half-maximal high affinity uptake) in forebrain synaptosomes was determined to be 0.5 μM. IC_{50} values for ECA and EAA were estimated at 0.4 μM and 1.5 μM, respectively. In the presence of 0.1 mM physostigmine, the IC_{50} for EAA was increased approximately ten-fold, to 14 μM. Intracerebroventricular administration of 12 nanomoles EAA resulted in a significant reduction (27%: $p<0.05$, Student's t-test, $df=10$) in hippocampal high affinity choline uptake, three hours after treatment, whereas an equimolar dose of ECA did not (13% reduction relative to controls: $0.05<p<0.01$, Student's t-test, $df=10$) (controls=16.7±1.0 pmoles/4 minutes/mg protein; ECA=14.5±0.9; EAA=12.1±0.5). Thus, while ECA appeared to be several times more potent than EAA at inhibiting synaptosomal choline uptake *in vitro*, EAA was a more potent inhibitor of hippocampal choline uptake after intraventricular administration.

Table 1 shows lethality and levels of choline acetyltransferase (CAT), muscarinic receptor binding (QNB) and glutamic acid decarboxylase (GAD) activity, seven days after bilateral intracerebroventricular infusion of various doses of EAA. Treatment with either 4, 8 or 12 nanomoles EAA resulted in significant reduction in hippocampal CAT activity. Hippocampal muscarinic receptor (QNB) binding was not altered by any dose. (EAA was found to be an irreversible ligand for muscarinic receptors *in vitro*, $IC_{50}=6$ μM.) Glutamic acid decarboxylase activity was also unaffected by treatments.

Choline acetyltransferase (CAT) activity was measured in striatum, hippocampus and parahippocampal cortex at various times after 12 nanomoles EAA (Fig. 1). No reduction in CAT activity was seen in striatum or parahippocampal cortex at any time after treatment. A significant reduction (20%: $p<0.05$, Student's t-test, $df=10$) in hippocampal CAT activity was seen by three days after treatment. A further reduction (49%: $p<0.01$, Student's t-test, $df=10$) in hippocampal CAT activity was evident seven days after treatment, after which CAT activity appeared to stabilize over the subsequent 21 days.

Effects of EAA on Radial Arm Maze Behavior

Figure 2 shows acquisition of radial arm maze behavior. Mice performed quite well in this task, reaching mean performance levels of 7.5 correct choices out of the first eight attempts after 20 acquisition trials (corrected percent accuracy=80%). During acquisition training, it was observed that some mice adapted predictable response patterns or algorithms. For example, some mice developed the strategy of choosing adjacent arms in sequence. Another common algorithm was entry into every other arm in sequence. Mice which developed such predictable responding were classified as "pattern" performers. Others which showed no such consistent tendencies from trial to trial were classified as "variable" performers.

Mice were treated after acquistion training with either vehicle or 8, 12 or 16 nanomoles EAA. Retention testing began two weeks after treatment and differences between groups were tested for significance by the Student's t-test. There was no performance change for control mice during this two week interval (Fig. 3). Infusion of 8 nanomoles EAA had little effect on performance. A significant performance

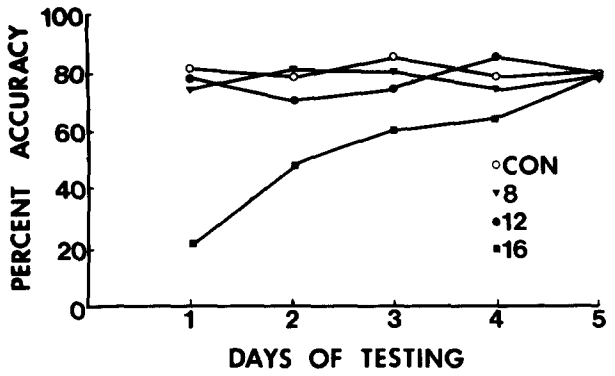

FIG. 5. Effects of EAA on radial arm maze performance in variable response group. Mice were classified as "variable" responders based on performance during last six trials of acquisition training, as described in the Method section. ○ Controls; ▼ 8 nanomoles EAA; ● 12 nanomoles EAA; ■ 16 nanomoles EAA.

TABLE 2

CHOLINE ACETYLTRANSFERASE ACTIVITY* IN HIPPOCAMPUS AND PARAHIPPOCAMPAL CORTEX 28 DAYS AFTER 8, 12 OR 16 nmoles EAA

Treatment	Hippocampus	Parahippocampal CX
Control (n=15)	10.8 ± 0.4	9.3 ± 0.5
8 nmoles (n=15)	7.0 ± 0.7†	9.1 ± 0.6
12 nmoles (n=12)	5.1 ± 0.8‡	8.5 ± 0.5
16 nmoles (n=11)	4.3 ± 1.2§	8.9 ± 0.4

*Activity expressed as μmole/hr/gram tissue ± standard error.

†Significantly different from control ($p<0.001$, Student t-test, $df=28$).

‡Significantly different from control ($p<0.001$, Student's t-test, $df=25$).

§Significantly different from control ($p<0.001$, $df=24$) and significantly different from 8 nmole group ($p<0.05$, $df=24$).

deficit ($p<0.025$, $df=25$) was seen on Day 1 following 12 nanomoles EAA. No significant deficits were shown in subsequent days at this dose. More profound behavioral deficits were seen after 16 nanomoles EAA. Significant performance deficits were seen with 16 nanomoles EAA for the first three days of retention testing as compared to controls (Day 1: $p<0.001$, $df=24$; Day 2: $p<0.001$, $df=24$; Day 3: $p<0.025$, $df=24$). By Days 4 and 5, however, no significant performance deficits were observed. Thus, EAA-induced hippocampal cholinergic dysfunction produced significant radial arm maze retention deficits which recovered in three days or less.

Figures 4 and 5 show the relative effects of toxin treatment on performance in "pattern" and "variable" response groups. A significant retention deficit was shown by the pattern response group on Day 1 following both 8 nanomoles ($p<0.05$, $df=14$) and 12 nanomoles ($p<0.05$, $df=16$) EAA, after which no significant differences were evident. No significant differences were shown in the variable response group following either 8 or 12 nanomoles EAA for any of the retention test days. However, significant and parallel deficits were shown for Days 1, 2 and 3 following 16 nanomoles EAA in both the pattern (Day 1: $p<0.05$, $df=14$; Day 2: $p<0.05$, $df=14$; Day 3: $p<0.05$, $df=14$) and variable (Day 1: $p<0.01$, $df=34$; Day 2: $p<0.05$, $df=34$; Day 3: $p<0.05$, $df=34$) response groups. Thus, the pattern response group appeared somewhat more susceptible to the behavioral toxicity of intraventricular EAA at the lower doses but both groups were similarly affected by the highest dose.

Control (vehicle-infused) mice in the pattern response group showed excellent retention of response strategy over the two week period between treatment and retention testing. However, it was observed that EAA treatment tended to disrupt the characteristic algorithms established during acquisition training. Such algorithms were completely abandoned (8 of 8 trials) on Day 1 of testing following 16 nanomoles EAA. While some EAA-treated mice redeveloped algorithms during testing, in some cases a different algorithm was "relearned." Perseveration (repetition of a sequence of choices) was evident in some mice treated with 16 nanomoles EAA.

Mice were sacrificed after 10 retention trials and CAT activity measured in hippocampus and parahippocampal cortex. Table 2 shows that hippocampal CAT activity was significantly reduced 28 days after either 8 nanomoles ($p<0.001$, Student's t-test, $df=28$), 12 nanomoles ($p<0.001$, Student's t-test, $df=25$) or 16 nanomoles ($p<0.001$, Student's t-test, $df=24$) EAA. In addition, 16 nanomoles EAA resulted in significantly greater reduction in hippocampal CAT activity than 8 nanomoles EAA ($p<0.05$, Student's t-test, $df=24$). No reduction in parahippocampal cortex CAT activity was seen after either dose.

DISCUSSION

The proposed site of action of N-ethyl-choline aziridinium (ECA) is at the presynaptic site for choline transport (high affinity choline uptake) on cholinergic terminals [11, 25, 27]. *In vitro*, ECA was 3–4 times more potent than the corresponding toxic acetylcholine analog, EAA, at inhibiting this uptake process. However, EAA was a more potent inhibitor of hippocampal high affinity choline uptake than ECA following intraventricular administration. Higher lethality was seen after intraventricular administration of ECA compared to equivalent doses of EAA (data not shown). Together, these data suggest that EAA is a more specific cholinergic neurotoxin than ECA following intraventricular administration.

Intraventricular administration of EAA (12 nanomoles) resulted in extensive and persistent reduction in hippocampal choline acetyltransferase activity, three to 28 days after treatment (Fig. 1). Hippocampal-selective toxicity has also been reported following intraventricular treatment with ECA [21,35]. Hippocampal hypersusceptibility to these toxins following intraventricular administration is most likely a result of this region's proximity to the injection site and the normal flow of cerebrospinal fluid within the ventricular system. In preliminary experiments, infusion of dye (0.5% cresyl violet) resulted in primarily staining of the paraventricular surface of dorsal and ventral hippocampus. However, there may be brain-regional differences in susceptibility to the persistent toxicity induced by these compounds [26].

Such toxin which causes persistent and specific central cholinergic dysfunction in experimental animals is a valuable tool for studying the role of cholinergic processes of higher cognitive functions. Information processing in radial arm

maze behavior is thought to be analogous to such processes utilized in human serial learning [30]. Human serial learning, which shares many common characteristics with radial arm maze behavior, is consistently impaired after treatment with anticholinergic agents [9,40]. The effects of EAA-induced cholinergic dysfunction on radial arm maze behavior in mice were therefore examined.

Contrary to the study by Mizumori *et al.* [28], these results showed that this strain of mice (CD-1 outbred albino) could acquire eight-arm radial maze behavior (Fig. 2). Mice treated with 12 or 16 nanomoles EAA showed significant performance deficits (compared to vehicle-injected controls) which were correlated with significant reductions in hippocampal CAT activity. The time-dependent loss of hippocampal CAT activity suggested that permanent changes in hippocampal cholinergic activity resulted from EAA treatment, but behavioral deficits were temporary (one to three days). We have observed similar temporary deficits in radial arm maze performance in mice treated with the toxic choline analog ECA (22 nanomoles, ICV injection, data not shown). Related may be the result that rats showed transient deficits in a reinforced T maze alternation discrimination following medial septal area lesions [17].

Behavioral deficits following EAA treatment were unlikely to result from the effects of the toxin on nonmnemonic processes such as motivation, arousal or attention. During the five days of retention testing, mice received water only as reinforcement within the maze and it was thus assumed that all animals were highly motivated to obtain the reinforcer. It was observed that all treated animals readily entered arms and consumed the available liquid. While drugs which either increase or decrease arousal (amphetamine and pentobarbital, respectively) may affect running times in the maze, accuracy in rat radial arm maze performance was unaffected by such treatments [10]. We have previously shown [35] that ECA-treated mice showed significant passive avoidance retention deficits which were associated with reductions in hippocampal choline acetyltransferase activity similar to that shown herein. Decreased arousal or attention would be predicted to result in the opposite, i.e., if the mouse was not aroused, attentive or mobile, reaction times in such a task would be slower. Similar passive avoidance deficits have been reported in rats following intraventricular administration of ECA [42].

Olton [30] proposed that animals use two different types of memory to perform accurately in the radial arm maze in much the same way that humans accomplish serial learning [48]. Reference or long-term memory retains information about the maze which is useful for all trials, e.g., the fact that reinforcement is available at the end of each arm. Animals (described herein as "pattern" performers) which develop response chains or algorithms appear to rely primarily on reference memory for solving the maze [30]; an example would be always turning right upon exiting each arm. Alternatively, short-term or working memory retains information which is useful only during a particular trial, e.g., knowledge of which arms have already been entered during that trial. Considerable evidence implicates the hippocampal formation in mediation of working memory in rats [19, 30–32]. Moreover, working memory in the rat appears selectively altered by anticholinergic agents [45,47].

Performance deficits were temporary following EAA treatment and mice treated with 16 nanomoles EAA reacquired maze performance almost identically to the original acquisition. This result was true both in mice which showed obvious response algorithms ("pattern" response group) and in mice which showed no such predictable patterns of response ("variable" response group). It is generally considered that both reference and working memory components are necessary for accurate performance in the radial arm maze [30]. However, it is not clear that the mice in these studies were utilizing spatial working memory to solve the task. In the case of the "pattern" responders, these mice apparently relied on a response strategy to solve the maze. This strategy was conserved over trials and thus, was considered a component of reference memory. The performance deficit in this group of animals following toxin treatment therefore implicates a deficit in reference memory. While there were no overt response algorithms in the "variable" response group, subtle long-term memory strategies cannot be discounted. In addition, it is assumed that all multiple-trial learning paradigms require memory for information which is pertinent over all trials, i.e., the "rules of the game" which allow the animal to perform the task. Interference with such memory would result in loss of the learned behavior, as seen herein.

Together, these data suggest that EAA-treated mice forgot how to solve the task but reacquired the learned behavior during retention testing. This suggests a long-term (reference) memory deficit was associated with toxin-induced hippocampal cholinergic lesion. This is in contrast to the reported effects of intraventricular ECA on rat radial arm maze behavior. Both Walsh *et al.* [42] and Jarrard *et al.* [20] observed persistent performance deficits in radial arm maze behavior following ECA treatment. Permanent deficits following such treatment suggested that treated animals remembered how to solve the task but could no longer remember which arms had been entered in a particular trial (short-term memory deficit). However, it should be noted that persistent behavioral deficits in these other studies could have resulted from a reference as well as working memory deficit. Somehow working memory, such as which arms have just been entered, has to be utilized to arrive at a decision. This utilization and decision process must be part of a long-term reference memory system; this is how acquisition occurs in the first place. In summary, both working and reference memory are essential for proper performance in any memory task. This then creates the possibility that deficits in either can disrupt memory performance. The experiments in this article have an advantage over most other experiments in that a subgroup of mice revealed their reference memory algorithms through a particular pattern of performance. Thus in this case reference memory was directly observable and measured.

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