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Intracortical AF64A: Memory Impairments and Recovery From Cholinergic Hypofunction

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MOUTON, P. R., E. M. MEYER AND G. W. ARENDASH. *Intracortical AF64A: Memory impairments and recovery from cholinergic hypofunction.* PHARMACOL BIOCHEM BEHAV **32**(4) 841–848, 1989.—The long-term effects of intracortical AF64A (ethylcholine mustard aziridinium ion) treatment on presynaptic cortical cholinergic markers and cognitive function in the rat were investigated. Two 1.0 μl infusions of AF64A (1 nmole/μl) or vehicle were placed bilaterally into the fronto-parietal cortex. At 3 weeks postinfusion, AF64A-treated animals were found to be deficient in passive avoidance memory retention. During weeks 4 through 10, AF64A-treated animals were markedly deficient in the extinction (memory) phase, but not the acquisition (learning) phase of 2-way active avoidance behavior. Cortical acetylcholine synthesis and high-affinity choline uptake were significantly decreased in AF64A-treated animals at 24 hours, 3 weeks, and 10 weeks following infusion. At 6 months after AF64A treatment, however, cortical cholinergic markers were not reduced compared to controls. These data indicate that memory deficits are observed during a period of cortical cholinergic hypofunction induced by cortical AF64A infusions and that a recovery from such hypofunction occurs by 6 months after these infusions.

Intracortical AF64A | Ethylcholine mustard aziridinium ion | Cholinergic hypofunction | Recovery | Nucleus basalis | Alzheimer's disease | Learning/memory | Acetylcholine

THE nucleus basalis of Meynert (NBM) of the basal forebrain contains neurons which provide the primary cholinergic innervation to the human cortex. Considerable evidence suggests that a loss or dysfunction of these neurons in senile dementia of the Alzheimer's type (SDAT) is directly causative to the cortical cholinergic hypofunction and memory deficits characteristic of this disease (3,10). A presynaptic cholinergic dysfunction appears to be indicated since presynaptic cholinergic markers (e.g., choline acetyltransferase (CAT) activity, acetylcholinesterase (ACHE) activity, high-affinity choline transport (HAChT), and acetylcholine synthesis) are found to be consistently reduced in the Alzheimer brain at autopsy (5, 6, 37).

A major cholinergic pathway in the rat brain, homologous to the "NBM to cortex" pathway in humans, originates in the basal forebrain's nucleus basalis magnocellularis (nBM) and is responsible for most of the extrinsic cholinergic innervation to the fronto-parietal cortex (11,23). In this regard, excitotoxic lesions of the nBM eliminate the "nBM to cortex" cholinergic pathway and thereby induce cortical cholinergic hypofunction and learning/memory deficiencies which mimic to a considerable degree the neurochemical and memory dysfunction of SDAT (1, 15, 33, 47). These data suggest that the nBM-lesioned rat may be an appropriate animal model for the cortical cholinergic hypofunction of SDAT. However, excitotoxic lesions of the nBM do not selectively eliminate cholinergic neurons comprising the "nBM to cortex" pathway since noncholinergic neurons in the nBM region, as well as neurons in adjacent brain areas (such as the globus pallidus), are also destroyed.

Another strategy for the development of an animal model for SDAT based on a cholinergic hypofunction would be to selectively impair cholinergic function of nBM neurons; specifically, at their neocortical projection sites where acetylcholine synthesis and metabolism occur. Choline analogs, such as ethylcholine mustard aziridinium ion (AF64A), have been shown in vivo and in vitro to induce a selective and irreversible inhibition of high-affinity choline transport (HAChT) (13, 24, 38, 39). Since HAChT carriers are specific for cholinergic terminals (12,22), and are rate-limiting for the synthesis of acetylcholine in cholinergic neurons (30,43), AF64A can produce a selective hypofunction of presynaptic cholinergic markers (41,46).

Previous in vivo studies using AF64A in rodents indicate that this agent may indeed be useful in the development of animal models for cholinergic hypofunction and cognitive dysfunction. Intracerebroventricular (ICV) AF64A infusion can induce memory deficits and significantly lower hippocampal levels of acetylcholine (13, 19, 46), HAChT (13,27), hemicholinium-3 binding (45),

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and CAT activity (27,45), while affecting cortical cholinergic markers only at high doses (13, 27, 46). In addition, intrastriatal administration of AF64A results in a specific decrease in striatal CAT activity and memory retention dysfunction (41). It should be pointed out, however, that inappropriately high doses of AF64A have the potential to cause extensive nonspecific tissue damage at or near infusion sites. In this regard, it has been suggested that the nonspecific tissue damage induced by AF64A may at least partially account for some of the behavioral and neurochemical effects that have been observed after intraventricular infusion of AF64A (25,46).

Our own studies with AF64A have involved cortical infusions of the agent to restrict its action to cholinergic terminals in the neocortex—the projection site of the "nBM to cortex" cholinergic pathway. At three weeks following intracortical AF64A infusions, we have found significant reductions in cholinergic markers (HAChT, acetylcholine synthesis and release, and hemicholinium-3 binding) within the cortex, as well as a widespread decrease in cortical AChE staining and a deficit in memory retention during passive avoidance testing (32). Furthermore, we found AF64A's effects to be specific for the cholinergic system in that no effects on cortical GAD activity, monoaminergic markers, or neuropeptide levels were observed (32). Moreover, the dose of AF64A infused (1 nmol/μl) resulted in minimal necrosis at infusion sites (4% of fronto-parietal cortex), avoiding the potential for nonspecific effects of AF64A which may occur at inappropriately high doses (19,40). We have, thus, suggested that low doses of AF64A at multiple infusion sites provide an optimal means of observing this agent's specific pharmacological effects in the neocortex, while ensuring a minimum level of incidental damage to surrounding neural tissue.

However, it is not known what time course exists for the cholinergic hypofunction and behavioral deficits observed after infusion of such low doses of AF64A into the rat cortex. The present study used multiple, intracortical AF64A infusions at the previously determined appropriate dose (i.e., 1 nmol/μl) to investigate both short- and long-term effects of AF64A treatment on cortical cholinergic markers, as well as effects of this agent on learning/memory abilities during both passive avoidance and 2-way active avoidance testing. We now report that such AF64A-treated rats exhibited both short- and long-term, but not permanent, decreases in presynaptic cholinergic markers within the cortex, as well as memory dysfunction during the period of cholinergic hypofunction.

METHOD

Subjects

All animals used in these experiments were Sprague-Dawley rats weighing 275–350 grams and were maintained on a 14 hr light/10 hr dark cycle. Water was available ad lib throughout the experiments, as was food (Purina Rat Chow).

General Protocol

Animals receiving AF64A or vehicle infusions were included in one of three experimental groups. Animals in Group 1 were analyzed biochemically for cortical cholinergic markers at 24 hours or 10 weeks postinfusion; those animals sacrificed at 10 weeks for biochemical analysis were behaviorally tested for passive avoidance at 3 weeks postinfusion. Animals in Group 2 were tested for passive avoidance at 3 weeks following infusion and were sacrificed immediately thereafter for cortical cholinergic marker determinations. Animals in Group 3 were tested for passive avoidance behavior at 3 weeks postinfusion, followed by active avoidance during weeks 4 through 10 postinfusion. These animals were sacrificed 6 months after infusion and their cortices analyzed for cholinergic markers.

AF64A Preparation

AF64A was synthesized from acetyl-AF64 according to (18) and frozen at −70°C until the day of surgery. At that time, the structure and purity of acetyl-AF64 was verified by index of refraction, infrared spectrometry, and nuclear magnetic resonance analyses. Acetyl-AF64A was rapidly dissolved in distilled water (2.0 mg/ml). The pH was adjusted to 11.5 with a small amount of 10 N NaOH (1.0 μl/ml) and maintained between 11.5 and 11.8 for 30 minutes by addition of 1 N NaOH (10 μl/ml). The pH was reduced to 7.4 with 6 N HCl (1.5 μl/ml). Distilled water was added to bring the final concentration of AF64A to 1.0 nmol/μl. Solutions of AF64A were then allowed to sit at room temperature for 60 minutes and were infused intracerebrally within 6 hours of cyclization. For the preparation of vehicle (control) solutions on the day of surgery, 2 ml of distilled water was adjusted to pH 11.5 with 10 N NaOH and processed exactly like AF64A to a final pH of 7.4.

Surgery

Using Nembutal (sodium pentobarbital) anesthesia (50 mg/kg, IP), animals received two cortical infusions of AF64A-HCl or vehicle into each hemisphere of the brain (4 infusions total). The dose of AF64A was 1 nmol/μl at each site—an amount we previously showed to result in minimal, nonspecific damage in the neocortex (32). Infusions were done at a rate of 0.5 μl/min utilizing a 10.0 μl Hamilton microliter syringe mounted on a Trent Wells stereotaxic apparatus; the syringe was left in place for 5 minutes following infusion and then slowly withdrawn. Stereotaxic coordinates at two levels in the fronto-parietal cortex were: Level I—1.85 mm anterior to bregma, 3.8 mm lateral to midline, and 1.6 mm ventral to the dura mater; and, Level II—2.0 mm posterior to bregma, 4.0 mm lateral, and 1.6 mm ventral to the dura mater.

Neurological Examination

To rule out any possible effects of cortical infusions on motor system function, animals in Group No. 3 were tested for movement and postural abilities (31) at 16 weeks postinfusion.

Behavioral Testing

Passive avoidance. The passive avoidance apparatus consisted of a rectangular Plexiglas box, divided into two compartments by a metal wall, and having a metal grid floor. A sliding guillotine door was located in the common wall separating the two compartments. One of the two chambers in the box was illuminated, the other was dark.

Three weeks after AF64A or vehicle infusions, animals were placed in the lighted compartment of the apparatus and allowed 30 seconds to acclimate to the surroundings. The guillotine door between the compartments was then raised and a timer started. After the rat moved into the darkened chamber though the opened door, the door was closed. Typically, rats move from the lighted to the dark compartment after only a few seconds latency (T_0) because of their inherent preference for a dark environment. The rat was given a 2-second, 2-mA foot shock and then removed from the apparatus and confined to a holding cage for 60 seconds. After this period, the rat was placed into the lighted compartment of the apparatus once again; the sliding door between compartments was opened and a timer started. Latency time to place all four paws in the dark chamber was recorded up to 5 minutes. An animal's

ability to learn and remember the shock after 60 seconds by not entering the dark chamber is considered a measure of learning and/or short-term memory. Similarly, 24 hours after receiving the shock, rats were again placed into the lighted chamber and allowed to orient for 30 seconds; the door was opened and the animals were given a five-minute opportunity to enter the dark chamber. At 24 hours after receiving the floor shock, latency time to enter the dark chamber is an indicator of long-term memory retention of the initial shock trial; superior memory is ascribed to those animals refusing to enter or doing so only after a long latency.

Two-way active avoidance. The two-way active avoidance apparatus consisted of a standard rectangular shuttle box with Plexiglas walls, the floor of which was a metal wire grid. A tone could be sounded through a device located in the ceiling of the shuttle box.

Active avoidance testing was divided into an acquisition (learning) and an extinction (memory) phase. The classical conditioning paradigm used in this test involved the sequence of a 1-second tone and a 4-second delay followed by a 1.5-mA foot shock. A conditioned avoidance response (CAR) occurred when the animal moved to the opposite side of the box during the time between the tone's initiation and the shock. If the animal did not move across the box during this period, the shock was automatically activated (up to a maximum of 15 seconds) until the animal "escaped" to the other side. Rats were given 10 trials per day. If the acquisition criteria of 80% or greater CARs on two consecutive days was achieved, the animal was started on extinction testing. For the extinction phase of testing, the tone was sounded but no shock was applied if the animal did not move across the box (i.e., did not commit a CAR). Thus, extinction can be considered a measure of the animal's general memory ability; normally, a steady decline in CARs as a function of time occurs during extinction when the tone is no longer paired with a foot shock. A performance criteria of 60% CARs on two consecutive days during the 30 days of acquisition testing had to be achieved by animals for inclusion in the extinction data analyses. Extinction trials were discontinued after extinction criteria was met (two consecutive days of 0% CARs) or after 10 days of testing.

Neurochemical Analysis

Synaptosome preparation. Animals were decapitated at 24 hr, 3 weeks, 10 weeks, and 6–7 months after cortical infusions of either AF64A or vehicle. Their fronto-parietal cortices were rapidly dissected out and placed in 0.32 M sucrose containing 10 μM Tris buffer (pH 7.4) and 100 μM EDTA. Synaptosomes were prepared essentially as described previously (29) and resuspended in oxygenated (5 min with 95% O_2/5% CO_2) Krebs-Ringer bicarbonate (KR) buffer, pH 7.4, with 8–12 mg protein/ml.

(^{3}H)-Choline uptake and (^{3}H)-ACh synthesis. Synaptosomes were incubated in oxygenated KR (0.4–0.8 mg protein/ml) at 37°C for 5 min with either 1 μM or 20 μM (^{3}H)-choline. Other samples were treated identically, except that 5 μM hemicholinium-3 was added to block high-affinity choline uptake. The samples were placed on ice for several minutes and then centrifuged at 25,000 × g for 5 minutes at 4°C. The pellets were surface washed twice with KR containing 100 μM eserine, and then lysed with 100 μM eserine in water. The lysate was assayed for total (^{3}H)-choline uptake and newly synthesized ACh as described previously (30). This radioenzymatic assay involved phosphorylating the (^{3}H)-choline with ATP via choline kinase, followed by ion-pair extraction of (^{3}H)-ACh bound to tetraphenylboron into butrylnitrile. The recovery of (^{3}H)-choline (which coextracts with ACh) was over 90%, and the residual unphosphorylated (^{3}H)-choline (which coextracts with ACh) was less than 5% of the original (^{3}H)-choline, as determined by recovery tubes run concomitantly. The newly synthesized total (^{3}H)-choline uptake and (^{3}H)-ACh were measured via liquid scintillation spectrophotometry. Each choline concentration was performed in duplicate. High-affinity choline uptake was determined by subtracting the two averaged hemicholinium-3-treated values from those without hemicholinium-3 for each animal at each choline concentration. Low-affinity choline uptake was defined as that seen in the presence of hemicholinium-3.

Chemicals. All radioactive chemicals and drugs (except AF64A) were obtained form Sigma Chemical Co., St. Louis, MO. (^{3}H)-choline (80 Ci/mmol) and (^{3}H)-hemicholinium-3 (120 Ci/mmol) were purchased from New England Nuclear Co., Boston, MA.

Protein assays. Protein levels were approximated using the Biorad assay, which involves the staining of peptide bonds with Coomasie Blue (7).

Statistical Analyses

Biochemical and behavioral data are expressed as mean ± SEM. Analysis of variance (ANOVA) was used to determine group differences, except for passive avoidance data in which equal error variance cannot be assumed; therefore, a nonparametric Kruskal-Wallis analysis of passive avoidance was done. Active avoidance data were analyzed for acquisition and extinction using repeated measures ANOVA, with post hoc testing of individual block differences by the method of least significant differences (LSD). Probability values less than 0.05 were considered significant.

RESULTS

Histology

Figure 1 shows several histological sections from an AF64A-treated rat to indicate the location of anterior and posterior infusion sites within the fronto-parietal cortex. Based on our previous report (32), the dose of 1 nmol AF64A in 1 μl results in minimal nonspecific tissue damage at the infusion sites, averaging only about 4% of the total fronto-parietal cortical area for all infusion sites combined in any given animal. Vehicle infusion produced a limited necrosis at cortical injection sites that was not significantly different from that observed after infusions of 1 nmol/μl AF64A. This dose of AF64A induces a qualitative reduction in AChE staining throughout most (75%) of the fronto-parietal cortex (32), while not affecting AChE staining in subcortical structures (Fig. 1B and C). It therefore appears that the corpus callosum is an effective barrier for preventing any significant diffusion of cortically-infused AF64A to subcortical areas such as the corpus striatum, septum, and hippocampus.

Behavioral Testing

Neurological examination. All AF64A- and vehicle-treated animals tested normal on a variety of neurological tests for movement and postural abilities. Included in the examination were: 1) tremor while stationary or in motion; 2) head and body elevation at rest or in motion; 3) postural symmetry and body muscle tonus; 4) locomotor activity; 5) hopping and righting reflexes; and 6) balance and traversal of a narrow beam. Therefore, no evidence for a general deleterious effect of intracortical AF64A on rat motor function was found.

Passive avoidance. No difference in entry latency was present between AF64A- and vehicle-treated groups for the acquisition (T_0) trial, indicating the groups were not differentially motivated in the step-through task to enter the dark chamber prior to receiving the shock (Fig. 2). At 60 seconds after the foot shock,

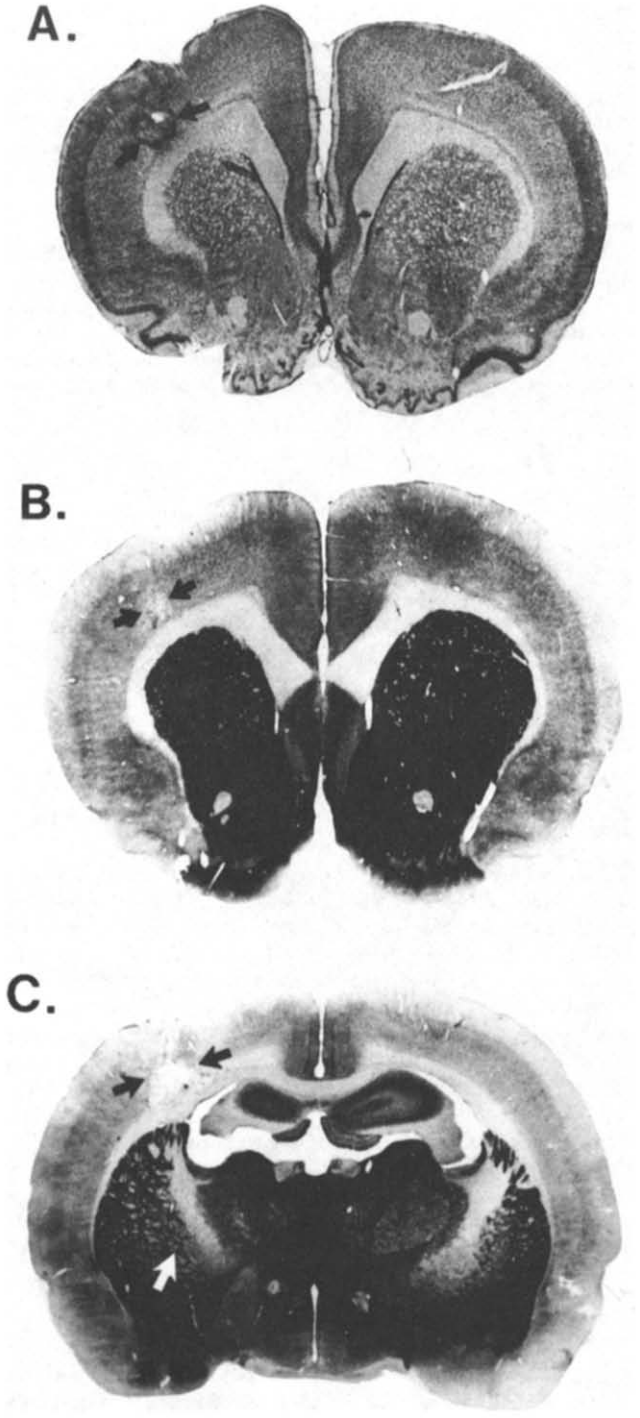

FIG. 1. Coronal brain sections through the largest extent of necrotic damage (dark arrows) resulting from a unilateral anterior cortex infusion of AF64A (1 nmol/μl) done 3 weeks prior to sacrifice. (A) A thionin-stained brain section showing no qualitative effect of the AF64A infusion on cortical cell numbers. (B) An adjacent AChE-stained brain section demonstrating decreased cortical AChE staining on the infusion side and no decreased staining subcortically in the caudate-putamen. (C) An AChE-stained brain section of the same animal at a posterior infusion site. Light arrow indicates location of the nucleus basalis magnocellularis (nBM).

there was again no difference in latencies to enter the dark chamber between AF64A- and vehicle-treated animals, with both

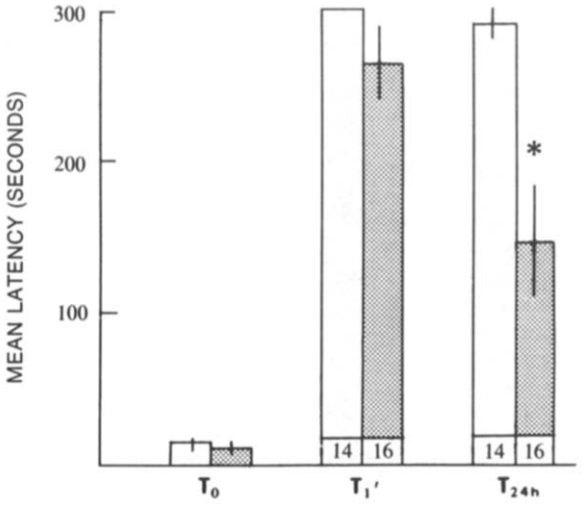

FIG. 2. Effects of bilateral AF64A (stippled) or vehicle infusions into the fronto-parietal cortex on multiple trial passive avoidance behavior at 3 weeks postinfusion. T_0, $T_{1'}$, T_{24} refer to preshock, 60-second, and 24-hour latencies, respectively. Asterisk indicates significantly different entry latency from vehicle-infused controls; *$p<0.001$.

groups showing high entry latencies. However, during the 24-hour postshock test (Fig. 2), AF64A-treated animals were markedly deficient in memory retention compared to vehicle-treated control rats since their entry latencies were significantly shorter ($p<0.001$).

Active avoidance. In the acquisition phase of testing in which the animal must learn to pair the tone and the footshock, no significant overall group differences or group by blocks interaction were seen between AF64A and control groups (Fig. 3). However, both AF64A and control groups did show a significant effect of training, $F(5,90)=54.80$, $p<0.001$.

For extinction testing, 80% of the AF64A-treated (n=8) and 90% of the vehicle-treated animals (n=9) achieved the performance criteria of 60% conditioned avoidance responses (CARs) on two consecutive days of acquisition testing and were, therefore, included in the extinction data analysis. During the extinction phase of testing, an overall group difference was observed, $F(1,15)=5.45$, $p<0.05$, as was an extinction difference by blocks, $F(4,60)=13.70$, $p<0.01$; thus, both groups reached extinction in the task and this extinction occurred at a significantly greater rate in the AF64A group compared to the control group (Fig. 3). Furthermore, post hoc LSD analysis of groups by individual blocks during extinction showed memory of the task to be significantly decreased for AF64A-treated compared to control animals on blocks 1 and 5 of extinction testing.

Neurochemical Effects of AF64A

At both 1 and 20 μM of added (^{3}H)-choline, acetylcholine synthesis in AF64A-treated animals was significantly decreased at 24 hours (−33% and −18%), 3 weeks (−43% and −27%), and 10 weeks (−21% and −25%) following cortical infusions (Fig. 4). In contrast, no differences in cortical acetylcholine synthesis were observed in AF64A-treated rats at 6 months postinfusion using either 1 or 20 μM of radiolabelled choline. Analyses of cortical high-affinity choline transport (HAChT) at 1 μM (^{3}H)-choline concentration showed AF64A-treated animals to have significantly decreased HAChT at the 24-hour (−44%), 3-week (−31%), and 10-week (−24%) time points. At the less specific 20 μM (^{3}H)-choline concentration, cortical HAChT was signifi-

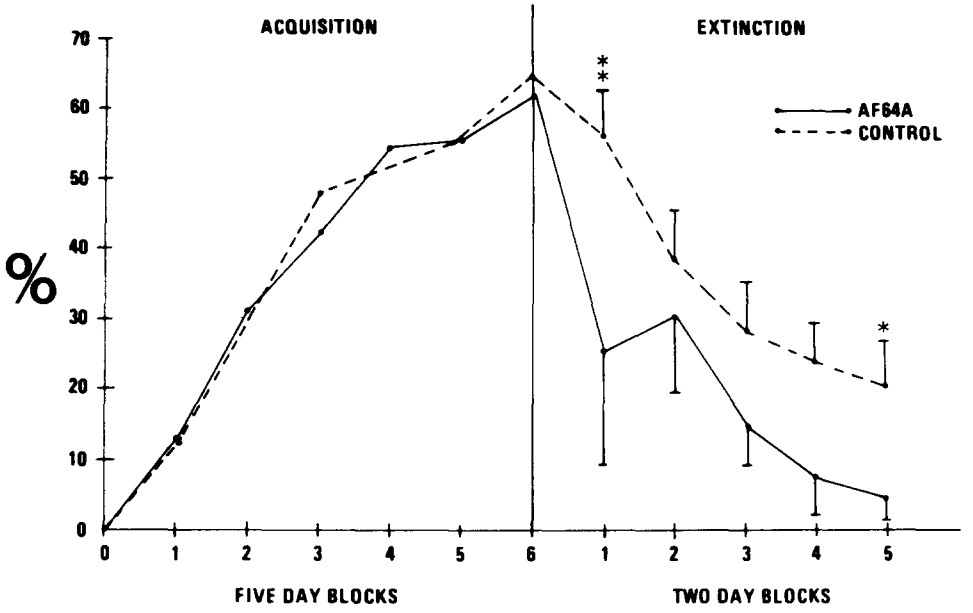

FIG. 3. Effects of intracortical AF64A infusion on 2-way acquisition and extinction active avoidance behavior. Data for 30 days of acquisition testing are presented in six 5-day blocks and 10 days of extinction data are presented in five 2-day blocks. % = mean percentage conditioned avoidance responses. Asterisks indicate significantly fewer CARs for AF64A-treated animals compared to vehicle controls for individual time blocks; $*p<0.05$, $**p<0.01$.

cantly suppressed only at 24 hours and 3 weeks following cortical infusion of AF64A. AF64A had no affect on HAChT by 6 months postinfusion, irrespective of whether 1 or 20 μM (^{3}H)-choline was added. Using 1 or 20 μM (^{3}H)-choline, low-affinity choline transport (LAChT) was significantly decreased at both 24 hours and 3 weeks postinfusion (Fig. 4). However, at the 10-week and 6-month time points, no differences in LAChT were seen, irrespective of whether 1 or 20 μM (^{3}H)-choline had been added. Similarly, CAT activity, measured at the 6-month time point, failed to show significant differences between AF64A- and vehicle-treated groups (3.1 ± 0.3 and 2.9 ± 0.2 nmol/mg/15 min, respectively).

DISCUSSION

A dysfunction of the "NBM to cortex" cholinergic pathway appears to be well established in the pathogenesis and symptomology of SDAT (4,10). In this context, the choline analog AF64A has been proposed as a useful agent to develop an animal model for the cholinergic hypofunction and memory deficits of SDAT (14). To induce preferentially a hypofunction of neocortical cholinergic terminals, we utilized intracortical infusions of AF64A. Our results indicate AF64A can induce a long-term cholinergic hypofunction in the cortex and behavioral impairments at least through 10 weeks postinfusion; however, a recovery of cortical cholinergic markers occurs by 6 months after such infusion.

These results are consistent with our previous work (32), in which we reported selective deficits in cortical cholinergic markers (HAChT, ACh synthesis, ACh release, HC-3 binding) and impaired passive avoidance memory retention at 3 weeks following intracortical AF64A infusion. Both of our studies utilized a dose of 1 nmol AF64A in 1 μl at 4 discrete sites in the fronto-parietal cortex. To avoid potential problems of nonspecificity at inappropriately high AF64A doses (19,25), our dose was carefully selected based on: 1) a minimal amount of nonspecific tissue damage at infusion sites, 2) no effect on cortical GAD activity, cortical monoaminergic markers, or cortical levels of the neuropeptides somatostatin and neuropeptide Y, and 3) a diffusion to 75% of the frontoparietal cortex, as evidenced by decreases in acetylcholinesterase staining (32). The present study sought to define in a more detailed manner the time course for AF64A-induced cholinergic hypofunction in the cortex and to determine any relationship that may exist between this hypofunction and the cognitive performance of treated rats in several tasks.

Neurochemically, we found that cortical cholinergic function (i.e., acetylcholine synthesis and HAChT) was most significantly affected from 24 hours through 3 weeks post-AF64A infusion. By 10 weeks, levels of cholinergic markers, though still significantly decreased, began returning to control levels. By 6 months after intracortical AF64A, a recovery of cortical cholinergic markers had occurred since ACh synthesis and HAChT were no longer different from controls. Due to its structural similarity to choline and the presence of the aziridinium ring, AF64A can selectively and irreversibly inhibit choline transport in cholinergic terminals, presumably by binding to HAChT carriers located on cholinergic terminals (12, 39, 40). Since HAChT is restricted to cholinergic neurons and is tightly linked to acetylcholine synthesis (16, 22, 30, 43), such an inhibition can result in a prolonged cholinergic hypofunction, as the present study indicates. These findings concur with earlier studies showing that: 1) AF64A acts as an irreversible inhibitor of HAChT on synaptosomes (39); 2) intraventricular AF64A decreases acetylcholine release and HAChT in hippocampal slices (24); and, 3) intraventricular infusions of AF64A can result in reductions in hippocampal and frontal cortex acetylcholine levels (46). It should be mentioned, however, that these later studies utilized relatively high doses of AF64A intracerebroventricularly, as compared to the low doses used in the present study by the intracortical route.

This is the first study to demonstrate recovery from a cholinergic hypofunction induced by AF64A infusions. One likely

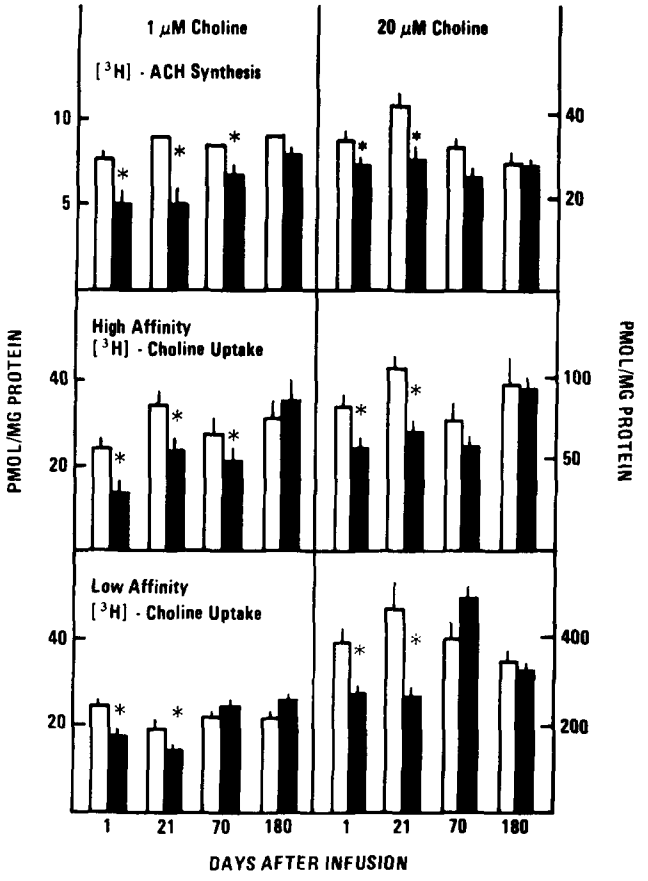

FIG. 4. (^{3}H)-Acetylcholine synthesis, high-affinity (^{3}H)-choline uptake, and low-affinity (^{3}H)-choline uptake following bilateral AF64A or vehicle infusion into the fronto-parietal cortex at 24 hours, 3 weeks, 10 weeks, and 6 months prior to sacrifice. Measurement was by liquid scintillation spectrophotometry following preincubation with either 1 μM or 20 μM (^{3}H)-choline. Asterisks indicate significant differences between AF64A- and vehicle-treated groups at $p<0.05$ or lower. The brains of 5 animals were analyzed for each time point.

explanation for such a recovery would involve gradual replacement of high-affinity choline transport carriers in presynaptic cholinergic terminals following AF64A infusion. Alternatively, this neurochemical recovery may reflect compensatory collateral sprouting of cortical cholinergic afferent, as has been shown to occur in hippocampus following incomplete fornix transection (15). It is not known whether intracortical AF64A is acting on terminals of extrinsic, or intrinsic cholinergic neurons or both (21).

No differences were seen in intracortical CAT activity six months after intracortical AF64A infusions. This finding is consistent with our earlier findings at the 3-week post-AF64A infusion time point (32) in which intracortical AF64A infusion did not result in decreased cortical CAT activity. An inability of AF64A to affect CAT activity would be predicted from studies showing that the inhibition of high-affinity choline transport by AF64A does not include any direct effects on CAT activity (2) and the affinity of AF64A for CAT is 1000 times less than that for the HAChT system (27).

The AF64A-induced passive and active avoidance deficits observed within the time period of AF64A's most profound effects on cortical cholinergic markers strongly suggest the involvement of cholinergic terminals from extrinsic nBM neurons since interruption of the "nBM to cortex" pathway through nBM lesioning results in learning and memory impairments. With respect to passive avoidance behavior, we confirm our earlier findings of a deficit in memory retention 3 weeks after intracortical AF64A treatment (32). Since lesions of the nBM result in similar deficits (1, 4, 15), the passive avoidance results of the present study are consistent with an AF64A-induced hypofunction of the "nBM to cortex" cholinergic pathway. AF64A-induced deficits in step-through passive avoidance tasks have been previously reported (8,46), but it is significant that substantially higher doses of AF64A were infused in these studies via the intracerebroventricular route, thus affecting a number of CNS cholinergic populations and leaving open the possibility that nonspecific damage to the septo-hippocampal pathway was responsible for these AF64A-induced memory deficits (19). Additionally, impairments in passive avoidance behavior following the intrastriatal infusion of AF64A have been reported (41), suggesting a role for striatal cholinergic neurons in cognitive processes.

Through the use of the 2-way active avoidance task, which involves both an acquisition (learning) and extinction (memory) component, we find that the effects of intracortical AF64A appear to be restricted to extinction; that is, AF64A-treated animals exhibited a memory dysfunction by extinguishing a learned behavior faster than control animals. In contrast, following lesions of the nBM, we (1) and others (15,26) have found deficiencies in both acquisition and extinction components using the same active avoidance paradigm. Although these dysfunctions in active avoidance behavior after nBM lesioning are probably due, in large part, to an interruption of the "nBM to cortex" cholinergic pathway, the present AF64A study suggests that the acquisitional dysfunction induced by nBM lesions may be due to either a destruction of noncholinergic neurons in the nBM and/or destruction of neurons in the adjacent globus pallidus and corpus striatum—two structures involved with passive avoidance behavior (15, 36, 42). Our results with cortical AF64A infusion indicate that only the memory phase of active avoidance behavior is affected by an AF64A-induced cholinergic hypofunction in the neocortex.

Qualitatively at least, intracortical AF64A can induce a similar reduction in presynaptic cholinergic markers (except for CAT activity) as is seen in senile dementia of the Alzheimer's type (SDAT); this, by affecting what may be a primary site of dysfunction, namely the cortical cholinergic terminal (34,35). Since several studies have reported either a loss or atrophy of neurons in the NBM of SDAT brains (3, 10, 34, 35), it may be that some cholinergic neurons comprising the "NBM to cortex" pathway of SDAT brains die while others survive in a dysfunctional state. If so, intracortical AF64A may have the potential to mimic this condition since we have recently found that 3 weeks following intracortical AF64A treatment, 18% of cholinergic neurons in the rat nBM have died and the surviving neurons are substantially atrophied (Mouton and Arendash, in review).

In summary, these data confirm our earlier study showing cortical cholinergic hypofunction and memory impairments at 3 weeks following intracortical AF64A treatment and further extend those findings by indicating that cholinergic markers are significantly decreased along a time course from 24 hours through at least 10 weeks postinfusion; by 6 months, presynaptic cholinergic markers show a recovery to control levels. Furthermore, cognitive impairments observed during the period of cortical cholinergic hypofunction suggest that a direct and specific relationship exists between these impairments and the AF64A-induced hypofunction.

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