The Biochemical and Ultrastructural Examinations in Central Cholinergic Damage of the Rat Induced by the Intraperitoneal Administration of AF64A

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Accepted August 24, 1990

Abstract—Ethylcholine mustard aziridinium ion (AF64A), a synthesized cholinergic neurotoxin, was administered via intraperitoneal injection to the rat to study its effect on the central cholinergic nervous system. A single or consecutive daily injection of AF64A for 10 days resulted in a persistent reduction of acetylcholine (ACh) content in the several tested regions of the brain in the following order: hippocampus > cerebral cortex = striatum, the degree was the greatest in the hippocampus. Both resting and K+ stimulated release of ACh from the hippocampus were also significantly reduced 24 hr after a single injection of AF64A. Furthermore, daily injection of AF64A for 10 days induced a significant reduction of choline acetyltransferase (ChAT) activity in the homogenate obtained from the hippocampus but not from the cerebral cortex and striatum. ChAT activity in the crude synaptosomal fraction of the cerebral cortex was also significantly decreased. These results suggest that intraperitoneal administration of AF64A could induce cholinergic hypofunction more selectively in the nerve terminals. The high affinity choline uptake, which is located mainly on cholinergic nerve terminals, was not affected by the administration of AF64A. Any notable changes of ultrastructure in the cholinergic nerve terminals after the administration were not observed in all three regions examined. The present findings suggested that intraperitoneal administration of AF64A induces a specific damage of cholinergic nerve terminals by inhibiting ChAT activity. The cholinergic damage was most prominent in the hippocampus.

Choline mustard analogues have been suggested as a useful tool in developing an animal model of selective central cholinergic hypofunction (1). One of these analogues, ethylcholine mustard aziridinium ion (AF64A) has been reported to have persistent neurotoxic effects on the cholinergic nerve terminals (2-4). Administration of AF64A in vivo resulted in persistent reduction of several markers for cholinergic neurons including acetylcholine (ACh) content, ACh release, choline acetyltransferase (ChAT) activity and high affinity choline uptake (HACU) activity (2-7). Alterations in these biochemical features are accompanied by marked deficits in cognitive behavioral tasks (6, 8-10). The persistent neurotoxic effects of AF64A on the cholinergic neurons have been expected as a promising drug to develop a model for Alzheimer's disease which is characterized by a progressive, chronic cognitive dysfunction of the central cholinergic system (11). Almost all studies on the effect of AF64A in vivo have been carried out by its intraventricular administration or administrations into restricted brain regions. However, such administration of AF64A into the brain might expose the injection site to a higher dose of the drug (12) and thus cause non-specific lesions including a circumscribed necrotic region at the injection site (9, 13, 14), because AF64A has a potent alkylating action by a highly reactive...
aziridinium ion capable of nucleophilic attack (11, 15).

In the present study, we administered AF64A intraperitoneally to examine its effect on the central cholinergic nervous system in order to exclude the non-specific effects and investigated the effects of AF64A on several regions of the brain by morphological and biochemical methods. Results obtained by biochemical methods were focused on the degree of the alteration in cholinergic markers among several different regions of the brain. Examinations by electron-microscopy were carried out in order to clarify the ultrastructural damage in the neurons caused by intraperitoneal administration of AF64A.

The results suggested that AF64A administered intraperitoneally distributed evenly in the brain at lower concentration and induced persistent selective neurotoxic effects on the central cholinergic neurons.

Materials and Methods

Animals: Male Wistar rats (Shizuoka Laboratory Animal Center) approximately 50 days old and weighing between 160 and 200 g were used in the following experiments. The rats were housed in groups of 3 and maintained in a temperature-controlled colony room with a 12 hr light/dark cycle. Water and food were freely available at all times.

Preparation and administration of AF64A: AF64A was freshly prepared from ethylcholine mustard (AF64) picrate by the method of Fisher et al. (16). Briefly, AF64 picrate was dissolved in saline, and picrate was removed by passing through a small column containing the anion exchange resin (Dowex 1 x 4 Cl type, 200–400 mesh). The pH of the solution was then adjusted to 7.4 with solid NaHCO₃ and allowed to stand at room temperature for at least 60 min before administration to obtain sufficient conversion of AF64A to the aziridinium form. Aziridinium ion formation was determined by a thiosulfate ion consumption-titration method (17). The dose of AF64A reported in this paper was corrected using the conversion-rate determined by this titration method. AF64A was administered intraperitoneally in a single (23.7 µmol/kg) dose or by daily injections for 10 days (initial dose 21.0 µmol/kg/day, maintenance dose 3.1 µmol/kg/day for 9 days). Control rats were given saline.

ACh content: Rats were killed by microwave irradiation (5 kW, 2,450±30 MHz, 1.0–1.1 sec) focussed on the head to prevent post-mortem changes in ACh content. Cerebral cortex, hippocampus and striatum were excised and weighed. ACh was extracted from three regions of the brain as described by Proscott et al. (18). Tissue samples were placed in glass-teflon homogenizer containing 9 volumes of formic acid-acetone (1 N formic acid : acetone 3:17) and homogenized. The homogenates were kept ice-cold for 30 min and then centrifuged at 10,000×g for 20 min. The supernatants were lyophilized and stored at −20°C until the assay. The ACh content of the samples was estimated by bioassay on the guinea pig ileum.

ACh release: After decapitation, the bilateral hippocampus was excised and cut into four blocks on the ice-cold plate. The blocks were placed into a 3-ml incubation chamber, thermostated at 37°C and continuously oxygenated with 98% O₂ and 5% CO₂, containing 2 ml of Krebs solution of the following composition: 128 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.6 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM glucose. Physostigmine salicylate (50 µM) was added to the Krebs solution. High-K⁺ (23.4 mM) Krebs solution was kept iso-osmotic by appropriate changes in the Na⁺ concentration. After the blocks of the tissue were superfused at a rate of 0.6 ml/min for 30 min to allow equilibration, the perfusion was stopped, and the bath fluid was replaced with fresh Krebs solution to determine the spontaneous ACh release. The bath fluid was also replaced with Krebs solution containing 23.4 mM KCl when high-K⁺-stimulated ACh release was determined. After incubation of 30 min, the bath fluid was collected to assay the amount of ACh. The collected samples were gently shaken with one tenth volume of Amberlite XAD-2 for 20 min and filtrated to remove endogenous prostaglandins that might be present in the samples (19).

The net amount of ACh released by high-K⁺ was calculated by subtracting the amount of spontaneous release from the total amount
released by high K+ Release of ACh was expressed as pmol/g wet tissue/min.

Assay of ACh: ACh in the sample was determined by bioassay against ACh on the longitudinal muscle strip from guinea pig ileum as described by Yagasaki et al. (20). The substance in the sample was identified as ACh by demonstrating that it was destroyed by boiling in alkali and that its action was antagonized by atropine.

ChAT activity: 1) In homogenate preparation: Each region of the brain was weighed and homogenized in 10 volumes of ice-cold 10 mM EDTA (pH 7.4) solution containing 0.5% (v/v) Triton X-100. The supernatant after centrifugation at 100,000 x g for 30 min was used for the assay of ChAT activity. 2) In synaptosomal preparation: Cerebral cortex was homogenized in 10 volumes of ice-cold 0.32 M sucrose, and a crude synaptosomal fraction (P2) was prepared as described by Whittaker (21). Homogenates were centrifuged at 1,000 x g for 10 min. The supernatant after centrifugation at 100,000 x g for 40 min, and the resulting pellet (P2) was resuspended in 10 volumes of ice-cold 10 mM EDTA (pH 7.4) solution containing 0.5% (v/v) Triton X-100. The supernatant after centrifugation at 100,000 x g for 30 min was used for the assay of ChAT activity. 3) ChAT activity in the homogenates or synaptosomal fraction was measured by radiochemical assay according to the method of Fonnum (22). [1-14C] Acetyl-CoA was used as the substrate and sodium tetraphenylboron was used to isolate labeled ACh. Ten microliters of samples were added to 4 μl of substrate solution containing 50 mM sodium phosphate buffer (pH 7.4), NaCl (300 mM), ChCl (8 mM), EDTA (20 mM), eserine salicylate (0.1 mM), and [1-14C]acetyl-CoA (0.2 mM, 5 μCi/ml). After an incubation for 15 min at 37°C, the reaction was stopped by cooling the samples in ice. The reaction mixture was transferred into a vial by washing out with 5 ml of 10 mM sodium phosphate buffer (pH 7.4). Two milliliters of tetraphenylboron-acetonitrile solution (5 mg/ml) was added to extract ACh into an organic phase. In addition, 10 ml of toluene scintillation fluid, containing 5 mg diphenyloxazole and 2 mg 1,4-bis-(4 methyl-5-phenyloxazole-yl) benzene per 10 ml of toluene, was added and mixed well. After leaving the sample in the cold room for 3 hr, radioactivity was counted in a liquid scintillation spectrometer.

HACU: Choline uptake was measured by the method of Simon et al. (23) with some modifications. The crude synaptosomal fraction (P2) was prepared from the cerebral cortex, hippocampus and striatum, as described above. One hundred microliters of the synaptosomal suspension in Na+-free Krebs-Ringer (NaCl was replaced by sucrose and NaHCO3 was replaced by 15 mM Tris HCl (pH 7.4)) was added to 0.89 ml normal Krebs-Ringer and then pre-incubated for 5 min at 37°C while being aerated with 95% O2 and 5% CO2. Reaction was started by addition of 10 μl [Me-14C]choline (1 μM, 54.5 nCi/ml). After incubation for 4 min at 37°C, the reaction was stopped by rapid cooling on ice, and then the mixture was passed through a Sartorius membrane filter (SM 11606, pore size of 0.45 μm). After washing twice with 10 ml of ice-cold Krebs-Ringer, the filter was placed in a vial, soaked in 0.1 ml of soluene-350 for 24 hr and then added to the scintillation fluid. [14C]Choline uptake of the preparations was calculated by subtracting the radioactivity in the experiment under the Na+-free condition from that in the normal and expressed as pmol/mg protein/4 min.

Electron microscopic observation: The rats were anesthesied with pentobarbital (50 mg/kg, i.p.). Brain tissues were fixed by perfusion with 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.4, rapidly removed and then separated into the cerebral cortex, hippocampus and striatum on an ice-cold plate. These tissues were cut into small rectangular pieces and fixed for further 2 hr at 0°C by the same mixture. After washing with 0.1 M sodium phosphate buffer, tissues were postfixed for 2 hr in 2% osmium oxide in the same buffer in an ice-cold bath. These samples were dehydrated and embedded in Epon 812. Thin sections were mounted on slit grids, contrasted with uranyl acetate and lead citrate and examined in a Hitachi H-600 electron microscope.

Protein assay: Protein was measured by the method of Lowry et al. (24).

Data analysis: All data are given as means±
S.E.M. The statistical significance was evaluated by Student’s t-test.

Results

In the case of intraperitoneal daily injections of AF64A for 10 days (initial dose 21.0 μmol/kg/day, maintenance dose 3.1 μmol/kg/day for 9 days), body weight in rats treated with AF64A had decreased by 10% at 4 days after injection of the initial dose. Thereafter, AF64A treated-rats gained weight in a manner similar to that in saline treated-rats. Rats in the AF64A group displayed a transient syndrome consisting of piloerection, lethargy, atoxia and epistaxis. This syndrome had almost disappeared about 5 days after injection of the initial dose.

Effect of AF64A on ACh content in various brain regions of the rat: Intraperitoneal administration of AF64A produced a persistent decrease of brain ACh content. ACh contents in several regions of the brain determined 24 hr and 10 days after an intraperitoneal single injection of 23.7 μmol/kg AF64A are shown in Table 1. AF64A significantly decreased ACh content in the cerebral cortex (decreased by 10% at 24 hr and 8% at 10 days), hippocampus (23 and 12% decrease at 24 hr and 10 days, respectively) and striatum (5 and 12% decrease at 24 hr and 10 days, respectively). The effect was most prominent in the hippocampus. Table 1 also shows the ACh content determined in the same regions as those described above 24 hr and 20 days after intraperitoneal daily injections of AF64A for 10 days (initial dose: 21.0 μmol/kg, maintenance dose: 3.1 μmol/kg). This treatment with AF64A also significantly reduced ACh content in all three brain regions at both 24 hr and 20 days after the last injection. The significant decrease of ACh contents in the cerebral cortex (decreased by 15% at 24 hr and 13% at 20 days), hippocampus (19 and 17% decrease at 24 hr and 20 days, respectively) and striatum (17 and 10% decrease at 24 hr and 20 days, respectively) shows that the effect of AF64A persisted even at 20 days after the last injection.

Effect of AF64A on ACh release from the hippocampus of the rat: The effect of AF64A on resting and K⁺-stimulated release of ACh from the superfused blocks of hippocampal tissue are shown in Fig. 1. The increase of [K⁺]₀ to 23.4 mM in the medium increased ACh release from a resting level of 70.2±1.1 to 261.9±8.9 pmol/min/g wet tissue in saline treated rats. A single injection of AF64A caused a significant decrease in both resting

Table 1. Influences of intraperitoneal administration of AF64A on the ACh content in several regions of rat brain

| Treatment (i.p.) | Days after last injection | ACh content (nmol/g wet tissue) |  |
| --- | --- | --- | --- |
|  |  | Cortex | Hippocampus | Striatum |
| Single injection |  |  |  |  |
| Saline | 1 | 19.2±0.1 (6) | 22.7±0.7 (6) | 53.2±0.5 (6) |
| AF64A | 23.7 μmol/kg | 1 | 17.3±0.3 (6) | 17.4±0.6 (6) | 50.5±0.7 (6)** |
| Saline | 10 | 19.1±0.3 (6) | 21.7±0.3 (6) | 52.8±1.0 (6) |
| AF64A | 23.7 μmol/kg | 10 | 17.6±0.2 (6) | 19.2±0.2 (6) | 46.3±0.5 (6)** |
| Daily injection for ten days |  |  |  |  |
| Saline | 1 | 19.2±0.2 (6) | 22.8±0.4 (6) | 52.6±1.3 (6) |
| AF64A | 21.0 μmol/kg ×1 +3.1 μmol/kg ×9 | 1 | 16.3±0.1 (6) | 18.4±0.2 (6) | 43.9±0.9 (6)** |
| Saline | 20 | 19.3±0.2 (6) | 22.4±0.4 (6) | 51.7±0.8 (6) |
| AF64A | 21.0 μmol/kg ×1 +3.1 μmol/kg ×9 | 20 | 16.8±0.3 (6) | 18.5±0.2 (6) | 46.5±1.1 (6)** |

Each value represents the mean±S.E.M. for the number of rats shown in parentheses. Significant differences from the control value were determined by Student's t-test (**P<0.01).
(11% decreased) and K+-stimulated (20% decreased) release of ACh from hippocampal tissue 24 hr after the treatment.

**Effect of AF64A on ChAT activity:** ChAT activity in homogenates from several regions of the brain determined 24 hr after daily intraperitoneal injections for 10 days are shown in Fig. 2. Treatment with AF64A resulted in a marked decrease in ChAT activity in the hippocampus but not in the cerebral cortex and striatum. ChAT activity in the crude synaptosomal fraction of the cerebral cortex was significantly decreased 24 hr after the AF64A treatment for 10 days (Table 2).

**Effect of AF64A on HACU:** HACU in the synaptosomal fraction obtained from several regions of the brain determined 24 hr after the AF64A treatment for 10 days are shown in Fig. 3. There was no significant difference between activities of HACU in the preparations obtained from AF64A treated-rats and the controls in all three regions.

**Effect of AF64A on ultrastructure of the hippocampal neurons:** The data shown in Table 1 and Fig. 2 reveal that damage in the cholinergic system was greater in the hippocampus. However, the ultrastructural features observed in the hippocampus 24 hr after the AF64A treatment for 10 days did not show any notable changes in the ultrastructures of nuclear, mitochondrion, nerve terminal, synapse and synaptic vesicle (Fig. 4). Also, no ultrastructural changes were observed in the cerebral cortex and striatum (not shown).

**Discussion**

It has been suggested that cholinergic hypofunction induced by administration of AF64A into the brain was due to the selective and irreversible inhibition of the HACU system in the cholinergic nerve terminals (11). In vitro studies with the synaptosomal fraction showed that the reduction in ChAT activity by AF64A was closely associated with the reduced HACU system (25) and that the inhibition of HACU by AF64A was effectively prevented by incubation with choline (26) or hemicholinium-3 (HC-3) a potent HACU inhibitor (27). Furthermore, recent studies in vivo showed that the pretreatment with HC-3...
Fig. 2. Influences of intraperitoneal administration of AF64A on ChAT activity in homogenates of several regions of rat brain. AF64A was injected daily for 10 days. Rats were killed 24 hr after the last injection. *P<0.05.

Fig. 3. Influences of intraperitoneal administration of AF64A on HACU in the P₂ fraction of several regions of rat brain. AF64A was injected daily for 10 days. Rats were killed 24 hr after the last injection.
Fig. 4. Influences of intraperitoneal administration of AF64A on the ultrastructures in the rat hippocampus. AF64A was injected daily for 10 days. a) Normal nucleus in a hippocampal neuron, 24 hr after the last injection. (×4,000) Scale bar=2.5 μm. b) Terminals of normal synapse in the hippocampal neuropil, 24 hr after the last injection. (×30,000) Scale bar=0.5 μm.

or its analogue, a bis 4-methylpipridine (A-4), prevented the decrease in ACh content and decrease in activity of the HACU, and the working memory impairments induced by AF64A (28, 29). Thus, AF64A seems to exert its effect on cholinergic neurons through the interaction with the carrier of HACU (5). AF64A appears to be transported by HACU systems into the nerve terminals and to inhibit ChAT or other related enzymes in the choline metabolism at lower concentrations (25, 30, 31). At higher concentrations, AF64A appears to irreversibly inhibit the HACU by progressive alkylation of the carrier of HACU (26, 32). In our studies, the intraperitoneal administration of AF64A induced the reduction in ChAT activity without affecting the HACU system. Our result is different from other reports where the intraventricular or the intracerebral administration of AF64A resulted in persistent reduction of the HACU activity. It seems that the discrepancy of these effects is due to the different distribution of AF64A in the brain between the intraperitoneal and the central administration. It is likely that cholinergic damage observed in the present study is due to the inhibition of ChAT activity in the nerve terminals and not due to the inhibition of the HACU system via the irreversible alkylation process by AF64A.

The interaction of AF64A or choline mustard aziridinium ion (ChM Az) with ChAT has been investigated in many laboratories. Solubilized ChAT was relatively insensitive to inhibition by AF64A (19) or ChM Az (31), although ChAT in the intact synaptosome was rapidly and irreversibly inactivated by incubation of the preparation with lower concentrations of these analogues (25, 32). These studies have suggested that synaptosomal ChAT coupled with the carrier of HACU was most sensitive to alkylation by AF64A.

The activity of CAhT is detected throughout the cholinergic nerve cell (33). The hippocampus contains numerous cholinergic nerve terminals (34), and the cerebral cortex contains a moderate number of cell bodies of the intrinsic cholinergic neurons (35). AF64A induced a marked reduction of ChAT activity in homogenates obtained from the hippocampus, but not in those of the cerebral cortex or striatum in the present study. However, ChAT activity in the synaptosomal fraction obtained from the cerebral cortex was significantly reduced (Table 2). It is likely that a substantial portion of the total ChAT activity in the homogenates obtained from the cerebral cortex is located in cell bodies rather than nerve terminals. These results suggest that AF64A produces a prominent inhibition of ChAT activity in the nerve terminal.

In the present study, ACh content in three brain regions and both resting and K+-stimulated release of ACh from hippocampal tissue blocks were significantly reduced by the treatment with AF64A (Fig. 1). These decreases may be due to the reduction in ACh synthesis, resulting from the inhibition of ChAT activity. There was no difference in the effect of AF64A on ACh content between a
single and consecutive daily injection for 10 days. It is possible that AF64A influences the turnover of ChAT-enzyme even in a single injection although the details are still unknown. Potter et al. (36) suggested that AF64A-induced reduction of ACh release is due to decrease in the population of cholinergic nerve terminals and a decrease in the ACh content in nerve terminals. Mouton et al. (37) also suggested that AF64A-induced decrease in ChAT activity is due to degeneration of the cholinergic neurons. However, there was no notable changes in the ultrastructures in our electron microscopic observations.

In summary, the present study indicates that the intraperitoneal administration of AF64A induces a long-term inhibition of ChAT activity in the cholinergic nerve terminals in the brain without any significant effects on the HACU system and any notable changes in the ultrastructure. Intraperitoneal administration of AF64A can be used to produce damage on central cholinergic nerve terminals as well as the central administration.

Acknowledgments: The authors are indebted to Mitsubishi Kasei Corporation for the generous supply of AF64 picrate.

References

1 Fisher, A. and Hanin, I.: Choline analogs as potential tools in developing selective animal models of central cholinergic hypofunction. Life Sci. 27, 1615–1634 (1980)

2 Leventer, S., McKeag, D., Clancy, M., Wulfert, E. and Hanin, I.: Intracerebroventricular administration of ethylcholine mustard aziridinium ion (AF64A) reduces release of acetylcholine from rat hippocampal slices. Neuropharmacology 24, 453–459 (1985)

3 Mantione, C.R., Zigmond, M.J., Fisher, A. and Hanin, I.: Selective presynaptic cholinergic neurotoxicity following intrahippocampal AF64A injection in rats. J. Neurochem. 41, 251–255 (1983)

4 Vickroy, T.W., Watson, M., Leventer, S.M., Roeske, W.R., Hanin, I. and Yamamura, H.I.: Regional differences in ethylcholine mustard aziridinium ion (AF64A)-induced deficits in presynaptic cholinergic markers for the rat central nervous system. J. Pharmacol. Exp. Ther. 235, 577–582 (1985)

5 Mantione, C.R., Fisher, A. and Hanin, I.: Possible mechanisms involved in the presynaptic cholinergic toxicity due to ethylcholine aziridinium (AF64A) in vivo. Life Sci. 35, 33–41 (1984)

6 Walsh, T.J., Tilson, H.A., Dehaven, D.L., Mailman, R.B., Fisher, A. and Hanin, I.: AF64A, a cholinergic neurotoxin, selectively depletes acetylcholine in hippocampus and cortex, and produces long-term passive avoidance and radial-arm maze deficits in the rat. Brain Res. 321, 91–102 (1984)

7 Mantione, C.R., Fisher, A. and Hanin, I.: The AF64A-treated mouse: possible model for central cholinergic hypofunction. Science 213, 579–580 (1981)

8 Chrobak, J.J., Hanin, I. and Walsh, T.J.: AF64A (ethylcholine aziridinium ion), a cholinergic neurotoxin, selectively impairs working memory in a multiple component T-maze task. Brain Res. 414, 15–21 (1987)

9 Jarrard, L.E., Kant, G.J., Meyerhoff, J.L. and Levy, A.: Behavioral and neurochemical effects of intraventricular AF64A administration in rats. Pharmacol. Biochem. Behav. 21, 273–280 (1984)

10 Nakamura, S., Nakagawa, Y., Kawai, M., Toyama, M. and Ishihara, T.: AF64A (ethylcholine aziridinium ion)-induced basal forebrain lesion impairs maze performance. Behav. Brain Res. 5, 331–358 (1988)

11 Fisher, A. and Hanin, I.: Potential animal models for senile dementia of Alzheimer’s type, with emphasis on AF64A-induced cholinotoxicity. Annu. Rev. Pharmacol. Toxicol. 26, 161–181 (1986)

12 Kozlowski, M.R. and Arbogast, R.: Specific toxic effects of ethylcholine nitrogen mustard on cholinergic neurons of the nucleus basalis of Meynert. Brain Res. 372, 45–54 (1986)

13 Stwertka, S.A. and Olson, G.L.: Neuropathology and amphetamine-induced turning resulting from AF64A injections into the striatum of the rat. Life Sci. 38, 1105–1110 (1986)

14 Villani, L., Contestabile, A., Mignani, P., Poli, A. and Fonnun, F.: Ultra-structural and neurochemical effects of the presumed cholinergic toxin AF64A in the rat interpeduncular nucleus. Brain Res. 379, 223–231 (1986)

15 Sandberg, K., Schnaar, R.L., McKinney, M., Hanin, I., Fisher, A. and Coyle, J.T.: AF64A: an active site directed irreversible inhibitor of choline acetyltransferase. J. Neurochem. 44, 439–445 (1985)

16 Fisher, A., Mantione, C.R., Abraham, D.J. and Hanin, I.: Long-term central cholinergic hypofunction induced in mice by ethylcholine aziridinium ion (AF64A) in vivo. J. Pharmacol.
Exp. Ther. 222, 140–145 (1982)

17 Sandberg, K., Schnaar, R.L. and Coyle, J.T.: Method for the quantitation and characterization of the cholinergic neurotoxin, monoethylcholine mustard aziridinium ion (AF64A). J. Neurosci. Methods 14, 143–148 (1985)

18 Proscott, D.J., Hildebrand, J.D., Saes, J.R. and Jewett, S.: Biochemical and developmental studies of acetylcholine metabolism in the central nervous system of the moth Manduca sexta. Comp. Biochem. Physiol. 56, 77–84 (1977)

19 Keirse, M.J.N.C. and Turnbull, A.C.: Extraction of prostaglandins from human blood. Prostaglandins 4, 607–617 (1973)

20 Yagasaki, O., Takai, M. and Yanagiya, I.: Acetylcholine release from the myenteric plexus by prostaglandin E2. Japan. J. Pharmacol. 33, 521–525 (1981)

21 Whittaker, V.P.: The isolation and characterization of acetylcholine containing particle from brain. Biochem. J. 104, 749–755 (1967)

22 Fonnum, F.: A rapid radiochemical method for the determination of choline acetyltransferase. J. Neurochem. 24, 407–408 (1975)

23 Simon, J.R., Atweh, S. and Kuhar, M.J.: Sodium-dependent high affinity choline uptake: a regulatory step in the synthesis of acetylcholine. J. Neurochem. 26, 909–922 (1976)

24 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951)

25 Rylett, B.J. and Colhoun, E.H.: Carrier-mediated inhibition of choline acetyltransferase. Life Sci. 26, 909–914 (1980)

26 Rylett, B.J. and Colhoun, E.H.: An evaluation of irreversible inhibition of synaptosomal high-affinity choline transport by choline mustard aziridinium ion. J. Neurochem. 43, 787–794 (1984)

27 Curti, D. and Marchbanks, R.M.: Kinetics of irreversible inhibition of choline transport in synaptosomes by ethylcholine mustard aziridinium. J. Membr. Biol. 82, 259–268 (1984)

28 Chrubak, J. J., Spates, M. J., Stackman, R. W. and Walsh, T. J.: Hemicholinium-3 prevents the working memory impairments and the cholinergic hypofunction induced by ethylcholine aziridinium ion (AF64A). Brain Res. 504, 269–275 (1989)

29 Potter, P. E., Tedford, C. E., Kindel, G. and Hanin, I.: Inhibition of high affinity choline transport attenuates both cholinergic and non-cholinergic effects of ethylcholine aziridinium (AF64A). Brain Res. 487, 238–244 (1989)

30 Barlow, P. and Marchbanks, R. M.: Effect of ethylcholine mustard on choline dehydrogenase and other enzymes of choline metabolism. J. Neurochem. 43, 1568–1573 (1984)

31 Rylett, R. J. and Colhoun, E. H.: Studies on the alkylation of choline acetyltransferase by choline mustard aziridinium ion. J. Neurochem. 44, 1951–1954 (1985)

32 Rylett, R. J.: Choline mustard: an irreversible ligand for use in studies of choline transport mechanisms at the cholinergic nerve terminal. Can. J. Physiol. Pharmacol. 64, 334–340 (1986)

33 Cuello, A. C. and Sofroniew, M. V.: The anatomy of the CNS cholinergic neurons. TINS 7, 74–77 (1984)

34 Ichikawa, T. and Hirata, Y.: Organization of choline acetyltransferase-containing structures in the forebrain of rat. J. Neurosci. 6, 281–292 (1986)

35 Fibiger, H. C.: The organization and some projections of cholinergic neurons of the mammalian forebrain. Brain Res. Rev. 4, 327–388 (1982)

36 Potter, P. E., Harsing, L. G., Jr., Kakucskas, I., Gaal, G. and Vizi, E. S.: Peripheral and central actions of AF64A (ethylcholine mustard aziridinium ion) on acetylcholine release, in vitro: comparison with hemicholinium. Neurochem. Int. 7, 1047–1053 (1985)

37 Mouton, P. R., Meyer, E. M., Dunn, A. J., Millard, W. and Arendash, G. W.: Induction of cortical cholinergic hypofunction and memory retention deficits through intracortical AF64A infusions. Brain Res. 444, 104–118 (1988)