4. Alteration of the striatal and hippocampal cholinergic systems following AF64A injection

AF64A injection produces serious lesion on cholinergic neurons. At the same time, however, AF64A is also reported to produce quite intense effects on other neurotransmitter systems and their metabolites. This may be due to non-specific tissue damage following comparably higher doses of AF64A injection. Thus, it is necessary to determine an appropriate dose of AF64A to make selective cholinergic lesion prior to behavioral testing.

Therefore, in Exp. 1, acetylcholinesterase (AChE) staining was employed to specify an appropriate dose of AF64A which could avoid non-specific tissue damages.

At the same time, neurochemical analysis is necessary to ascertain selective decrease in ACh levels following AF64A injection. Furthermore, ACh level following AF64A treatment is suggested to rise through some compensatory process the mechanism of which is unknown yet (Hanin, 1996). It is necessary to investigate whether there is a selective decrease in ACh levels in the injected region of the rat brain following AF64A treatment.

Therefore, in Exp. 2, HPLC technique was employed to determine ACh level 16 days after AF64A treatment.

4.1. Histology: Acetylcholinesterase staining [Exp. 1]

Methods

Detailed description of animals used and surgical procedure are described in 'general method'.

26 days after surgery, animals were anesthetized with 100 mg/kg sodium pentobarbital and perfused with 0.02 *M* phosphate buffered saline (PBS, pH 7.4) followed by a fixative containing 4 % paraformaldehyde and 0.2 % picric acid buffered to pH 7.4 by 0.1 *M* phosphate buffer (PB). Brains were removed and further immersed in the fixative for one day. The brains were placed in 0.1 *M* PB containing 20 % sucrose in 4 ℃ for 2 days. Frozen coronal sections (20 μm) were cut with cryostat microtome. The sections were washed out sufficiently with 0.1 *M* PBS and stored 1 day in 0.1 *M* PBS containing 0.1 % Triton-X at 4 ℃.

For AChE-staining, the brain sections were washed out with 0.1 *M* maleic acid buffer (MAB: pH 6.0) and incubated with 0.1 *M* MAB containing 36 μM acetylthiocholine iodide, 5μM potassium ferricyanide, 30μM copper sulfate and 50 μM sodium citrate at room temperature for 30 min. After the incubation, the sections were washed out with Tris-HCl buffer (50 m*M*, pH 7.6) and placed in Tris-HCl buffer containing 0.02 % 3,3'-diaminobenzidine and 0.3 % ammonium nickel sulfate. Then immediately, 30 % hydrogen peroxide (H_2O_2) was added into this solution at the rate of 15 μl per 100 ml, yielding a clear staining pattern of AChE-positive nerve elements. Stained brain sections were examined under a light microscope.

Results

Fig. 6 shows acetylcholinestrase staining of the striatum and hippocampus. AChE-positive dense terminals in the striatum and the hippocampus were observed in the saline-injected animals (A, C). In contrast, those AChE-positive dense terminals around syringe tracts in the striatum and hippocampus were decreased in many of the striatal 2.0 nmol/10μl (not shown) and 1.8 nmol/12μl (B) animals and the hippocampal 2 nmol/10μl (not shown) and 1.8 nmol/12μl (D) animals, respectively. Non-specific tissue damages in the striatum and hippocampus were detected in many of the striatal 5 nmol/10μl animals and hippocampal 5 nmol/10μl animals, respectively (not shown).

Discussion

2 nmol/10μl and 1.8 nmol/12μl of AF64A caused selective lesions to the cholinergic neurons. On the other hand, 5 nmol/10μl of AF64A produced non-specific tissue damages around the syringe tracts in many animals, and thus it was not regarded as an appropriate dose for the selective lesion of the cholinergic neurons in the current study. Therefore, the rat brains into which 1.8 nmol/12μl of AF64A was injected were further investigated in HPLC study whether other neurotransmitters were altered by AF64A injection.

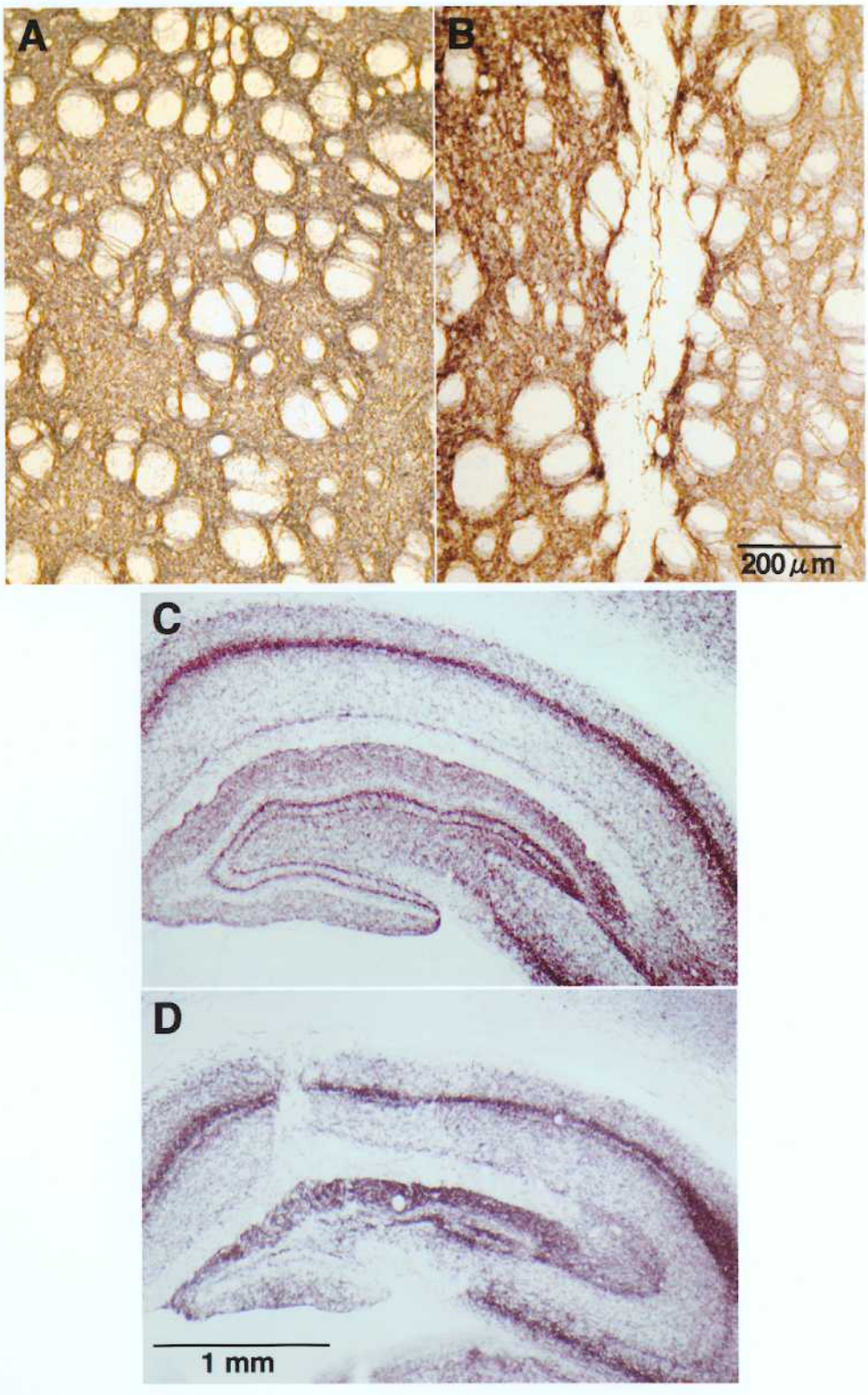

Fig. 6. Acetylcholinesterase (AChE) staining of the striatum (A,B) and hippocampus (C,D) 26 days after 1.8nmol/12μl of AF64A injection. AChE-positive fibers in the striatum were decreased in striatal AF64A injected (B) animals around syringe tracts compared to saline-treated (A) animals. As well as in the striatum, AChE-positive fibers in the hippocampus were decreased in hippocampal AF64A injected (D) animals around syringe tracts compared to saline-treated (C) animals. 2.0 nmol/10μl of AF64A treated animals produced similar decrease of AChE-positive fibers in the striatum and hippocampus (not shown).

4.2. Biochemical analysis: HPLC study [Exp. 2]

Methods

Detailed description of animals used and surgical procedure are described in general method. In HPLC study, animals of 1.8 nmol/12μl dose group, which were proven to show the least tissue damage, were investigated with regard to biochemical data.

The animals were sacrificed 16 days after surgery by head-focused microwave irradiation (0.9 s, 2450 MHz, 10 kW; New Japan Radio Co. Ltd.). The brains were rapidly removed and the striatum, hippocampus, and cortex were dissected out. The dissected materials were weighed and homogenized either in 1 ml of 0.1 N perchloric acid (PCA) containing 100 μM disodium ethylenediamine tetraacetic acid (EDTA-2NA) with 100 ng isoproterenol (ISO) (as internal standard) for the estimation of noradrenaline (NA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) or in 1 ml 0.1 N PCA containing 100 μM EDTA-2NA with 5 ng ethylhomocholine (EHC) (as internal standard) for the estimation of acetylcholine (ACh) and choline (Ch) using a Polytron homogenizer at 0 ℃. The homogenate was centrifuged at 15000 g for 20 minutes at 4 ℃. The supernatant (5 μl) was injected into HPLC system for the estimation of NA, DA, DOPAC, HVA, 5-HT, and 5-HIAA. For the estimation of ACh and Ch, 20 μl of the supernatant was injected into HPLC system.

Two HPLC systems were used in the present biochemical analysis. The HPLC system used for the determination of monoamines and their

metabolites (NA, DA, DOPAC, HVA, 5-HT, and 5-HIAA) consisted of an ECD-300 electrochemical detector (Eicom, Japan), a EP-300 chromatograph pump system (Eicom), a Model 7125 sample injector (Rheodyne), a DG-100 degasser (Eicom), and an ATC-300 column oven (Eicom). Monoamines and their metabolites were separated on an Eicompack MA-50DS column (2.1 ϕ × 250 mm; Eicom) which was kept 25 ℃ with the column oven. The working electrode was a WE-3G graphite electrode (Eicom) set at a detector potential of +0.75 V against an RE-100 Ag/AgCl reference electrode (Eicom). Mobile phase consisted of 70 m*M* citric acid, 100 m*M* sodium acetate, 1.1 m*M* sodium 1-octanesulfonate, 27 μM ethyenediaminetetraacetic acid, and 15 % methanol (v/v) at pH 3.9. The flow rate was 0.2 ml/min.

The HPLC system used for the determination of ACh and Ch consisted of an ECD-100 electrochemical detector (Eicom, Japan), an EP-100 HPLC pump (Eicom), a Model 7125 sample injector (Rheodyne), and a DG-100 degasser (Eicom). ACh, EHC, and Ch were first separated on an AC-GEL polymeric reversed-phase column (6.0 ϕ × 150 mm; Eicom). An AC-ENZYMPACK enzymatic post-column reactor with immobilized acetylcholinesterase (AChE) and Ch oxidase (Eicom) converted ACh and Ch to H_2O_2 and betaine. The separation column and enzyme reactor was kept at 33℃ using a CTC-100 circulation type column handy cooler (Eicom). The working electrode was a WE-PT platinum electrode (Eicom) set at a detector potential of +0.45 V against an RE-100 Ag/AgCl reference electrode (Eicom). Mobile phase consisted of 0.1 *M* phosphate buffer, pH 8.5, containing 0.82 m*M* sodiumdecanesulfonate and 0.59 m*M* tetram-

ethylammonium chloride. The flow rate was 1.0 ml/min.

Results

Levels of neurotransmitters and their metabolites 16 days after AF64A injection

The concentrations of ACh, DA, 5-HT, and their metabolites are shown in Fig. 7, Fig. 8, Fig. 9, and Table 2. When animals were injected with 1.8 nmol of AF64A in the striatum, only ACh concentration in the striatum decreased to the level of 76.9% of the saline-injected control in average (Fig. 7). Here, saline treated (Sal) group was administered AF64A either into the striatum or the hippocampus (the striatal and hippocampal saline treated groups). These animals of Sal groups were regarded as one Sal group in the statistical analysis since no statistical difference was found between these two Sal groups with regard to neurotransmitter levels in each region of the brain. A one-way ANOVA computed on ACh concentration in the striatum revealed a significant group effect [$F(2,17)=6.53$, $P<.01$]. Post hoc tests using Tukey-Kramer's method showed that striatal AF64A injection significantly decreased ACh concentration in the striatum as compared to the control ($p<.01$) and hippocampal lesion ($p<.01$) groups. On the other hand, striatal AF64A injection did not influence the levels of brain Ch, NA, DA, 5-HT, DOPAC, HVA, and 5-HIAA (Fig. 8, Fig. 9, and Table 2).

When animals were injected with 1.8 nmol of AF64A in the hippocampus, ACh concentration in the hippocampus decreased to the level of

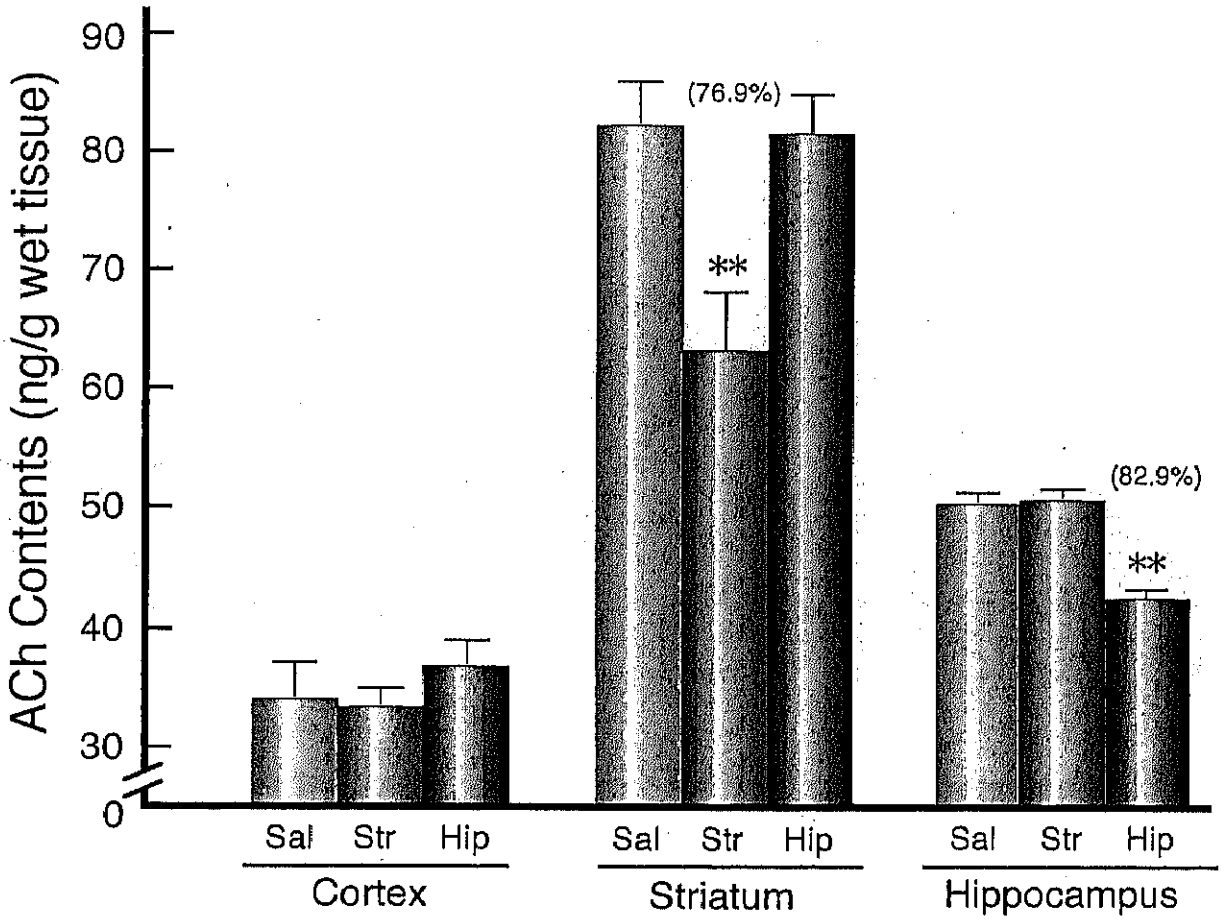

Fig. 7. Effects of AF64A treatment on ACh concentration in the cortex, striatum, and hippocampus of the rat. Data are expressed in ng/g tissue. Each value represents the mean and S.E.M. ** P<.01, compared to Sal group. The values in the parentheses indicate % changes to saline injection group. Sal: saline treatedgroup; Str: striatal AF64A treated group; Hip: hippocampal AF64A treated group.

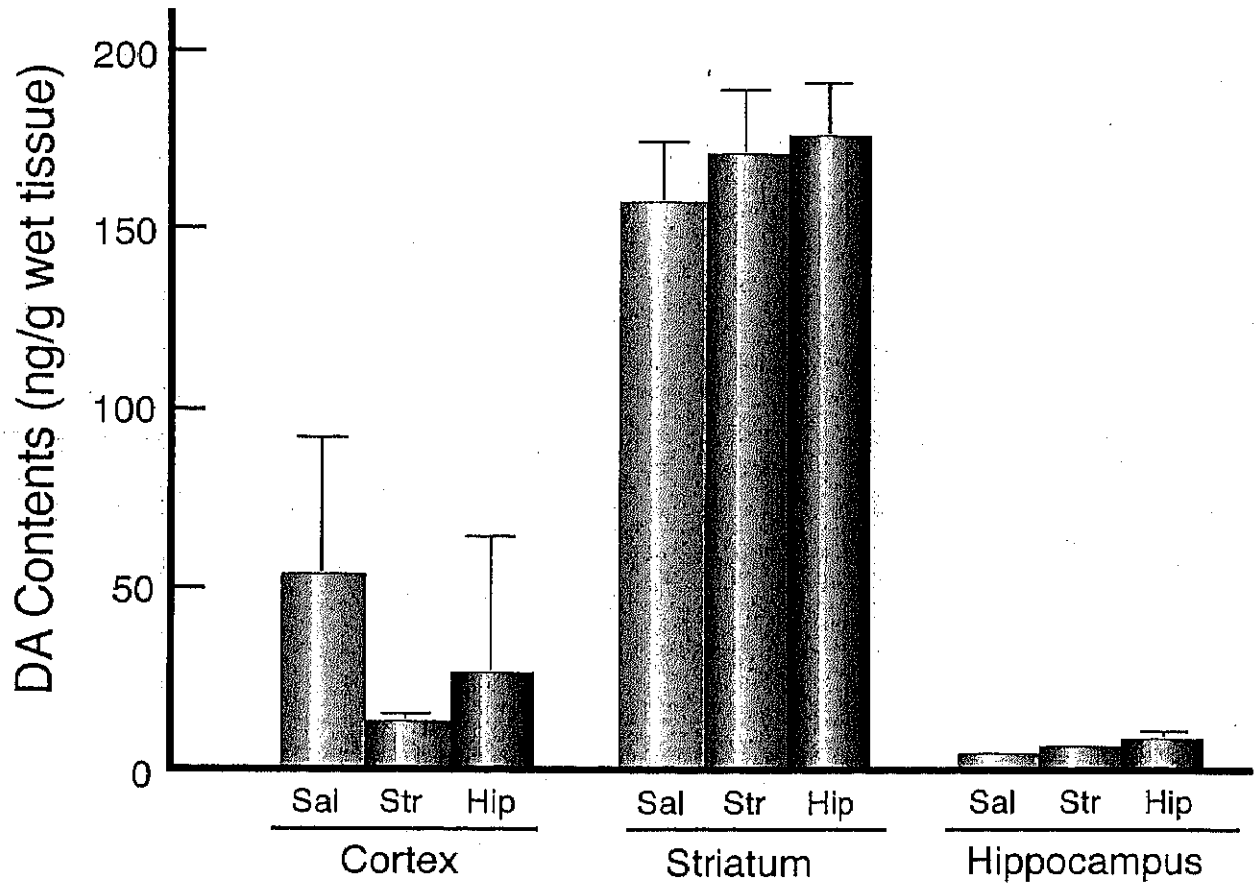

Fig. 8. Effects of AF64A treatment on DA concentration in the cortex, striatum, and hippocampus of the rat. Data are expressed in ng/g tissue. Each value represents the mean and S.E.M. See Fig. 7 for further information.

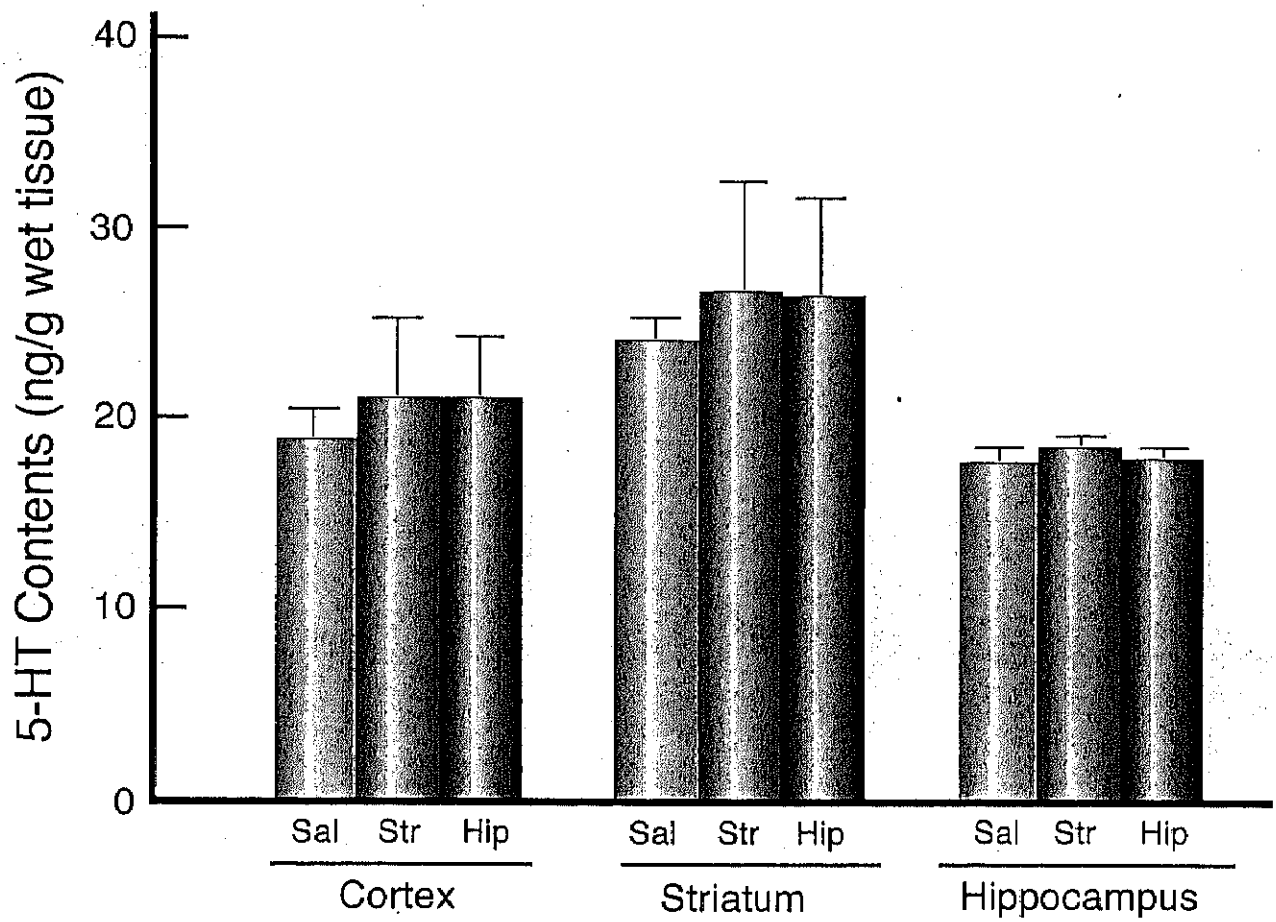

Fig. 9. Effects of AF64A treatment on 5-HT concentration in the cortex, striatum, and hippocampus of the rat. Data are expressed in ng/g tissue. Each value represents the mean and S.E.M. See Fig. 7 for further information.

Table 2. Effects of AF64A treatment on concentration of choline, NE, DOPAC, HVA, 5-HIAA in the cortex, striatum, and hippocampus of the rat. Data are expressed in ng/g tissue.

	Treatment	Ch	NE	DOPAC	HVA	5-HIAA
Cortex	Striatal-AF64A	26.1±2.6	182.6±21.1	38.1±4.2	58.5±4.3	104.4±6.6
	Hippocampal-AF64A	78.1±52.1	190.6±17.0	52.2±4.7	88.2±13.5	113.1±9.5
	Saline	26.4±2.4	178.8±14.6	39.1±6.5	55.4±18.6	108.8±7.1
Striatum	Striatal-AF64A	37.7±3.6	254.4±50.7	347.4±36.6	384.8±44.3	309.5±35.5
	Hippocampal-AF64A	44.1±12.8	223.0±22.7	378.7±28.8	375.3±38.7	258.0±13.3
	Saline	34.9±5.5	211.3±27.5	314.1±33.7	291.1±18.9	249.3±7.8
Hippocampus	Striatal-AF64A	50.1±6.1	273.9±21.9	21.5±2.4	26.7±5.4	183.2±6.4
	Hippocampal-AF64A	39.6±7.3	218.7±36.4	18.9±4.2	20.4±4.1	170.4±27.8
	Saline	39.6±4.1	249.8±10.1	16.1±1.4	21.2±7.5	174.2±10.3

Data are expressed in ng/g wet tissue; Each value represents the mean ±S.E.M.

82.9% of the saline injected control in average (Fig. 7). A one-way ANOVA computed on the concentration of ACh in the hippocampus revealed a significant group effect [$F(2,17)=35.62$, $P<.01$]. Post hoc tests showed that hippocampal AF64A injection significantly decreased ACh concentration in the hippocampus as compared to the control ($p<.01$) and striatal lesion ($p<.01$) groups. On the other hand, hippocampal AF64A injection did not significantly affect the levels of brain Ch, NA, DA, 5-HT, DOPAC, HVA, and 5-HIAA (Fig. 8, Fig. 9, and Table 2).

Discussion

In the present study, 1.8 nmol of AF64A into the striatum and hippocampus selectively decreased ACh levels in injected region.

Selectivity of AF64A to brain cholinergic systems has been controversial since Levy, Kant, Meherhoff, and Jarrard (1984) suggested the nonspecific effects of AF64A. Some findings support the hypothesis that AF64A is a selective cholinergic neurotoxin (Kozlowski & Arbogast, 1986; Sanberg, Hanin, Fisher, & Coyle, 1984), while others question the selectivity of AF64A (Levy et al., 1984; McGurk, Hartgraves, Kelly Gordon, & Buchter, 1987; Villani, Contestabile, Migani, Poli, & Fonnum, 1986). A brain microdialysis study also question the selectivity of AF64A on cholinergic neurons, because 2×8 m*M* of AF64A into the striatum have decreased not only ACh but also DA, GABA, and glutamate in striatal extracellular region (Meana, Johansson, Herrera-Marschitz, O'Connor, Goiny, Parkinson, Fredholm & Ungerstedt, 1992). The degree to which specific cholinergic insult can be induced depends on a variety of factors includ-

ing: the purity of the starting compound, the dose and concentration, and the injection rate and volume, among others (Chrobak et al., 1988). Serotonergic, noradrenergic, and dopaminergic parameters have been reported to alter following AF64A injection. However, these effects were transient, and the injection doses varied (Eva, Fabrazzo, & Costa, 1987; Hortnagl, Potter, & Hanin, 1987; Kilts, Breese, & Mailman, 1981). Hanin (1996) later discussed AF64A's effects on other neurotransmitter systems as transient secondary effects. In addition, the report that these effects were attenuated by inhibitors of choline transport (Potter, Tedford, Kindel, & Hanin, 1987) supports the idea that the effects can be attributed to cholinergic hypofunction.

In the present study, different doses, volumes, and even number of sites to which the drug was injected were tested. According to the pilot experiments, we adapted a dose of AF64A with which other neurotransmitters as well as ACh were not significantly decreased in the previous studies. AF64A is likely to show non-selective effect if it is administered with high doses. If it is diluted, however, it is likely that it fails to show a significant effect to decrease ACh levels to sufficient levels in the injected region. The total dose and volume administered in the present study was 1.8 nmol/12μl. AF64A was injected with a dose and volume of 0.45 nmol/3μl each in four injection sites in the striatum and hippocampus in order not only to avoid non-selective effects but also to avoid too limited selective effect of the drug. Consequently, the ACh levels in the injected region were selectively reduced without affecting other neurotransmitters such as DA, 5-HT, NA, Ch, and so on. The dose and volume adapted in

the present study decreased ACh levels to the levels of 65 to 85% of normal animals. If sites to inject AF64A or its injection volume or dose are risen for the purpose of strengthening the selective effect of the drug, however, the drug may induce several non-selective effects since increasing injection sites is highly a severe burden for the rat brain and more dose or volume may destroy tissues around syringe tracts.

Two nmol/10μl of AF64A injection, another dose condition carried out through two injection sites, could also avoid non-selective tissue damages around syringe tracts. Tissue damages around syringe tracts indicate non-selective effects of AF64A, thus, though it may be less selective than the dose condition of 1.8 nmol/12μl, 2 nmo/10μl-conditon is also the dose to selectively decrease ACh levels, presumably.