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Mechanisms of brain damage induced by Diisopropylfluorophosphate : Neurochemical and pharmacological approaches

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ABSTRACT

Mechanisms of brain damage induced by Diisopropylfluorophosphate : Neurochemical and pharmacological approaches

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Centrally mediated seizures and convulsions have been known as one of the toxic signs that occur following poisoning with organophosphate (OP) anticholinesterase agents such as diisopropylfluorophosphate (DFP). This study was designed to investigate the role of excitatory amino acid (EAA) systems in the initiation and the maintenance of OP-induced seizures, using the quantitative *in vivo* microdialysis in freely moving rats. Microdialysates were collected from the hippocampus of rat brain, treated with DFP (2.67 ㎎/㎏,

s.c.) alone, and/or atropine sulfate (15 mg/kg, i.m.) and procyclidine (30 mg/kg, i.m.). The protective effects of atropine, a muscarinic blocker, and/or procyclidine, an *N*-methyl-D-aspartate (NMDA) antagonist, against DFP were examined. DFP treatment increased levels of aspartate (Asp) and glutamate (Glu) significantly in the hippocampal persuate with the induction of seizures. Treatment of procyclidine could block the increased of Asp and Glu levels effectively induced by DFP. However, treatment of atropine could not give significant anticonvulsive effects against DFP-induced seizures. The increase of Asp and Glu levels by DFP were completely blocked by the combined treatment of atropine and procyclidine also. The histopathological findings for the hippocampus confirmed the above results. More effective protection was observed with the treatments of procyclidine alone or of both procyclidine and atropine than atropine alone against DFP-induced brain damage. Procyclidine was shown to be effective in DFP-induced seizures. Conclusively, NMDA antagonists maybe capable of controlling the OP-induced seizures and brain damages. It is reasonable that the use of combined treatment of anticholinergics and NMDA antagonists for the OP poisoning. NMDA antagonists are promising candidates as antidotes against central nerve system (CNS) intoxication by OP, acting through both as anti-excitotoxic and as anti epileptic mechanisms.

Key word: DFP, organophosphate, microdialysis, convulsion, seizure, NMDA, aspartate, glutamate, acetylcholinesterase, cholineacetyltransferase

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procyclidine (30 ㎎/㎏) were injected intramuscularly immediately after DFP (2.67 ㎎/㎏, s.c.) injection. The collection time was 30 minutes. Concentrations are expressed as % of mean baseline value. Data are expressed mean ± SD. Data are obtained from the average of 3 rats (*: $P < 0.05$).

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LIST OF ABBREVIATIONS

Asp, aspartate

AChE, acetylcholinesterase

ChAT, cholineacetyltransferase

CNS, central nerve system

DFP, diisopropylfluorophosphate

DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid)

EAA, excitatory amino acid

Glu, glutamate

NMDA, *N*-methyl-$_D$-aspartate

OPA, 1,2-phthalic dicarboxaldehyde

OP, organophosphate

PBS, phosphate-buffered saline

INTRODUCTION

Organophosphates (OP) represent a class of highly toxic compounds that includes chemical warfare agents, insecticides and pesticides. Most OPs are irreversible inhibitors of acetylcholinesterase (AChE), which results in the attenuation of the catalyzed hydrolysis of acetylcholine and the excessive accumulation of extracellular acetylcholine. They may give rise to a cholinergic crisis, leading to severe acute intoxication, manifested in man by diverse muscarinic, nicotinic symptoms and by central nerve system (CNS) effects; such as drowsiness, comas, convulsions (Koller et al., 1979; Moretto, 1998).

Acute OP poisoning has been reported to also cause intellectual and psychiatric sequelae, which may be transient or last several months (Namba et al., 1971; Sidell et al., 1974). Only certain OP are known to produce a delayed neuropathy (Abou-Donia, 1981). Sustained exposure to low levels of OP may lead to electroencephalographic alterations, insomnia, neurobehavioral abnormalities, disorders of memory and concentration, and to psychiatric sequelae (Dill et al., 1964; Duffy et al., 1979; Gershon et al., 1961; Korsak et al., 1977).

Centrally mediated seizures and convulsions are one of the toxic signs that occur following poisoning with OP anticholinesterase nerve agents such as soman (Ahlbom et al., 1995; Glenn et al., 1987; Mark et al., 1998; Misulis et al., 1987; Samuel et al., 1999; Shin et al., 1999; Testylier et al., 1999).

Originally, these convulsions were considered as a factor that complicated definitive treatments of the more immediate life-threatening effects (De Candole et al., 1953; Rickett et al., 1986). It has become apparent that these seizures rapidly progress to the status of epilepticus, and contribute to profound brain damage developed as a consequence of exposure to these highly toxic OP (Lemercier et al., 1983; McLeod et al., 1984; McLeod et al., 1985; Petras, 1981; Philippens et al., 1992; Singer et al., 1987). Effective management of OP-induced seizures is critical for immediate casualty treatment, and minimization of brain damage, as well as a rapid and full recovery from the effects of agent exposure (Dunn et al., 1989; Hayward et al., 1990; Martin et al., 1985; McDonough et al., 1989). The role of various neurotransmitter systems in the OP-induced seizures is not totally understood.

OP-induced seizures are thought to be initiated by the large accumulation of acetylcholine in brain, which occurs subsequently to the inhibition of brain AChE by the agents (Lallement et al., 1998; Lallement et al., 1992; Shin, 1982; Shin et al., 1997; Shin et al., 1999). The accumulation of acetylcholine in brain is capable of triggering recurrent seizure activity in susceptible brain circuits. Pretreatment, or early therapeutic treatment with anticholinergic drugs, will block the development of recurrent seizure activity and brain damage (Capacio et al., 1991; Green et al., 1977; Olney et al., 1990; Pazdernik et al., 1983).

Recent work has also emphasized the role of the excitatory amino acids (EAA) systems in the control of OP-induced seizures (Yoram et al., 1997). EAA antagonists that act on the *N*-methyl-$_D$-aspartate (NMDA) receptor, such as dizocilpine (MK-801), N-[1-(2-thienyl) cyclohexyl]-poperidine (TCP), and

procyclidine have been shown to be effective anticonvulsant when given before, or after, initiation of OP-induced seizures (Braitman et al., 1989; Price et al., 1989; Shin, 1990; Sparenborg, 1992). Thus, two different types of compounds involving two different neurotransmitter systems, anticholinergic and NMDA antagonists, all have been shown to be effective in alleviating the development of OP-induced seizures and brain damage.

On the basis of these findings, this study has hypothesized that several neurotransmitter systems become sequentially involved in OP-induced seizures.

The technique of *in vivo* microdialysis allows sampling and continuous monitoring of extracellular pools of neurotransmitters. By the microdialysis technique, the changes in extracellular neurotransmitter concentrations were observed as indirect evidences of neurochemical modifications during brain lesions, ischemia and hypoglycemia in animal models (Cartmell et al., 2000; Testylier et al., 1996; You et al., 1998).

This study was designed to investigate the role of EAA systems in the initiation and the maintenance of OP-induced seizures, using the quantitative *in vivo* microdialysis in freely moving rats.

MATERIALS AND METHODS

Materials

Diisopropylfluorophosphate (DFP) was purchased from Fluka Chemika (AG, Buchs, Switzerland). Atropine sulfate, procyclidine hydrochloride, L-aspartic acid, L-glutamic acid, 2-mercaptoethanol, paraformaldehyde, ethopropazin, rabbit serum and acetylthiocholine iodide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1, 2-phthalic dicarboxaldehyde (OPA) was purchased from Acros organics (New Jersey, USA). Methanol (HPLC grade) and tetrahydrofuran (HPLC grade) were purchased from Merck (Darmstadt, Germany). 3,3'-diaminobenzidine was purchased from Amnesco (solon, OH, USA).

Microdialysis experiments

Surgery

The experiments were performed on male Sprague-Dawley rats with a body weight of 250-300 g. The animals were housed under a 12/12 light day cycle. Light was on 06:00 to 18:00 h. The animals were allowed free access to water and standard food pellets in temperature-controlled

environments. The room temperature was continuously maintained at 25 ± 1℃. The animals were housed in individual cages for 3-5 days between implantation of a guide cannula and microdialysis sampling.

The rats were anesthetized with ketamine (30 ㎎/㎏, i.m.) plus xylazine (3 ㎎/㎏, i.m.) and placed on a stereotaxic apparatus (David Kopf). During the experiments, body temperature was continuously monitored and maintained at 37.5℃ with a thermostatically controlled heating pad connected to a rectal probe. The head was shaved, the skin of the skull was incised along the midline. After exposure of the skull and drilling of a burr hole, a guide cannula (Bioanalytical system Inc. West Lafayette, Indiana, USA) was stereotaxically implanted into the right hippocampus according to the atlas of Paxinos and Watson (1998). Stereotaxic coordinates from the bregma for the position of the tip of the guide cannula were: anteriorly 3.3 ㎜, laterally 2.6 ㎜, ventrally 3.5 ㎜ from the dura. The guide cannula was attached with dental cement (Bioanalytical system Inc. West Lafayette, Indiana, USA) to the bone with 3 small stainless steel screws. A dummy cannula was inserted in place of a microdialysis probe until the day before experiments. After surgery, the rat was housed in a large acrylic bowl and was allowed a minimum of 48 hours recovery time before starting the microdialysis experiments.

Microdialysis procedure

The awaking rat was placed in the acrylic bowl chamber. A microdialysis probe (regenerated cellulose membrane, 2 ㎜ in length, 0.32 ㎜ outer diameter, Bioanalytical system Inc. West Lafayette, Indiana, USA) was carefully inserted

through the guide cannula into the hippocampus of the hand-held conscious rat. The inlet of the microdialysis probe was connected to Bee Hive Controller and microsyringe pump (Bioanalytical system Inc. West Lafayette, Indiana, USA) with a syringe containing the perfusion solution. The outlet of the microdialysis probe was connected to a refrigerated fraction collector (temperature 4℃, BAS HoneyComb, Bioanalytical system Inc. West Lafayette, Indiana, USA). The microinfusion pump and the refrigerated fraction collector were located outside the acrylic bowl chamber. All new probes were perfused with 50 % ethanol/water for 1 hour to remove residual glycerol in the membrane. The microdialysis probe was perfused with filtered and degassed high K^+ Ringer's solution at a flow-rate of 2 $\mu\ell$/min using a with Bee Hive Controller and microsyringe pump. The composition of the high K^+ Ringer's solution (in mM) was Na^+ 51, K^+ 100, Ca^{2+} 2.3, and Cl^- 155.6. Each sample was collected during 30 min in an vial placed in the refrigerated fraction collector (4℃). For DFP injections, a catheter mounted on a hypodermic needle was introduced the subcutaneous part under of the rat and the needle was fixed with acrylic cement to the implant on the skull. Each of the treatment drugs administered one single dose. The LD_{50} for subcutaneous injections of DFP in rats was determined from literature (Sivain et al., 1989) to be 2.67 ㎎/㎏ and DFP was dissolved in corn oil. Atropine sulfate (15 ㎎/㎏) and procyclidine (30 ㎎/㎏) were injected intramuscularly immediately after DFP injection.

Glutamate and aspartate analysis

Extracellular levels of aspartate (Asp) and glutamate (Glu) were measured by reversed-phase column liquid chromatography with fluorescence detection methods (Burce et al., 1988; Lindroth et al., 1979). For the RF-10A_{XL} fluorescence detector (Shimadzu Corporation, Kyoto, Japan), the excitation wavelength was set at 330 ㎚, the emission cut-off filter at 418 ㎚. All mobile phases were degassed by a DGU-14A degasser (Shimadzu Corporation, Kyoto, Japan). Separations were performed on a Nova-Pak C_{18} ODS column : 4.6 × 150 ㎜, 4 ㎛ (Waters). Gradient elution comprising an LC-10AT (Shimadzu Corporation, Kyoto, Japan) mixing a binary gradient according to the following scheme: 0-100/0; 2-100/0; 4-0/100; 6-100/0 (min-%A/%B). Mobile phase A consisted of 92.5 % 0.1 M sodium acetate buffer (pH 6.95), 5 % methanol, 2.5 % tetrahydrofuran. Mobile phase B consisted of 97.5 % methanol, 2.5 % tetrahydrofuran. The flow-rate was total 1.2 ㎖/min. OPA solution was prepared by dissolving 27 ㎎ of OPA in 1 ㎖ of methanol and 10 ㎕ β-mercaptoethanol and 9 ㎖ sodium tetraborate buffer (0.1 M, pH 9.3) were added. The dialysis samples were pre-derivatized with OPA solution as follows: a 11 ㎕ of derivatization reagent was added to 22 ㎕ of microdialysate which was vortex-mixed for 2 min at 4℃. The injection volume was 30 ㎕.

Histology

Tissue preparation

Rats were deeply anesthetized with ketamine (30 ㎎/㎏, i.m.) plus xylazine (3 ㎎/㎏, i.m.) and perfused through the heart via a peristaltic pump with heparinized saline (1000 IU/1000 ㎖, 25 minutes), followed immediately by a freshly fixative solution of 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) (300 ㎖, 25 minutes). The brains were removed rapidly and fixed by immersion in 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature for 4 hours. After fixation, each brain was placed in a 20 % sucrose solution in 0.1 M phosphate buffer (pH 7.4) at 4℃ for 12 hours. Fixed brains were then mounted in optimal cutting temperature freezing plate (BFS series Freezing stage, Physitemp, U.S.A) cryostat embedding medium (Jung tissue freezing medium, Leica Ins., Heidelberger, Germany). The tissue was sectioned on a Leica microtome (Leica RM 2125 RT, Leica Ins., Heidelberger, Germany) at 30 ㎛. Sections were placed in phosphate-buffered saline (PBS), washed once with PBS, and stored at 4℃ until use.

Cresyl violet staining

For best results, the sections were stained the same day. Tissues were immersed for 5 minutes in each of the following: xylene, xylene, 100 %

alcohol, 100 % alcohol, 95 % alcohol, and 70 % alcohol. They were dipped in distilled water and stained in 0.5 % cresyl violet for 2 minutes. They were differentiated in water for 3-5 minutes and then dehydrated through 70 % alcohol, 95 % alcohol, 100 % alcohol, and 100 % alcohol. They were then put in xylene and gelatin coated slider coverslipped.

Histochemistry for acetylcholinesterase (AChE)

The AChE staining procedure was done according to a histochemical reaction described by Koelle and Friedenwald (Koelle et al., 1949). Briefly, the sections were rinsed (3 times, 10 minutes) at room temperature with phosphate buffer (pH 7.4), then rinsed incubated in reaction medium (4 mM cupuric sulfate, 16 mM Glycine, 50 mM sodium acetate with 4 mM acetylthiocholine iodide as substrate for AChE and 0.14 mM ethopropazine as the inhibitor of non-specific esterase activity; pH 5.0) for 15 hours at room temperature. Following incubation, sections were again rinsed (2 times, 1 minute) in distilled water and then reacted (10 minutes, constant agitation) in a freshly prepared solution of 1 % sodium sulphide (pH 7.5). The staining reaction was terminated by rinses in distilled water and phosphate buffer. The staining sections were mounted on slides, dehydrated and coverslipped in gelatin-coated sliders.

Immunohistochemistry for cholineacetyltransferase (ChAT)

Immunohistochemical staining was preformed according to the avidin-biotin peroxidase complex (ABC) method as previously described (Armstrong et al., 1983; Sakaguchi et al., 1991; Satoh et al., 1985). This study used a commercially available monoclonal antibody against ChAT. The procedure to visualize ChAT immunoreactivity was as following: Incubation in 1 % normal rabbit serum, diluted in 0.1 M PBS containing 0.25 % triton X-100, for 30 minutes. Overnight incubation (37℃) in the antibody to ChAT, diluted 1:50 in 0.1 M PBS containing 1 % normal rabbit serum and 0.25 % triton X-100. Rinse in PBS, 10 minutes with two changes. Incubation in biotinylated goat anti-rat IgG (Vector), diluted 1:200 in PBS, containing 1 % normal rabbit serum and 0.25 % triton X-100, for 60 minutes at 37℃. Rinse in PBS, 10 minutes with two changes. Incubation in PBS containing avidin-biotin peroxidase complex (Vector, 60 minutes prior to use), 60 minutes at 37℃. Rinse in PBS, 10 minutes with two changes. Incubate in 3,3'-diaminobenzidine solution (0.4 % in 0.1 M PBS, pH 7.4) containing 30 % hydrogen peroxide for 10 minutes. Rinse and mount on gelatin coated slide glass.

Data analysis

The levels of Asp and Glu were determined by comparing the peak areas of microdialysate chromatograms to those of each external standard. Changes

in the concentration of Asp and Glu in hippocampal persuate were expressed as a percent of the measured before drug administration over 30 minutes control period which started 1 hour after probe implantation. Linear regression analysis was performed to test the existence of a statistically significant linearity of the calibration curves. Statistical comparisons were made using analysis of variance and Student's t-test as indicated at the appropriate points in the text. Differences between the groups were regarded as significant if $P < 0.05$. Data expressed as mean $\pm$ SD values.

RESULTS

A schematic diagram of the results of the probe placement in the right hippocampus is provided in Fig. 1. The hatched bar indicates the area on the coronal sections of the rat brain hippocampus lesioned by probes. Recovery of probes used in the present study was tested *in vitro* using a standard solution of Asp and Glu before insertion of and after removal of the probes. Individual probe recovery (%), based on the mean of 3 dialysate samples collected before and after using the probe, was calculated by dividing the concentration of Asp and Glu in the dialysate by the concentration of the Asp and Glu in the standard solution and multiplying it by 100, respectively. *In vitro* probe recovery for Asp and Glu were 12.11 ± 8.54 % and 15.03 ± 21.91 %. Fig. 2. shows the typical chromatograms of standard Asp and Glu (0.125 pmol/㎖) (Fig. 2.A), and of hippocampal microdialysate from rat brains (Fig. 2.B). The detection limit of 0.125 fmol/㎖ is achievable by operating the fluorescence detector at maximum excitation energy and use of the photomultiplier signal. Linear relationships for Asp and Glu concentrations were obtained with correlation coefficients not less than 0.97 after correction with the appropriate external standard (Fig. 3). The concentrations of Asp and Glu in microdialysate were determinated by comparison to their standard peak areas.

After DFP injection, rats began to display full motor convulsions within 30 minutes. This motor activity was observed during 2 days after DFP injection

(data not shown).

The basal level of Asp and Glu in the microdialysate was 7016 ± 785 pM (n=3) and 952 ± 46 pM (n=3), respectively. The effects of DFP administration on the extracellular level of Glu in hippocampus are shown in Fig. 4.A. DFP administration increased Glu levels during the first 30 minutes about 79 % compared to the control and a maximum increase of about 224 % was reached in 2.5 hours. The effects of atropine, a muscarinic antagonist, on extracellular Glu levels in hippocampus are shown in Fig. 4.B. When atropine and DFP are administrated, Glu levels were not significantly different from that of DFP treated group. The effects of procyclidine, muscarinic and NMDA antagonist, on extracellular Glu levels in hippocampus are shown in Fig. 4.C. When procyclidine and DFP are administrated, Glu levels decreased during the first 1 hour and 30 minutes of administration to about 132 % to that of control level, and maximum decrease about 168 % was reached after 2.5 hours. This decrease was maintained over 5 hours following DFP and procyclidine administration. The effects of atropine and procyclidine on extracellular Glu levels in hippocampus are shown in Fig. 4.D. When DFP, atropine and procyclidine were administrated, Glu levels decreased during the first 30 minutes of administration to about 26 % of control, and maximum decrease was about 222 %, which was in reached 2.5 hours. This decrease was maintained over 5 hours. The effects of DFP administration on extracellular Asp levels in hippocampus are shown in Fig. 5.A. When DFP was administrated, Asp levels increased during the first 30 minutes of administration to about 101 % to that of the control, and a maximum increase of about 312 % was reached in 2 hours. The effects of atropine on extracellular Asp levels

in hippocampus are shown in Fig. 5.B. When DFP and atropine were administrated, Asp levels decreased during the first 1 hour of administration to about 79 % control level, and maximum decrease about 231 % was reached 3 hours. These decrease were maintained over 5 hours following DFP plus atropine administration. The effects of procyclidine on extracellular Asp levels in hippocampus are shown in Fig. 5.C. In case of procyclidine and DFP administration, Asp levels decreased during the first 1 hour of administration to about 73 % of control level, and maximum decrease about 182 % was reached 3.5 hours. These decrease was maintained over 5 hours following DFP plus procyclidine administration. The effects of atropine and procyclidine on extracellular Asp levels in hippocampus are shown in Fig. 5.D. When DFP, atropine and procyclidine administrated, Asp levels decreased during the first 30 minutes of administration to about 26 % of control level, and maximum decrease about 221 % was reached 3.5 hours. These decreases of Asp and Glu levels by DFP were maintained over 5 hours following by the combined treatment of atropine and procyclidine.

Cresyl violet staining was used to visualize neuronal cell death. The neurons stained with cresyl violet were reduced by DFP treatments especially in the medial CA_1 and CA_3 region of the hippocampus (Fig. 6.B). The medial CA_1 and CA_3 region of the hippocampus were vulnerable to DFP-induced brain injury. Procyclidine and atropine plus procyclidine treated groups were protecting the reduction of neuronal numbers or neuronal damage in the medial CA_1 and CA_3 region of the hippocampus but atropine treated group was not protected (Fig. 6.C, D, E). The typical AChE-positive fiber band could be seen in the outer molecular layer (Fig. 7.A). Rats treated with DFP showed severe

damage to the outer molecular layer (Fig. 7.B) and in other treatments, rats showed damage to the outer molecular layer (Fig. 7.C, D, E). The typical ChAT-positive neuron could be seen in the caudoputaman at the bregma 26 ㎜ (Fig. 8.A). In DFP treated group, ChAT-positive neurons showed significant changes of neuron loss. In contrast, the procyclidine and procyclidine plus atropine administration group showed similar results to the control group.

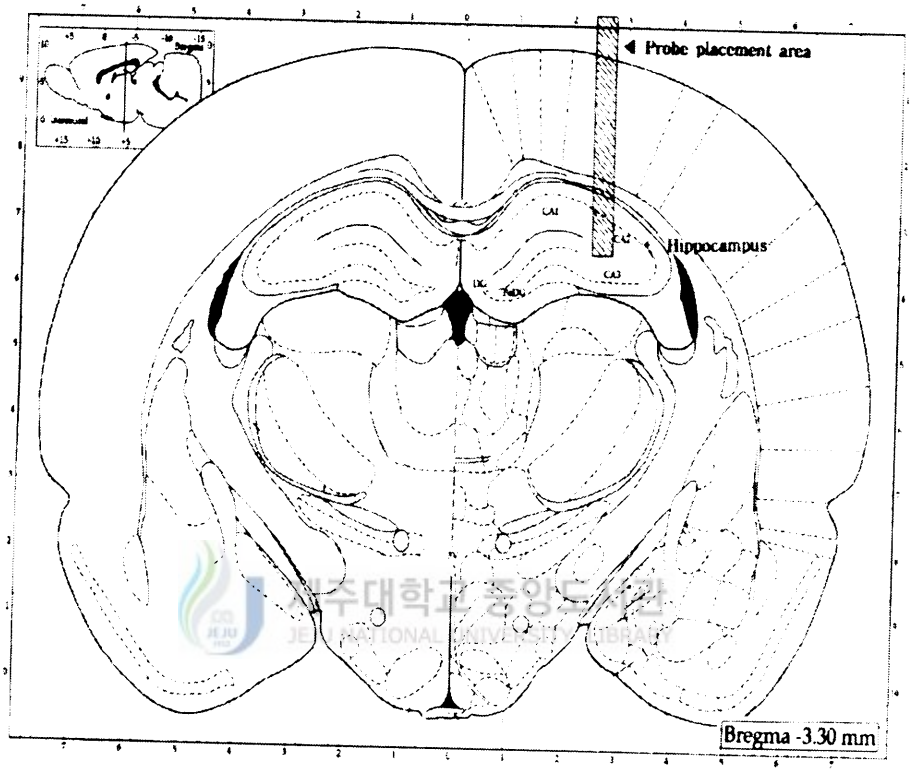


Fig. 1. Diagram showing the probe placement area in the hippocampus. Stereotaxic coordinates from the bregma for the position of the tip of the probe were: anteriorly 3.3 ㎜, laterally 2.6 ㎜, ventrally 3.5 ㎜ from the dura.

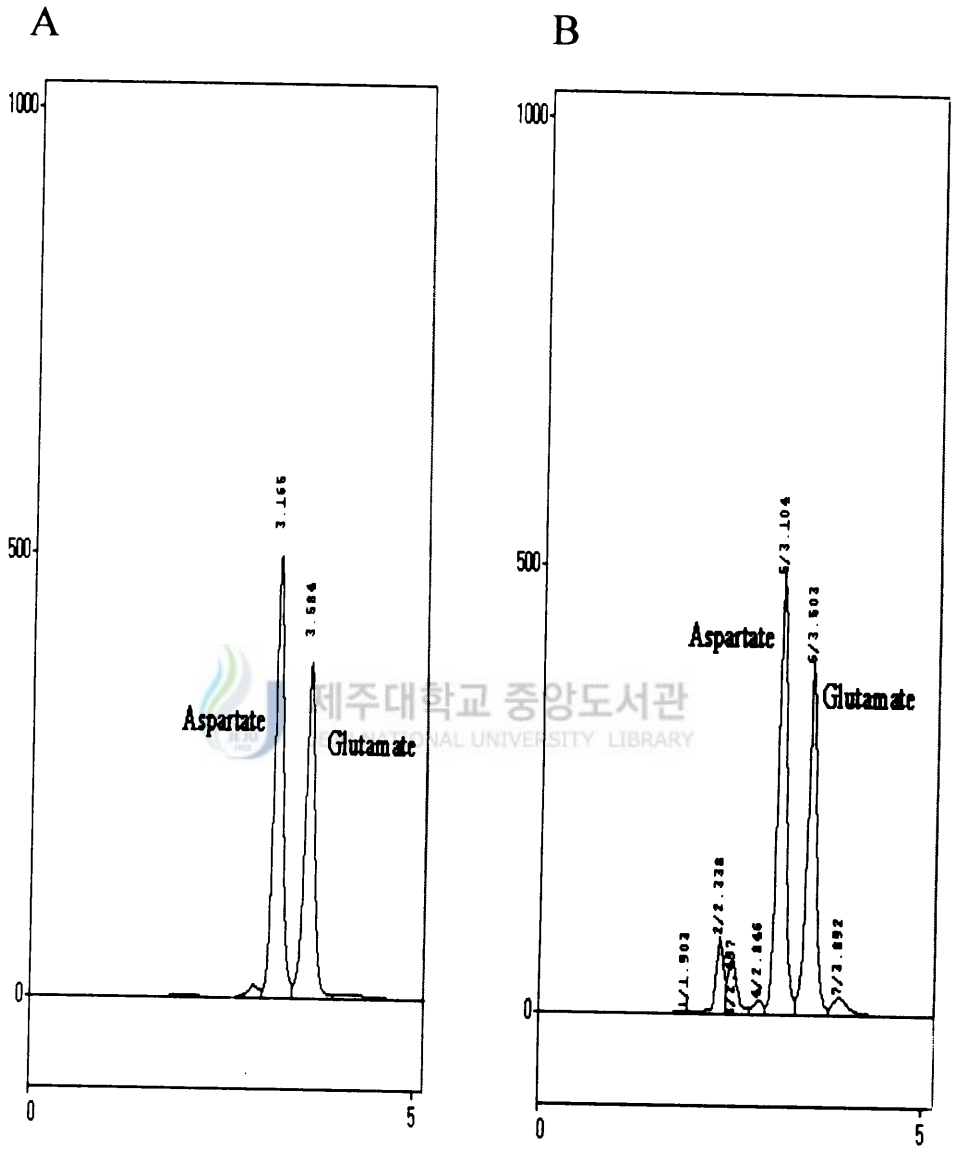
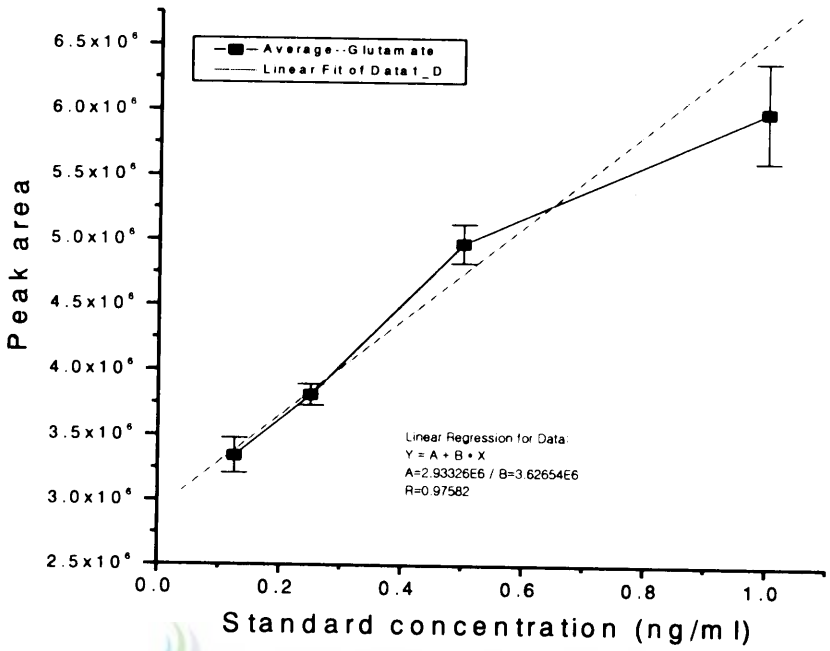


Fig. 2. Typical chromatograms of (A) standard Asp and Glu (0.125 pmol), (B) a hippocampal microdialysate (5$\mu\ell$) collected from a rat brain post DFP administration (2.67 mg/kg, s.c.)

Fig. 3. Linear relationship between peak area and concentration for standard solutions of Glu (A), and Asp (B). Standard solutions (4 concentrations, 3 points per concentration) were prepared by diluting the stock solutions with a microdialysis buffer. Data are corrected with the appropriate external standard and are expressed mean ± SD.

A



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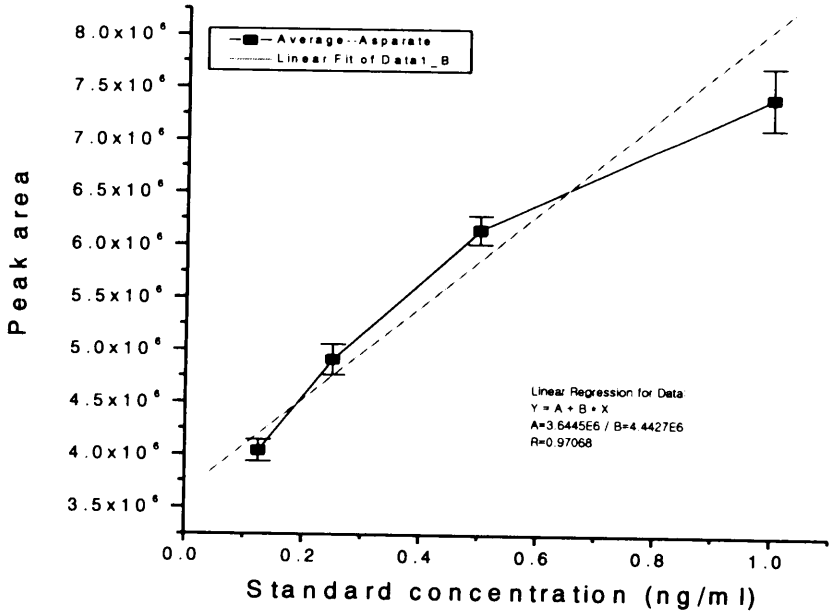


Fig. 4. Time course of Glu levels in microdialysate collected from hippocampus of awaking rats before and after drug treatments. A, Control group and DFP treated group: B, DFP treated group and DFP plus atropine treated group: C, DFP treated group and DFP plus procyclidine treated group: D, DFP treated group and DFP + atropine + procyclidine treated group. Atropine (15 ㎎/㎏) and procyclidine (30 ㎎/㎏) were injected intramuscularly immediately after DFP (2.67 ㎎/㎏, s.c.) injection. The collection time was 30 minutes. Concentrations are expressed as % of mean baseline value. Data are expressed mean ± SD. Data are obtained from the average of 3 rats (*: $P < 0.05$).

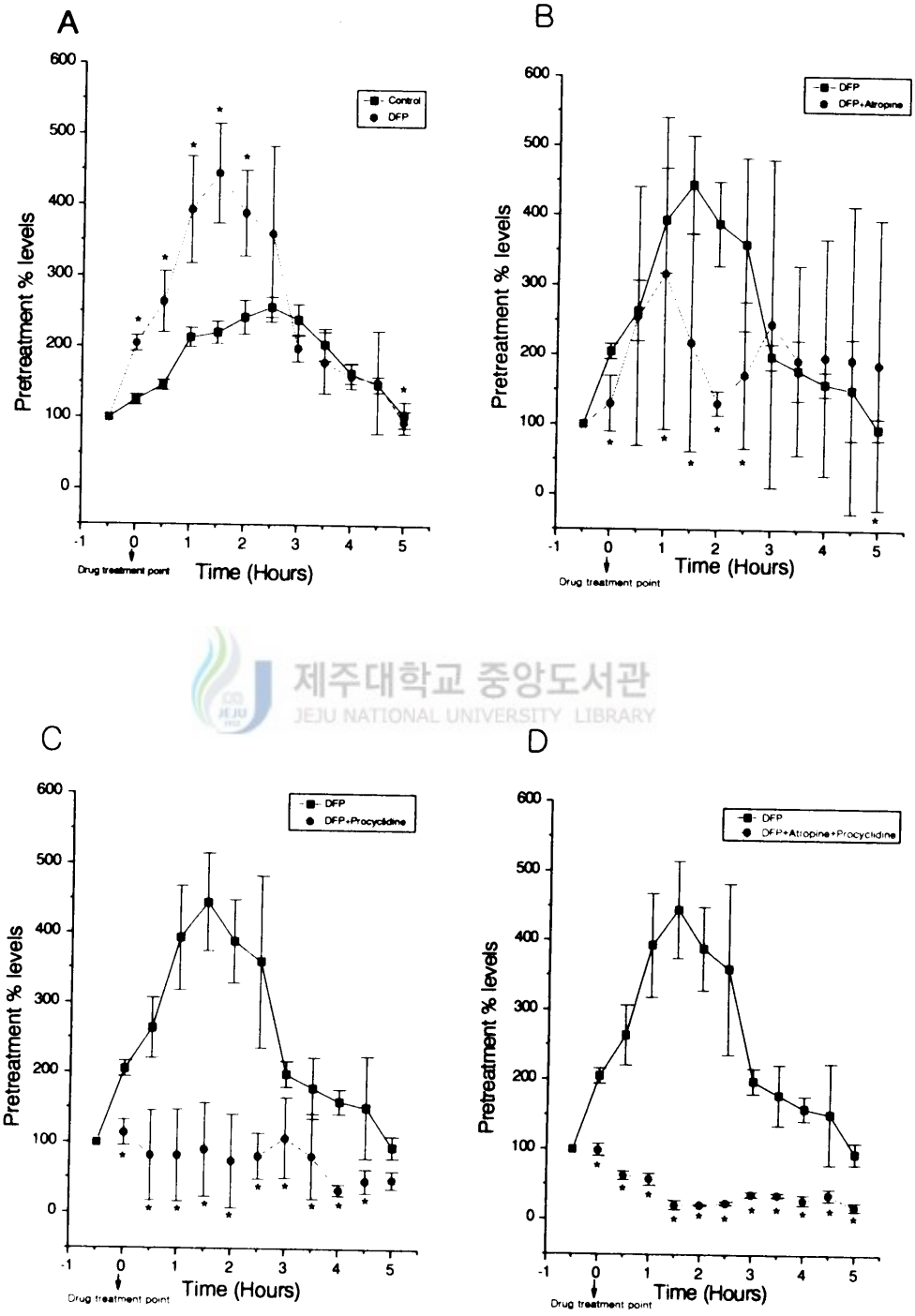


Fig. 5. Time course of Asp levels in microdialysate collected from hippocampus of awaking rats before and after drug treatments. A, Control group and DFP treated group: B, DFP treated group and DFP plus atropine treated group: C, DFP treated group and DFP plus procyclidine treated group: D, DFP treated group and DFP + atropine + procyclidine treated group. Atropine (15 ㎎/㎏) and procyclidine (30 ㎎/㎏) were injected intramuscularly immediately after DFP (2.67 ㎎/㎏, s.c.) injection. The collection time was 30 minutes. Concentrations are expressed as % of mean baseline value. Data are expressed mean ± SD. Data are obtained from the average of 3 rats (*: $P < 0.05$).

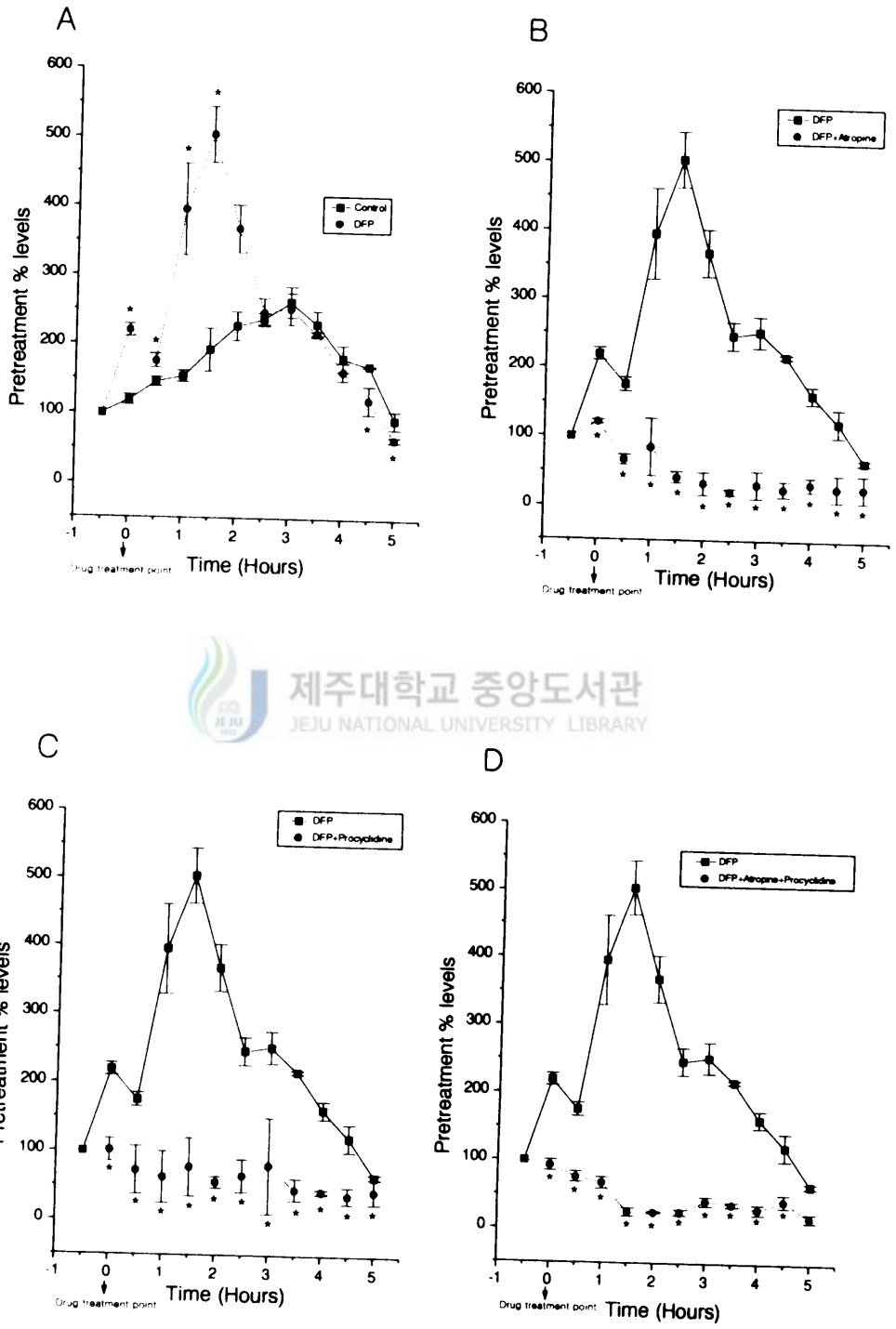


Fig. 6. Photomicrographs of the hippocampus stained with cresyl violet, showing the hippocampus 7th day after drug treatment (×13.2). A, Control group: B, DFP treat group: C, DFP plus atropine treat group: D, DFP plus procyclidine treat group: E, DFP + atropine + procyclidine treat group.

A

B

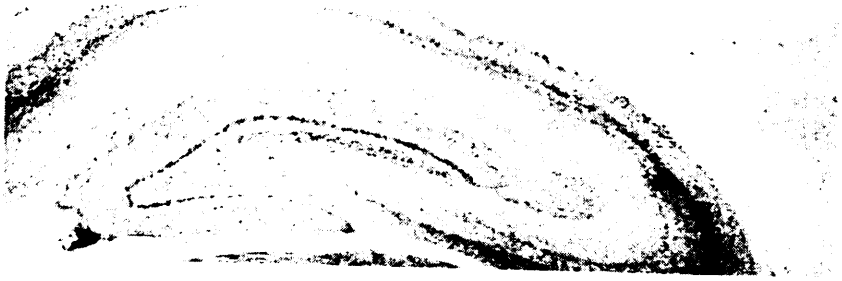
C

D

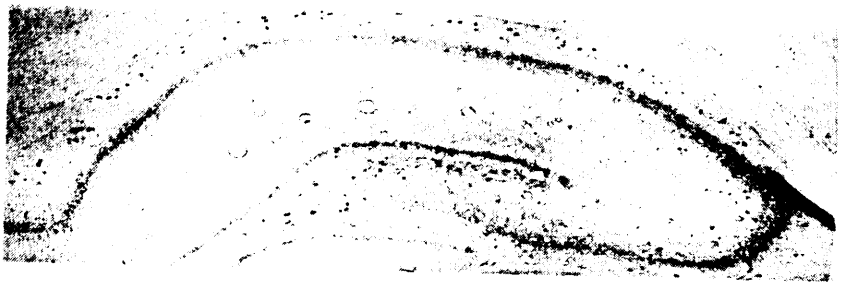
E

Fig. 7. Histochemical localization of acetylcholinesterase, showing the hippocampus 7 days after drug treatment (×13.2). A, Control group: B, DFP treat group: C, DFP plus atropine treat group: D, DFP plus procyclidine treat group: E, DFP + atropine + procyclidine treat group.

A



B

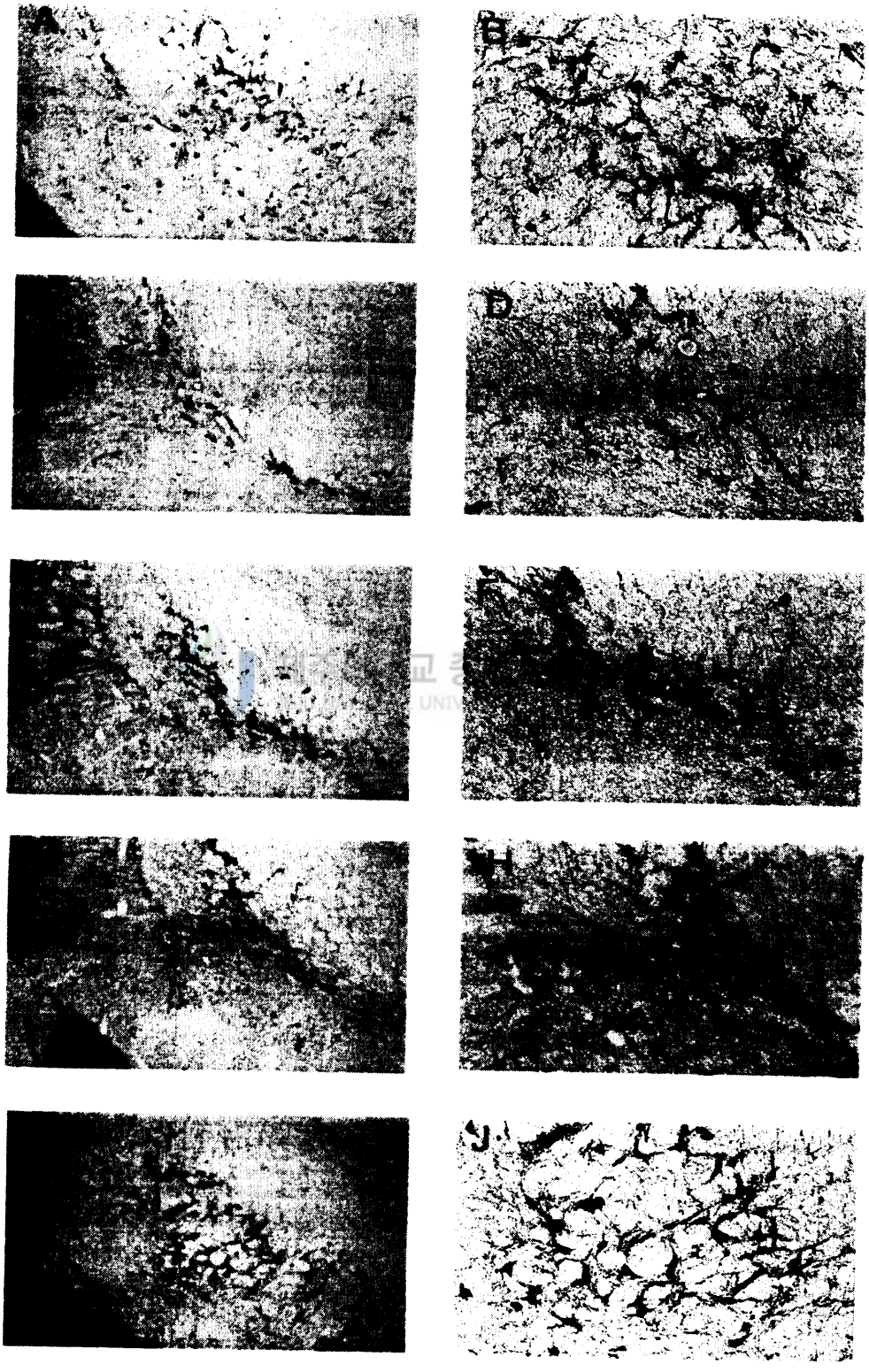


C

D

E

Fig. 8. Immunohistogram of choline acetyltransferase, showing the caudoputaman 7 days after drug treatment. A, B: Control group. C, D: DFP treat group. E, F: DFP plus atropine treat group. G, H: DFP plus procyclidine treat group. I, J: DFP + atropine + procyclidine treat group. (left ×13.2, right ×33)



DISCUSSION

The objective of this study was to characterize the roles of the EAA system in DFP induced seizure. The EAA system in OP-induced seizures and brain damage has received increasing attention following a report that NMDA antagonists exert anticonvulsant effects against nerve agent induced seizures when given as a pretreatment, or after seizures have been initiated (Sparenborg et al., 1992). The role of the NMDA receptor system in OP-induced seizures appears to be involved in the maintenance of seizure activity triggered by the initial cholinergic hyperstimulation and in the initiation of biochemical events that lead to neuropathology (Lallement et al., 1992). In previous experiments the rate of Asp and Glu release in samples of perfusate obtained by microdialysis was measured. This study's goal was to estimate the extracellular concentrations of Asp and Glu in hippocampus of DFP-induced seizure. After having established baseline under these conditions, this study described change and magnitude in time course induced by DFP. Following DFP administration, Glu levels increased during the first 30 minutes of administration to about 79 % of the control level, and a maximum increase of about 224 % of control level was reached in 2.5 hours. When DFP was administrated, Asp levels increased during the first 30 minutes of administration to about 101 % to that of the control and a maximum increase of about 312 % was reached in 2 hours. The results show that procyclidine, an NMDA antagonist, decreased significant Asp and Glu levels in DFP-induced seizures but atropine, a

muscarinic antagonist, not can precipitate significant anticonvulsant effects against seizures induced by DFP. The release of Asp and Glu in DFP-induced seizure were completely abolished by atropine plus procyclidine administration. The histopathological examination of hippocampal brain tissue confirmed the previous results since a greater protection was observed with the treatments of procyclidine or procyclidine plus atropine compared to the single treated with atropine. During DFP-induced seizure, Glu release induced a rapid activation of NMDA receptors in hippocampus and NMDA receptors are capable of the maintenance of seizure activity and brain damage. EAA antagonists that act at the NMDA receptor, an procyclidine, were shown to be effective as anticonvulsant and neuronal protections of DFP-induced seizures.

In summary, NMDA antagonists are capable of controlling DFP-induced seizures and they must be used in combination with the anticholinergic drug both to assure a maximal antiepileptic capacity and to prevent profound brain damage. NMDA receptor antagonists are promising candidates as antidotes against CNS intoxication by OP, acting through both as anti-excitoxic and anti-epileptic mechanisms. Today, as chemical warfare involving OP nerve agents is a realistic threat in many parts of the world, a search for efficient antidotes is essential. While further pharmacological studies are needed in order to establish the formula of choice and its delivery regimen, NMDA receptor antagonists may prove to be useful candidates for such an antidote.

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국 문 초 록

Diisopropylfluorophosphate에 의한 뇌 손상 기전에 대한 신경화학적 및 약리학적 연구

제주대학교 대학원

수의학과 수의 독성화학 전공

고 봉 우

(지도교수: 이 영 재)

중추신경 매개성 경련과 발작은 비가역적 콜린에스테라제 억제제인 유기인제 중독에 따른 독성증상 중의 하나로 뇌신경세포의 손상에 관여하는 것으로 알려져 있다. 본 연구에서는 유기인제에 의한 뇌 손상유발에 있어서 흥분성 아미노산계의 역할을 규명하고자 하였다. DFP (2.67 ㎎/㎏, s.c.) 투여하여 뇌 손상을 유발한 흰쥐에 있어서, 미세투석법 및 HPLC 분석법을 사용하여 뇌 해마부위에서의 aspartate 및 glutamate의 농도를 측정함으로써 경련유발에 따른 신경전달계의 변화를 관찰하였고, 이에 따른 신경조직의 변화를 관찰하였다. 치료약물로 항콜린 약물인 atropine (15 ㎎/㎏, i.m.)과 NMDA 수용체 길항제인 procyclidine (30 ㎎/㎏, i.m)을 각각 단독 또는 병합 투여하여 DFP에 의한 뇌신경독성에 대한 방어효과를 관찰

하였다. DFP를 투여한 흰쥐는 경련유발과 더불어 해마부위에서의 현저한 aspartate와 glutamate의 농도 증가를 보였으며, 병리조직학적으로 해마의 신경세포 감소를 보였다. DFP에 의하여 상승되어진 해마부위의 aspartate와 glutamate의 수준은 procyclidine에 의하여 현저히 억제되었으나, atropine에 의하여는 DFP 투여군과 유의한 차이를 보이지 않았다. procyclidine과 atropine 병합 투여시에는 procyclidine 단독 투여시와 동일한 효과를 관찰할 수 있었다. 조직염색소견에서도 DFP에 의하여 현저하게 소실되어진 신경세포는 atropine 단독 투여시보다 procyclidine 단독 투여군 또는 atropine과 procyclidine 병합 투여군에서 뚜렷한 신경세포의 손상 및 소실을 방어하는 것을 관찰할 수 있었다. 따라서 DFP에 의하여 유발된 경련은 흥분성 아미노산 신경전달계를 활성화시키고 이에 따라 뇌신경의 손상이 유발되는 것으로 생각된다. 특히 procyclidine은 DFP에 의한 경련유발과 뇌 세포손상을 효과적으로 방어할 수 있었으며, 이는 흥분성 아미노산계 중 NMDA 수용체가 DFP에 의한 뇌 손상기전에 관여됨을 시사한다. 이상의 결과로 DFP에 의하여 유발된 경련은 일차적으로 콜린성 신경계를 과도하게 자극하여 이에 따른 흥분성 아미노산의 과도한 분비를 유발하고 특히 NMDA 수용체를 활성화하여 뇌의 손상을 가져온다고 사료된다.

중요어: DFP, 유기인제, 경련, 발작, 미세투석법, *N*-methyl-D-aspartate, aspartate, glutamate, acetylcholine, acetylcholinesterase, cholineacetyltransferase