CHAPTER III

Research Methodology

Animals

Male Sprague-Dawley rats (National Animal Center, Salaya, Nakorn Pathom) weighing between 200-250 g were used in all experiments as experimental animals. Six animals were treated for each group of experiment. Animals were housed 2 per cage at 24±1°C under a 12-h light/dark cycle. All animal procedures were carried out in compliance with Mahidol University Code of Practice and the National Institutes of Health (USA) Guidelines for treatment of laboratory animals. The protocol for this study was approved by the Animal Research Committee of Naresuan University.

Methamphetamine administration

D-methamphetamine hydrochloride (Alltech, Palatine & DC, IL, USA) with permission of Ministry of Public Health was used for the experiment. After one week for acclimating to the environment, all animals were divided into three groups including control group, acute and chronic methamphetamine groups. The rats in acute methamphetamine group were received saline for 13 days and one intraperitoneal injection of 8 mg/kg methamphetamine on the fourthteen day (Segal and Kuczenski, 1997a). In the chronic methamphetamine group, rats were treated with intraperitoneal injection of 4 mg/kg/day methamphetamine for 14 days (Konradi et al., 1991; Segal and Kuczenski 1997b) and the control group, rats were treated with intraperitoneal injection of 0.9% saline for 14 days. Rats were sacrificed by cervical dislocation 24 h after the last injection and brains were rapidly removed. Brains were isolated according to the rat brain atlas (Paxinos and Watson, 1998).

Analysis of NMDAR1 and EAAT3 proteins by western blotting technique

The western blot (alternately, immunoblot) is a method to detect a specific protein in synthetic membranes, depending on interaction between antigen and antibody. This interaction is visualized by either direct or indirect immunoassay methods. In the present study, the expression of glutamate receptor (NMDAR1) and

neuronal glutamate transporter (EAAT3) were detected using indirect method. This method, an unlabeled primary antibody was added first to bind to the antigen and followed by adding a biotinylated-conjugated secondary antibody that was directed against the primary antibody. The step biotinylated-conjugated secondary antibody was used to localize avidin biotinylated horseradish peroxidase complex (ABC) and was visualized using peroxidase substrate, 3,3', 5,5'-tetramethylbenzidine (TMB).

Antibodies

Primary antibodies

NMDAR1 polyclonal antibody (G-8913, Sigma, Mo, USA) was used for specific detection of NMDAR1 proteins by immunoblotting technique. Anti-NMDAR1 receptor is antibody which is raised against a peptide mapping to the carboxyl terminus of rat NMDAR1 receptor. It is identical in the rat, mouse and human NMDAR1 receptor. EAAT3 goat polyclonal antibody (sc-7761, Santa Cruz Biotechnology, Inc., USA) was applied to specifically detect EAAT3 proteins by immunoblotting technique. Anti-EAAT3 transporter is an antibody raised against peptides mapping to the carboxyl terminus of EAAT3 of human origin, which is identical to corresponding rat sequences of EAAC1. β-actin mouse monoclonal antibody (sc-47778, Santa Cruz Biotechnology Inc, USA) is raised against a peptide mapping to carboxyl terminus of gizzard actin of chicken. It is used for β-actin detection in rat, mouse, human, chicken, cow, dog, pig and rabbit.

Secondary antibodies

In this experiment, goat anti-rabbit biotinylated-conjugated secondary antibody (Vectorstain ABC kit, Vector Laboratories, Burlingame, CA), was used for binding rabbit anti-NMDAR1 primary antibody. Rabbit anti-goat biotinylated-conjugated secondary antibody (Vectorstain ABC kit, Vector Laboratories, Burlingame, CA) was applied to bind with the goat anti-EAAT3 primary antibody. Horse anti-mouse biotinylated-conjugated secondary antibody (Vectorstain ABC kit, Vector Laboratories, Burlingame, CA), was used for binding mouse anti-β-actin primary antibody.

Tissue preparation

The hippocampal formation, frontal cortex and striatum were isolated according to the rat brain atlas (Paxinos and Watson, 1998). Tissue was homogenized in 12.0 volumes of 5 mM Tris-HCl and 20 mM NaCl, pH 8.0. The homogenate was centrifuged at 48,000 x g for 10 min, and the pellet was collected. The pellet was homogenized again in lysis buffer containing 50 mM Tris-HCl 8.0, 0.15 M NaCl, 0.1% SDS, 0.25% sodium deoxycholate, 1 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM PMSF. Protein concentration was determined in an aliquot of tissue lysate by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL., USA). The samples were stored at 4°C until assayed.

Protein assay

The bicinchoninic acid (BCA) assay is the method for determining protein concentration. This method is based on the conversion of Cu^{2+} to Cu^{+} under alkaline conditions. The Cu^{+} is then detected by reaction with BCA, exhibiting an intense purple which is strongly absorbed at a wavelength of 562 nm. The absorbance is measured with spectrophotometer or microplate reader. The protein content of unknown samples is determined by comparison with a standard curve prepared from known concentrations of protein.

Protein concentrations in the samples were assayed according to BCA method (Instruments of BCA proteins assay reagent kit, Pierce, Rockford, IL., USA) and the procedure was performed in 96-well plates. Protein standards were prepared from a solution of bovine serum albumin (BSA). The homogenate was diluted in lysis buffer and measurements were performed in triplicate. The BCA working reagent (WR), combination of 50 parts of BCA reagent A and 1 part of BCA reagent B, were added to the sample in proportion of one to eight (sample : WR). The solution was mixed and incubated for 30 min at 37°C. Absorbance was read at a wavelength of 540 nm by the microplate reader (Labsystem iEMS Reader MF) with Transmit Software Revision 4.5. A standard curve which is produced from BSA had concentrations between 0.25 mg/ml and 1.0 mg/ml. The protein content of unknown samples was determined using comparison with the standard curve for known protein concentrations.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for NMDAR1, EAAT3 and β-actin

Protein samples were prepared with an equal volume of 2x SDS-PAGE sample buffer containing 250 mM Tris buffer, pH 6.8, 2% SDS, 10% glycerol, 20 mM dithiothreitol and 0.01% bromophenol blue. The samples were boiled for 5 min to denature the proteins and completely unfolding them with SDS. The samples were then centrifuged at 48,000 x g for 3 min and the supernatant was collected. The supernatants containing 80 µg original proteins, which correspond to the appropriate results for NMDAR1 detecting (Figure 19), were analyzed on 4% stacking gel and 8% separating gel for 1.45 h at 75 constant voltages. For EAAT3, the supernatants containing 50 µg, chosen to give results within the linear range for EAAT3 estimations (Figure 21), were analyzed on 4% stacking gel and 10% separating for 2.15 h at 75 constant voltages. In control the efficiency for loading and transferring of NMDAR1 protein, the β-actin was used as an internal standard and applied in the same sample belong to NMDAR1, while a pooled control sample were used in multiple lanes on each gel as an internal standard to control for both within-gel and inter-gel variance on EAAT3 assay. The samples were run in one dimension along the gel. Proteins were moved toward the positive electrode (anode) and were separated depending on protein size, which smaller size can be moved faster than larger ones. Prestained SDS-PAGE standards (Biorad Laboratories, Hercules, CA) ranging from 6.9 to 201 kDa and Prestained SDS-PAGE standards (Pierce, Rockford, IL., USA) ranging from 17.3 to 205 kDa were run in parallel with sample of NMDAR1 and EAAT3, respectively.

Protein blotting for NMDAR1, EAAT3 and β-actin

Following electrophoresis, separated proteins were transferred from the eletrophoresis gels to polyvinylidene fluoride (PVDF) membranes (Amersham, Arlington Heights, IL, USA) with a semi-dry blotting apparatus (ECL semi-dry blotter, Amershams Biosciences, Co., USA) for 1 h 30 min at 120 mA. After blotting, the gels were stained with Coomassie Brilliant blue (Figure 15), while membranes were stained with Ponceau S to confirm complete transferring of protein from gel to membrane (Figure 18). Following staining, stained gels were destained with the

Coomassie blue destaining solution and dried on filter paper under vacuum (GD 2000 slab gel dryer, Amersham Bioscience, Co., USA) (Figure 16). The stained membranes were washed several times with phosphate-buffered saline (PBS) and stored dry at room temperature until detection.

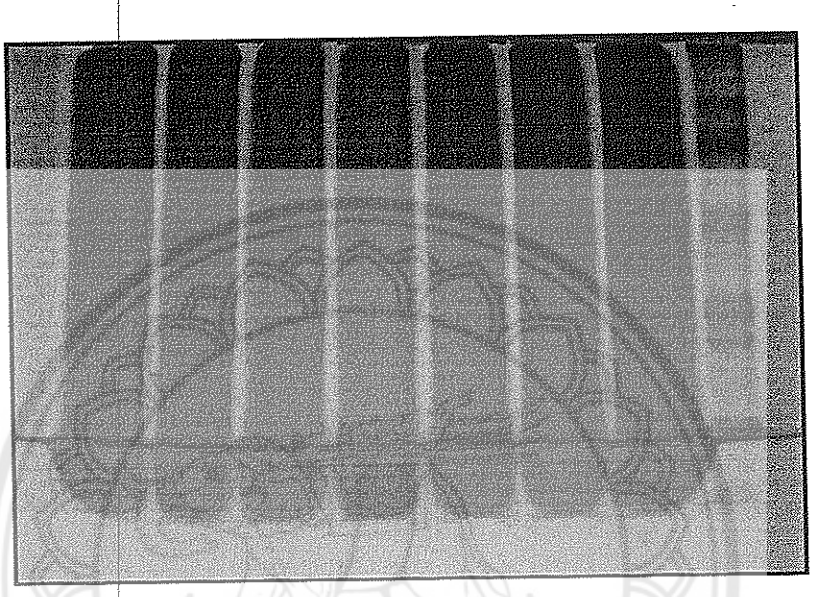

Figure 15 SDS-PAGE staining with Coomassie blue before electroblotting proteins onto PVDF membrane

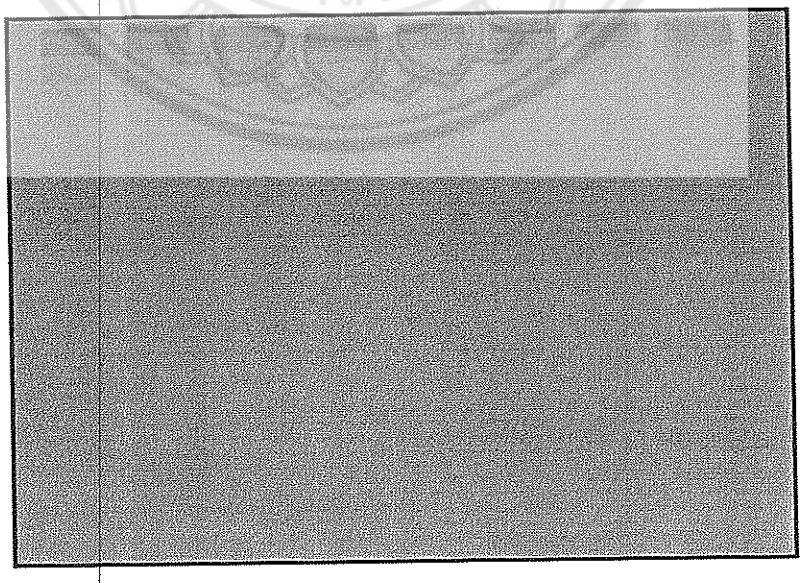

Figure 16 SDS-PAGE staining with coomassie blue after electroblotting proteins onto PVDF membrane

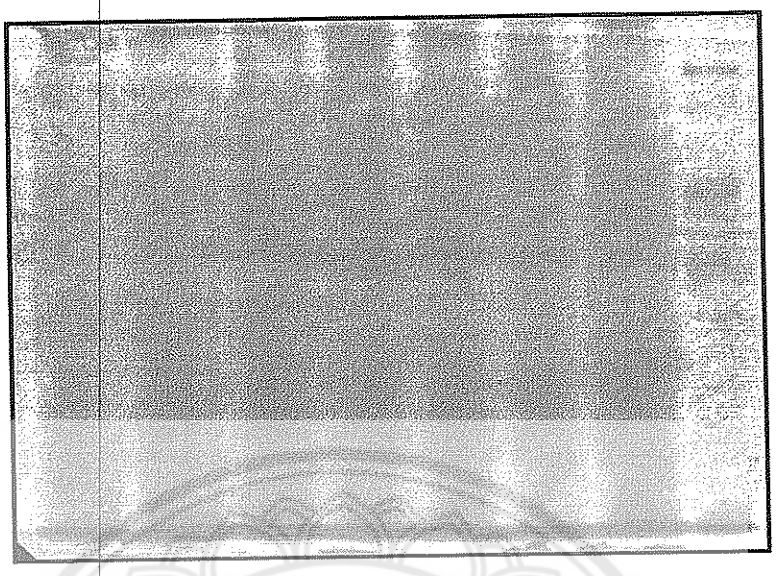

Figure 17 PVDF membrane after electroblotting procedure

Immunodectection of NMDAR1, EAAT3 and β-actin

The membranes were rehydrated by soaking in methanol and washed with PBS for several times to equilibrate them. The membranes were incubated with protein blocking solution containing 5% bovine serum albumin in PBS with 0.5% of Tween-20 (PBS-T) at room temperature for overnight. After blocking, the membranes were then incubated with primary antibodies for 4 h with rabbit anti-NMDAR1 or 3 h with goat anti-EAAT3 at room temperature. β-actin was labeled as internal standard using mouse anti-β-actin for 4 h at room temperature. All primary antibodies were used at a dilution of 1:500 in blocking solution, which demonstrated the results in the middle of the linear range as determined by densitometric analysis (Figure 20 and Figure 22). The membranes were washed three times for 5 min each with 0.5% PBS-T. This was followed by incubation with biotinylated secondary antibody diluted 1:200 in blocking solution for 2 h at room temperature. Following three washes for 5 min each with 0.5% PBS-T, the membrane was incubated for 1 h with 1:100 avidin biotinylated horseradish peroxidase complexes (ABC kit) in PBS. After washing with PBS-T 3 times and PBS 2 times for 5 min each, the protein immunoreactivity was visualized using 3,3',5,5'-tetramethylbenzidine (TMB) (Promega, Madison, USA) for 10 min, and followed by rinsed in distilled water for stopping the reaction. The developed membranes were allowed to dry and immediately scanned into a computer.

Quantitaive analysis

After the immunodetected membranes were scanned into a computer, integrated optical density (IOD) was measured using Scion Image program based on NIH image (v. Alpha 4.0.3.2; www.scioncorp.com; 2000-2001). The value is the sum of the optical densities of all pixels in the region divided by the number of pixels. All immunoreactivities bands were measured with the same dimension to obtain their integrated optical density following the method previously reported (Nudmamud-Thanoi and Reynolds, 2004). The densities of NMDAR1 within each experimental group were corrected for background to prevent variation between membranes before normalization against that of β-actin (Santa Cruz Biotechnology Inc, CA) (Sidiropoulou et al., 2001). In EAAT3 evaluation, densities of EAAT3 were corrected for background and applied a pooled control sample as an internal standard to control

for both within-gel and inter-gel variance. The average of values from two replicates for each subject was used for statistical analysis.

Statistical analysis

Data are integrated optical density of either NMDAR1 or EAAT3 immunoreactivity bands. All data were expressed as mean ± standard error of the mean (SEM), considering the control group as 100%. The data from acute and chronic methamphetamine groups were calculated as the percentage change from the control group. Statistical analysis was performed using one-way analysis of variance (ANOVA) with post hoc Dunnett test, Students'-t-test and Pearson correlation coefficient. A level of p-values less than 0.05 were considered statistically significant.

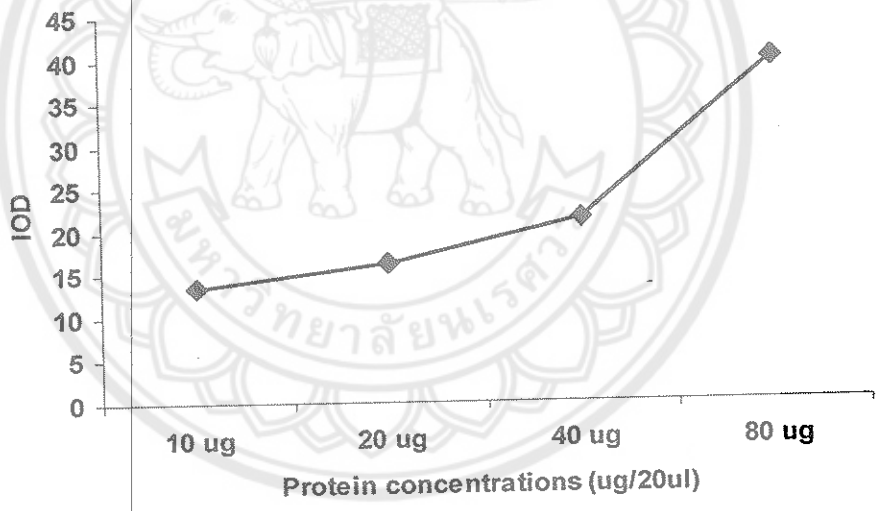

Figure 18 The relationship between IOD of NMDAR1-IR band and protein concentrations in the control rat brain sample

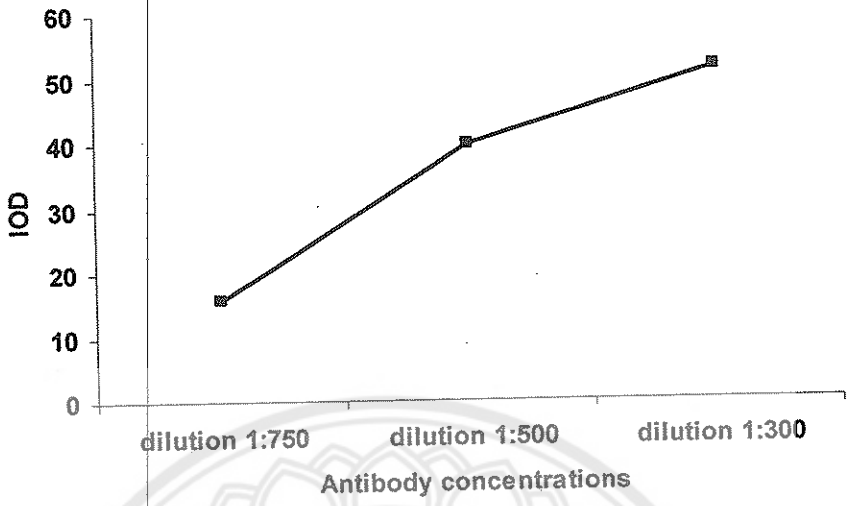

Figure 19 The relationship between IOD of NMDAR1-IR band and concentrations of rabbit polyclonal NMDAR1 antibody

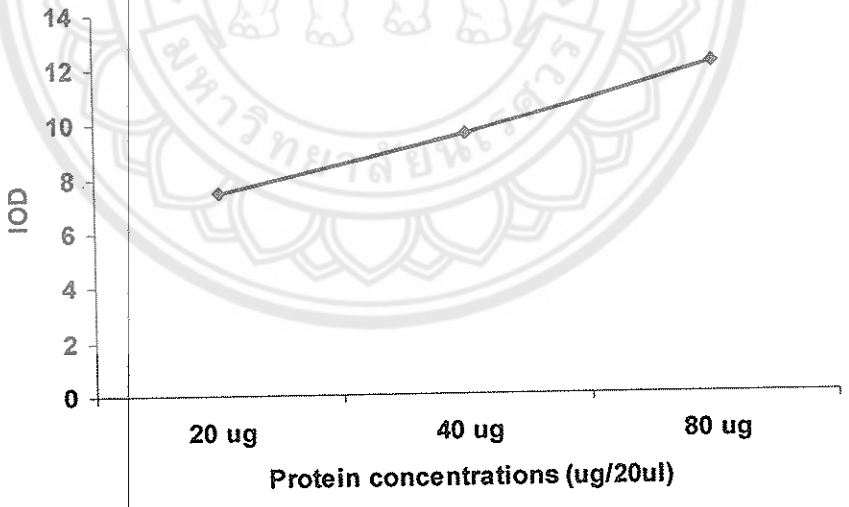

Figure 20 The relationship between IOD of EAAT3-IR band and protein concentrations in the control rat brain sample

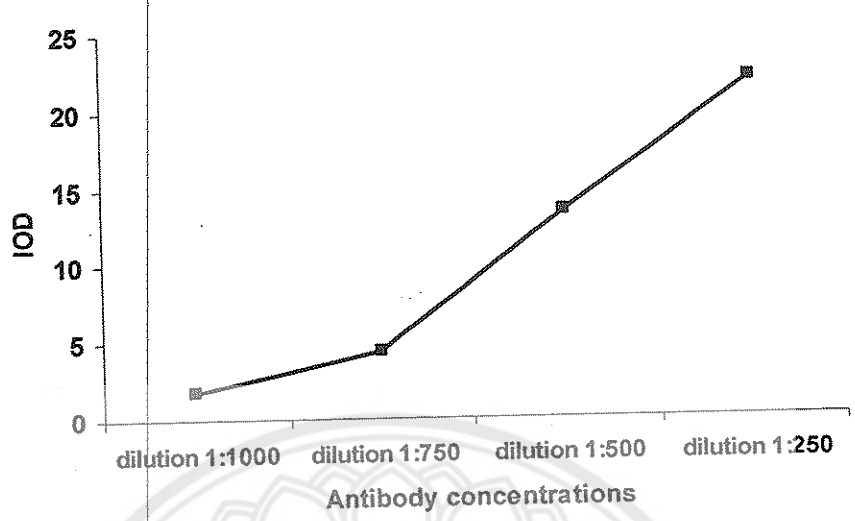

Figure 21 The relationship between IOD of EAAT3-IR band and concentrations of goat polyclonal EAAT3 antibody