CHAPTER II

MATERIALS AND METHODS

MATERIALS

Polyclonal antiserum against Fos (residues Ser-Gly-Phe-Asn-Ala-Asp-tyr-Glu-Ala-Ser-Ser-Arg-Cys) conjugated to bovine thyroglobulin(Code No.AB1584, Lot No. 17120013), biotinylated rabbit anti sheep IgG (Code No.AP147B, Lot No.17068030) and normal rabbit serum (Code No. S-20, Lot No.17090246) were purchased from Chemicon International, Inc., U.S.A. Avidin-biotin-peroxidase complex (Code No. PK 6100) was obtained from Vector labs, Burlingame,CA. 8-hydroxy-2-di-n-propyl-amino-tetralin (8-OH-DPAT) (Code No. H-8520) and 1,2,5-dimethoxy-4-iodophenyl-2-aminopropane (DOI) (Code No. D-101) were purchased from Sigma Chemical Company and Research Biochemical Inc., U.S.A., respectively.

METHODS

1. EXPERIMENTAL ANIMALS

Forty eight adult male Wistar rats weighing between 300-400 grams were utilized in this study. All of them were purchased from National Laboratory Animal Center, Mahidol University, Nakorn pathom and kept in the cages at the animal center of the Faculty of Medicine, Chulalongkorn University. The animals were kept under a photoperiod of 12 hours light and 12 hours darkness, with free access of food and water at least two weeks before experiment.

The animals were divided into 3 groups.

Groups 1 Control group :

Animals were injected with normal saline solution (NSS) intraperitoneally.(i.p.)

Groups 2 5-HT_1- agonist group :

Animals were injected with 8-hydroxy-2-di-n-propyl-amino-tetralin (8-OH-DPAT) in NSS at a dose of 3 mg/kg bw i.p.

Groups 3 5 HT_2-agonist group :

Animals were injected with 1,2,5-(dimethoxy-4-iodophenyl)-2-aminopropane (DOI) in NSS at a dose of 1 mg/kg bw i.p.

2. NOCICEPTIVE TEST

The two kinds of nociceptive test were used in this study. Both tests were performed on the three animal groups as described above.

2.1 Tail Flick test

The tail flick test was performed using " Tail Flick Analgesia Meter " (Harvard, U.K.). Tail flick test is a heat nociception test initially employed by D'Amour and Smith (1941) and further described in detail by Ren and Han (1986). The test is based on a segmentally mediated reflex movement of the tail. The distal 4 cm. portion of the rat tail was stained with a black ink marker pen in order to absorb a maximum amount of heat. The ventral side of the tail was then placed over the tail flick analgesia meter which the light beam of 2.5 ampere was focused. Time interval between heat exposure to tail flicking (tail flick latency) was measured . Five measurements were made in each animal with 3-minutes intervals and the mean value of these measurements was used for calculation.

The experiments were done by lightly anesthesizing the animals with sodium pentobarbital (25 mg/kg b.w. i.p.). The tail flick test was first performed on these animals, without any drug administration before the test. Three days after of the first test, the rats were retested for the second tail flick. At this second time, the animals were injected with normal saline, 8-OH-DPAT, and DOI in each groups thirty minutes prior to the test. The tail flick latency measure for two experiments were compared.

2.2 Formalin test

The model was performed by the injection of formalin into the forepaw of the animal in order to produce chemical inflammation. The experiments were done as the followings. Three groups of rats were pretreated with normal saline or 8-OH-DPAT or DOI. Thirty minutes after administration, the animals were anesthetized by ether inhalation and was injected with 100 µl of 5% formalin solution subcutaneously at the plantar surface of the left forepaw. Then, the formalin-induced nociceptive behaviors were observed by examining the total time (second) of the lifting (the rats lifted and flexed injected forepaw from ground), licking (the rats used their tongue to lick injected forepaw) and scratching (the rats represented sound forepaw to scratch injected forepaw) within 10 minutes (phase I represented tissue injury period) and 10-60 min (phase II representd inflammatory period) after formalin injection. The observation was recorded by video camera in order to count later.

Two hours after formalin injection, the animals were sacrificed for further immunohistochemistry study.

In order to identify that Fos did not evoke by injection, subcutaneous injection of 100 µl normal saline into the left forepaw.

3. IMMUNOHISTOCHEMICAL PROCEDURES

The animals from the tonic pain model were then detected for Fos expression by the immunohistochemical method.

3.1 Tissue preparation

Two hours after formalin injection, the animals were deeply anesthetized with pentobarbital at a dose of 50 mg/kg i.p. and perfused transcardially with 300 ml 0.1 M. phosphate buffer saline (PBS pH 7.4), and then with 500 ml of 4% paraformaldehyde fixative in 0.1 M phosphate buffer. After perfusion the the upper and 7^{th}, 8^{th} cervical and 1^{st} thoracic spinal cord level of rat were removed and postfixed in the same fixative overnight at 4°C. The tissues were cryoprotected overnight in 30% sucrose in 0.1 M phosphate buffer at 4°C. The tissue blocks were then frozen in crushed dry ice and sectioned in the axial plane in a cryostat at a thickness of 30 μm and kept in 0.1 M PBS until immunohistochemical process.

3.2 Immunohistochemical process

The immunostaining was performed on free-floating sections with a polyclonal antiserum raised in sheep against Fos, using the avidin-biotin-peroxidase complex method. First, the sections were rinsed for 10 min in PBS. They were then pretreated in 1% hydrogen peroxide (H_2O_2) in PBS for 10 min at room temperature to reduce the endogenous peroxidase. Prior to incubation in the primary antiserum, the sections were incubated in 5% normal rabbit serum in PBS-A (phosphate buffer saline contained 0.3% Triton X-100 and 1% bovine serum albumin-BSA) for 30 min at room temperature. This was followed by the incubation in the specific sheep anti Fos antiserum diluted 1:500 in PBS-A for 48 hours at 4°C and washing for 3x10 min in

PBS-B (phosphate buffer saline contained 0.25% Triton X-100 and 0.1% bovine serum albumin-BSA). The sections were incubated with biotinylated rabbit anti-sheep IgG diluted 1:400 in PBS-B for 1 hour at room temperature. After washing in PBS-B for 2 x 10 min and in PBS for 10 min, the sections were incubated at room temperature with the ABC-streptavidin-horseradish peroxidase complex diluted 1:250 in PBS for 1 hour at room temperature. The sections were then washed sequentially in PBS for 2 x 10 min, and in 0.05 M. Tris-HCl buffer (pH 7.6) for 10 min. Finally they were reacted for peroxidatic activity in a solution containing 0.025% 3,3′ diaminobenzidine (DAB) solution and 0.01% H_2O_2 in 0.05 M. Tris-HCl buffer (pH 7.6) for 30 min and rinsed in distilled water for 2 x 5 min. The sections were placed on gelatinized glass slides, dried and coverslipped the slides with permount.

The Fos protein-like immunoreactive cells (FLI) were examined and plotted under the light microscope from the sections C_7, C_8 and T_1 spinal level with boundaries of lamina I/II, III/IV and V of Rexed's lamination. For quantitative analysis all FLI were counted from eight tissue sections at random of each three spinal levels (C_7-T_1) for each rat.

5. STATISTICAL ANALYSIS

The effects of 8-OH-DPAT and DOI on total numbers of Fos-immunoreactivity neurons and behavior responses were compared by using one way analysis of variance (ANOVA) and then Bonferroni test for post hoc comparisons. Pair *t*-test was used to compare tail flick latency between with and without agonists pretreatment. In all comparisons, statistical significance were considered at the level of p value<0.05.