CHAPTER III

MATERIALS AND METHODS

Materials

A. Chemicals and Biochemicals

Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) (Merck)

Sodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$) (Scharlar)

Sodium chloride (NaCl) (Merck)

Potassium chloride (KCl) (Sigma)

Calcium chloride ($CaCl_2.2H_2O$) (Sigma)

Magnesium chloride ($MgCl_2 \cdot 7H_2O$) (Sigma)

Sodium bicarbonate ($NaHCO_3$) (Sigma)

Glucose (Sigma)

Normal saline for injection and for irrigation

Para chorophenylalanine (PCPA) (Sigma)

Sodium thiopental (Abbott)

Heparin (Leo)

Histamine (Sigma)

Bradykinin triacetate salt (Sigma)

Serotonin hydrochloride (Sigma)

Prostaglandin E_2 (Sigma)

Paraformaldehyde (Acros)

Sodium hydroxide (NaOH) (Merck)

Hydrochloric (HCl) (Merck)

Sucrose (Sigma)

OCT cutting medium (Richard-Allan Scientific)

Absolute ethanol

Hydrogen peroxide (Merck)

Normal horse serum (Gibco)

Gelatin (Fluka)

Permount (Fisher Scientific)

Rabbit anti-Fos polyclonal affinity purified antibody (sc-52; Santacruz)

Rabbit anti-pNR1 ser-896 immunoaffinity purified antibody (06-640; Upstate)

Rabbit anti-NR1 polyclonal antibody (sc-9058; Santacruz)

Antibody diluent (Dako)

Envision+ HRP anti-rabbit (Dako)

Liquid DAB substrate kit (Dako)

B. Materials

Syringes and needles (Nipro)

Cannula

Microscopic glass slides and cover slips

Stereotaxic apparatus

Perfusion apparatus

Dental drill with hand piece (Marathon, NSK)

Cryostat (Microm)

pH meter (Fisher Scientific)

Light microscope (Olympus)

Micropipette (Pipetteman)

Microcentrifuge tube

Refrigerator -20 °C and 4 °C

Hotplate

Cyclomixer (Clay Adams)

Magnetic stirrer (Corning)

Latex glove

Laboratory sealing film (Whatman)

Experimental design

In order to study the effect of dural stimulation on NMDA receptor expression, NMDA receptor phosphorylation, and trigeminal nociception in normal condition and the effect of serotonin depletion on those parameters, the experiments were designed as following diagram (figure 1).

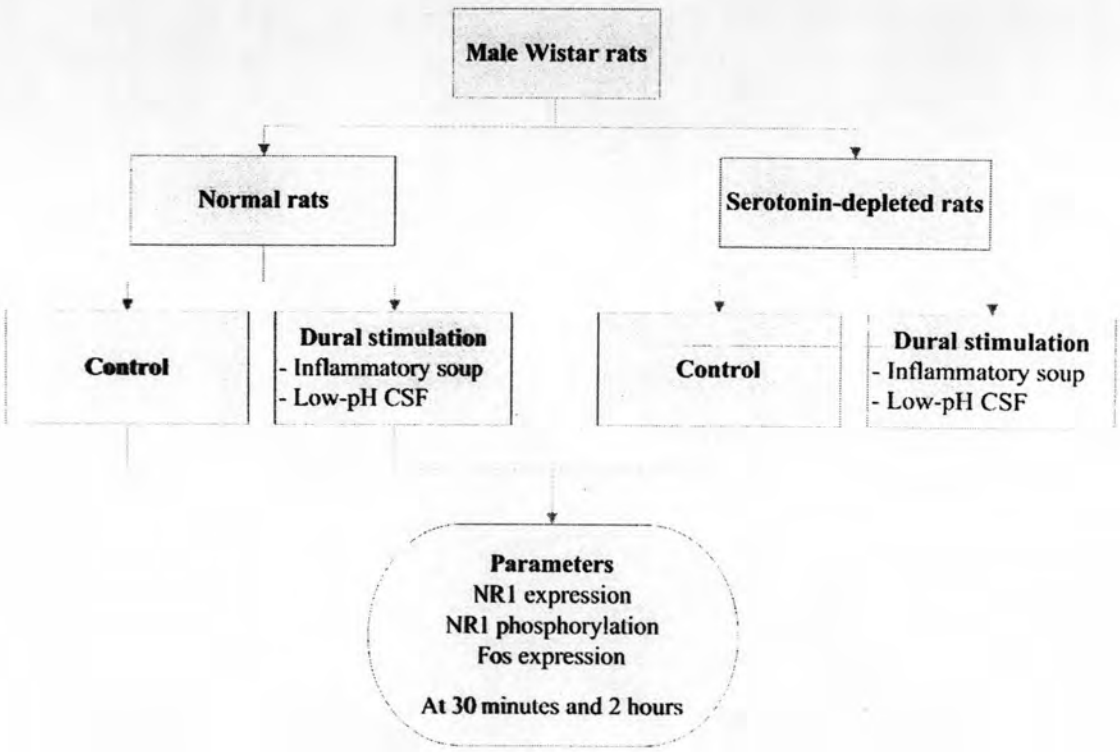

Figure 1 Experimental design

In this study, the male Wistar rats weighting 200-350 grams were divided into 2 groups: normal serotonin group and serotonin-depleted group. In normal serotonin group, physiological saline was administered (i.p.) three days before the operation, while in serotonin-depleted group, para-chlorophenylalanine (PCPA), a tryptophan hydroxylase inhibitor, was administered (i.p. 100 mg/kg body weight) three days before the operation. Each group was further divided into 2 subgroups: dural stimulation group and control group. Dural stimulation was performed by topical application of inflammatory soup (IS) or low-pH phosphate-buffered artificial cerebrospinal fluid (low-pH CSF, pH 4.7). Artificial cerebrospinal fluid (CSF) was applied in control group. In each subgroup, experiment was conducted at 30 minutes and 2 hours time

points. In the day of experiment, open skull operation was performed in all rats to expose dura, and then the chemicals were applied on the dura for 30 minutes. Thirty minutes and two hours after dural stimulation, rats were humanely killed and perfused with phosphate-buffered saline (0.01 M PBS) and 4% paraformaldehyde. Then, brainstems and spinal cords were collected for Fos, NMDA receptor NR1 subunit, and serine-896 phosphorylated NMDA receptor NR1 subunit immunohistochemical studies. Five rats per time point of each subgroup were used. However, the result from our pilot study showed that low-pH CSF was not effective in stimulating trigeminal nociception. Thus, in low pH group, the rats were performed only at 2-hour time point.

For clarification, the rats were divided into 4 subgroups as following:

1.) Normal rats without dural stimulation (n = 10).

In this group, the CSF was applied on exposed dura for 30 minutes. After 30 minutes and 2 hours, rats were humanely killed (n = 5 per time point).

2.) Normal rats with dural stimulation (n = 14).

The dural stimulation was performed in this group by topical application of IS or low-pH CSF on exposed dura for 30 minutes. In IS group, rats were humanely killed after 30 minutes and 2 hours (n = 5 per time point). In low pH group, rats were humanely killed after 2 hours (n = 4).

3.) Serotonin depleted rats without dural stimulation (n = 10).

After depletion of serotonin by PCPA injection three days before the operation, the CSF was applied on exposed dura for 30 minutes. After 30 minutes and 2 hours, rats were humanely killed (n = 5 per time point).

4.) Serotonin depleted rats with dural stimulation (n = 14).

After depletion of serotonin by PCPA injection three days before the operation, IS-or low-pH CSF was applied on exposed dura for 30 minutes in order to stimulate the dura. In IS group, rats were humanely killed after 30 minutes and 2 hours (n = 5 per time point). In low pH group, rats were humanely killed after 2 hours (n = 4).

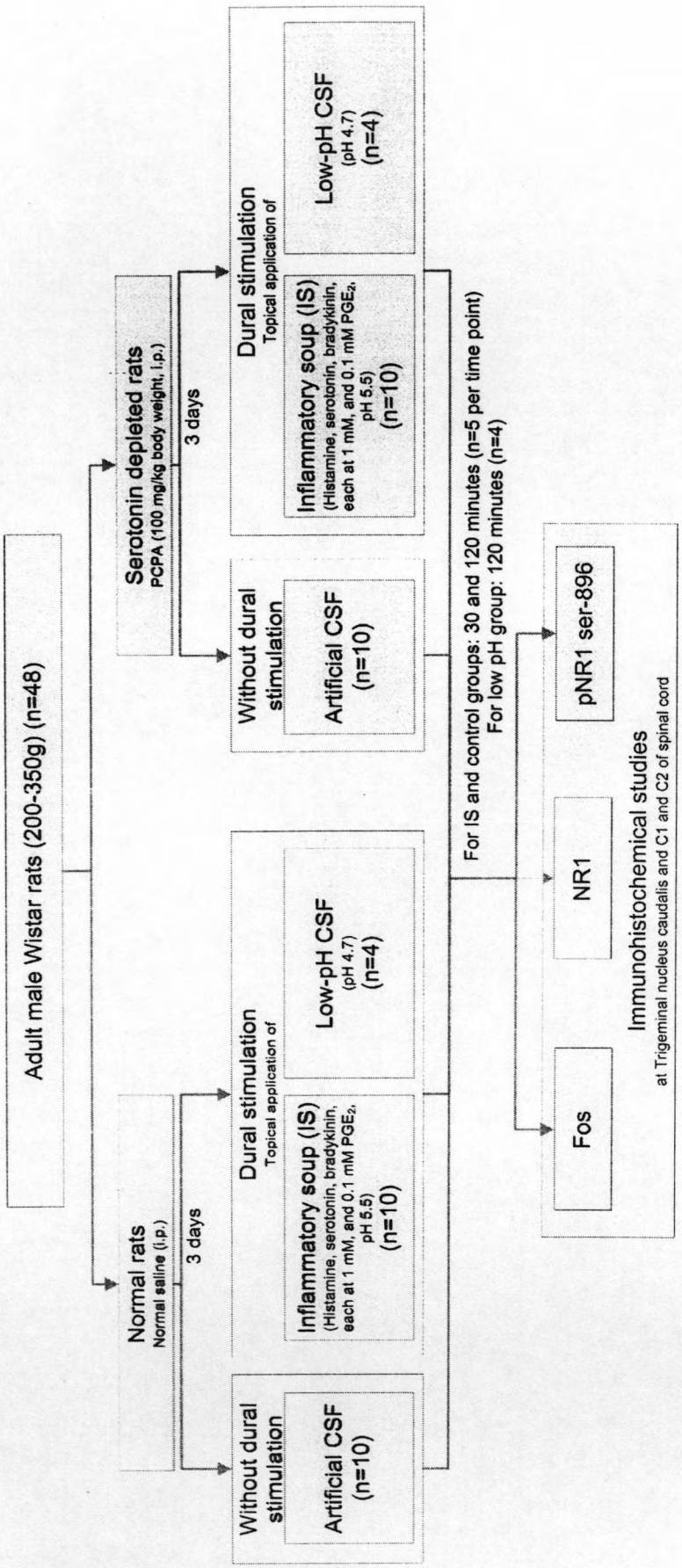

Figure 2 More detailed experimental design

Methods

A. Animal preparation

Adult male Wistar rats (National Laboratory Animal Center of Mahidol University, Thailand) weighting 200-350 g were housed in stainless cages with free access to food (regular dry rat food) and water. The animals were maintained in a temperature-controlled room with 12-hour dark/light cycle. They were allowed to acclimate to the housing environment at least 7 days before experiments.

B. Induction of serotonin depletion

In the serotonin-depleted groups, PCPA (100 mg/kg body weight) was administered intraperitoneally 3 days prior to the operation. This regimen causes a long-lasting reduction of serotonin, and can deplete serotonin level in the brain to less than 20 percent of normal amount [115]. The action of PCPA is that it irreversibly incorporates itself into the tryptophan hydroxylase to produce inactive enzyme [22, 105]. In our experiment, this regimen caused an up to 80% reduction of 5-HT in many brain areas as quantify by HPLC technique at 3 days after PCPA injection. In the control groups, same amount of physiological saline was injected intraperitoneally.

C. Surgical preparation

The rat was anesthetized with sodium thiopental (60 mg/kg, i.p.), and fitted with an intratracheal tube. Cannula was inserted into the femoral vein for later infusion of anesthetic (figure 3). Additional doses of anesthetics were given as required to maintain surgical anesthesia based on response to tail pinch. Rat was placed in a stereotaxic apparatus. To apply chemical stimulation onto the dura mater, the skull was exposed, and a craniotomy of 5-mm in diameter was performed at parietal bone (5 mm posterior to bregma and 3 mm lateral to midline; figure 4 and 5). The bone was carefully drilled using slow speed, saline-cooled technique in order to keep the dura underlying the bone intact and to minimize surgical irritation of the neurons.

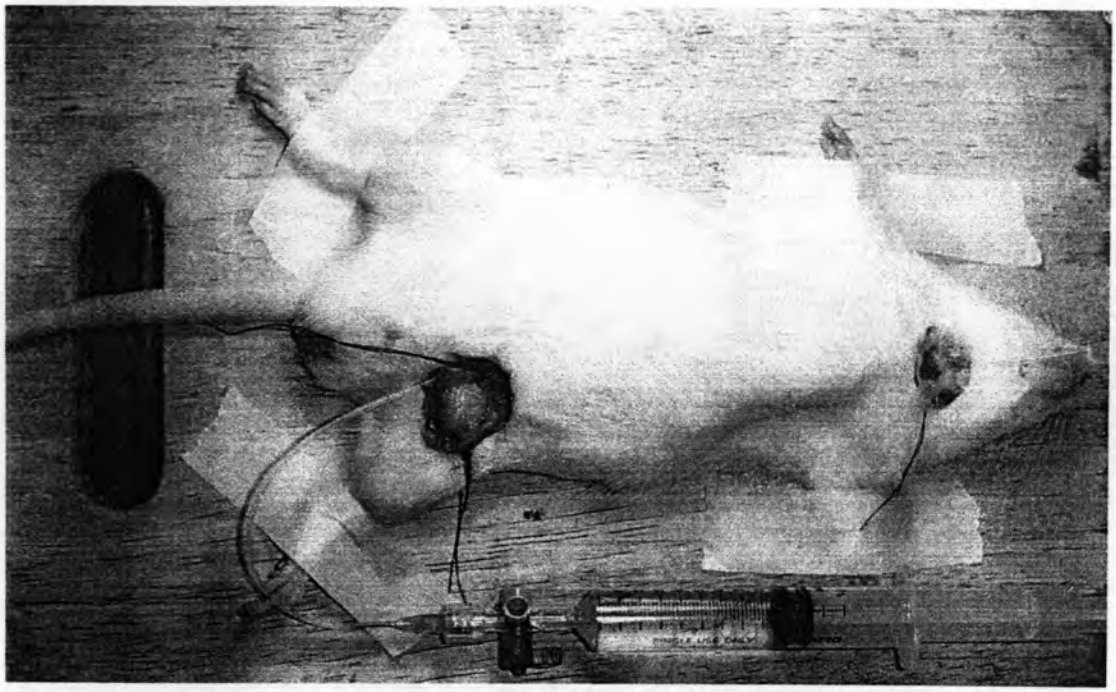

Figure 3 Tracheotomy and femoral vain cannulation. Tracheotomy was performed to assist ventilation and femoral vain cannulation was performed for later infusion of anesthetic.

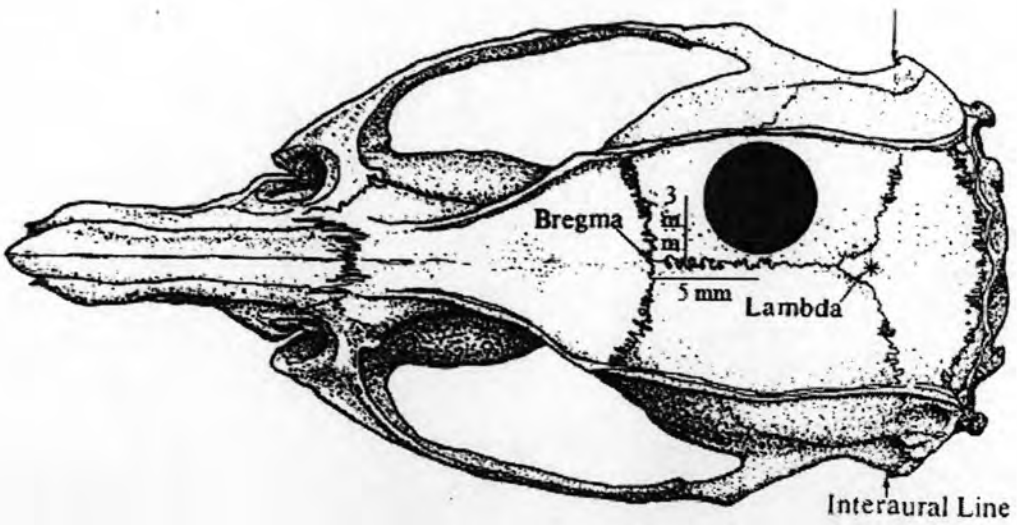

Figure 4 Craniotomy position on rat skull. A craniotomy of 5-mm in diameter was performed at parietal bone (5 mm posterior to bregma and 3 mm lateral to midline).

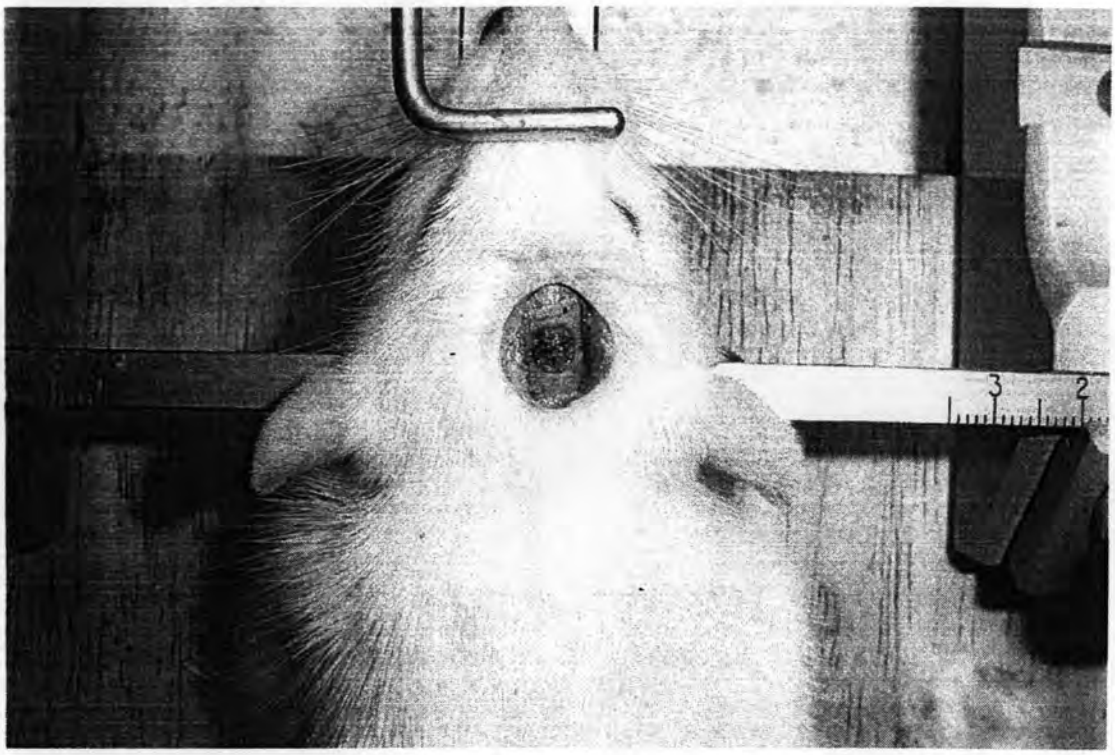

Figure 5 Open skull operation. The bone was carefully drilled using slow speed, saline-cooled technique.

D. Chemical stimulation

The head of the rat was adjusted to make it lying perfectly horizontal to prevent chemical spreading out of craniotomy. To activate trigeminal nociception, 30 µl of IS containing a mixture of inflammatory mediators (histamine, serotonin, bradykinin, each at 1 mM, and 0.1 mM prostaglandin E_2, pH 5.5) or low-pH phosphate-buffered CSF (low-pH CSF; pH 4.7) was topically applied on exposed dural surface for 30 minutes [55]. After that, the dura was washed with a CSF and kept moist with cotton soaked with a CSF.

E. Perfusion

After the stated survival time (30 minutes or 2 hours after the beginning of inflammation induction), rat was humanely killed with an excessive dose of thiopental. Rat was laid on its back and the thorax was carefully opened for access to the heart. Left ventricle was cut and a cannula of perfusion apparatus was inserted to the hole through ascending aorta (figure 6). Right atrium was cut open for drainage. Rat was perfused transcardially with 200 mL of heparinized PBS at room temperature (heparin:

0.01 M PBS = 0.25:100, pH 7.3 - 7.4) (about 3 minutes) or until blood-rich organ such as liver turned grayish-white. Then, 250 ml of 4% paraformaldehyde in 0.01 M PBS pH 7.2 - 7.3 at 4 °C was perfused (about 10 minutes). During perfusion, muscle tremor was seen on the limbs and tail. After perfusion, the brain, brain stem, spinal cord, and trigeminal ganglia were removed, and further fixed in 4% paraformaldehyde in 0.01 M PBS for 3.5 hours at 4 °C. Then, the samples were transferred and stored in 0.01 M PBS at 4 °C until continuing the next process.

F. Cryosectioning

Brain stem and spinal cord were cut into a 5-mm block including trigeminal nucleus caudalis, C1 and C2 of spinal cord (approximately -1 mm to -6 mm from obex; figure 7). Sample was immersed in 30% sucrose in 0.01 M PBS for 24 to 36 hours at 4 °C. Then, sample was placed on a stage, and Optimal Cutting Temperature (OCT) embedding medium was applied to completely cover the sample. After the OCT freezed solid at -20 °C, sample was coronally sectioned on a cryostat at 20 μm thick. The ribbons of sections were collected in 0.01 M PBS. Then, the sections were washed 3 times and stored in 0.01 M PBS before continuing the next process.

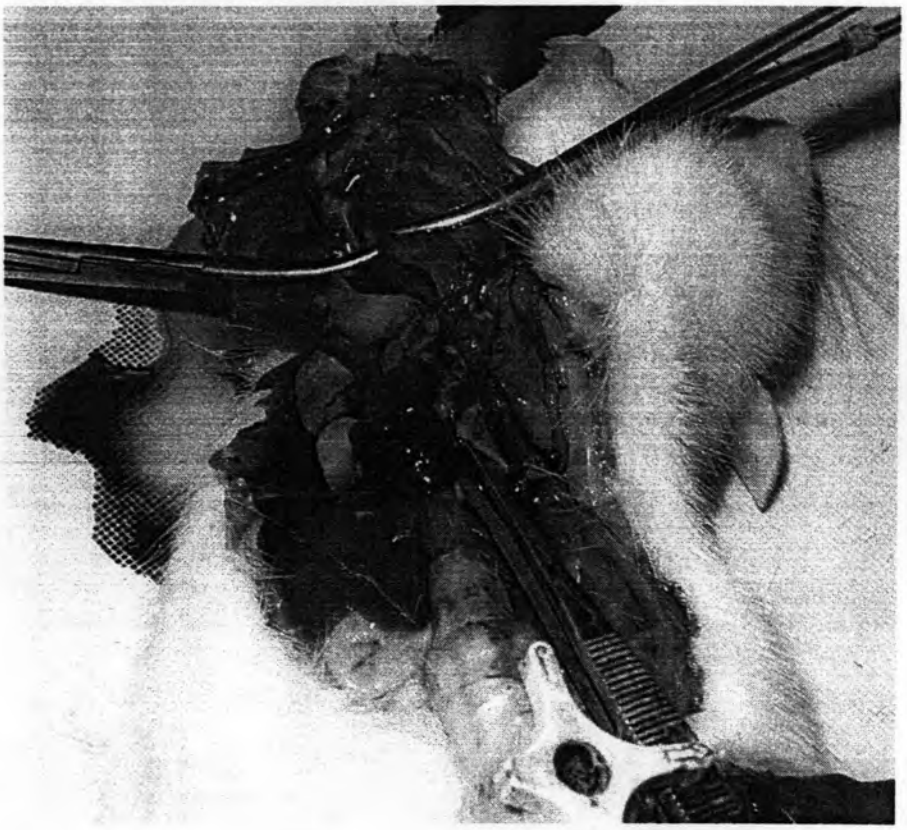

Figure 6 A cannula inserted to heart for perfusion. Left ventricle was cut and a cannula of perfusion apparatus was inserted to the hole through ascending aorta.

Figure 7 Brain stem and spinal cord block. Brain stem and spinal cord were cut into a 5-mm block including TNC, C1 and C2 of spinal cord (approximately -1 mm to -6 mm from obex).

G. Immunohistochemistry

All sections were randomly selected and collected in 3 glass vials (20 sections per vial) for Fos, NR1, and serine-896 phosphorylated NR1 (pNR1 ser-896) immunohistochemistry using free floating technique. All incubation temperature, if not stated, is room temperature and washing buffer is 0.01 M PBS.

1.) Fos immunohistochemistry

Sections were washed 3 times, 10 minutes each. Then, sections were incubated in 50% ethanol for 30 min and then in 3% hydrogen peroxide in 50% ethanol for 30 minutes to suppress endogenous peroxidase. To block nonspecific binding, sections were washed 3 times, 10 minutes each, and incubated for 1 hour in PBS containing 3% normal horse serum. Sections were then incubated for 24 hours at 4 °C with rabbit anti-Fos polyclonal antibody (Santacruz) diluted 1:500 in PBS containing 3% normal horse serum. After washing 3 times, 10 minutes each, sections were incubated for 45 minutes with Envision+ HRP anti-rabbit. After washing 3 times, 10 minutes each, sections were reacted with liquid DAB for 14 minutes. The reaction was terminated with two successive rinses in distilled water. Sections were mounted onto gelatin-coated slides, air dried for overnight, and coverslipped with Permount. The Fos-immunoreactive (-ir) cells were defined as those with a dark brown stain in their nuclei.

2.) NR1 and pNR1 ser-896 immunohistochemistry

Sections were washed 3 times, 10 minutes each. Then, sections were incubated in 3% hydrogen peroxide in PBS for 30 minutes to suppress endogenous peroxidase. To block nonspecific binding, sections were washed 3 times, 10 minutes each, and incubated for 1 hour in PBS containing 10% normal horse serum and 0.1% Triton X-100 and for another 1 hour at 4 °C in the same buffer. For NR1, sections were then incubated for 21 hours at 4 °C with rabbit anti-NR1 polyclonal antibody (Santacruz) diluted 1:150 in antibody diluent. For pNR1 ser-896, sections were then incubated for 42 hours at 4 °C with rabbit anti-pNR1 ser-896 antibody (Upstate) diluted 1:1,000 in antibody diluent. After washing 2 times, 10 minutes each, sections were incubated for 30 minutes with Envision+ HRP anti-rabbit. After washing 2 times, 10 minutes each, sections were reacted with liquid DAB for 10 or 9 minutes for NR1 or pNR1 respectively. The reaction was terminated with two successive rinses in distilled water. Sections were mounted onto gelatin-coated slides, air dried for overnight, and coverslipped with Permount. The staining pattern of pNR1 ser-896 is predominantly

perinuclear (endoplasmic reticulum region). Unlike pNR1, NR1 staining pattern is predominantly in the cytoplasm of the cells.

H. Quantification of Fos, NR1, and pNR1 ser-896 immunostaining

For Fos and pNR1 ser-896 slides, immunoreactive cells were manually counted under 10X objective lens of light microscope by the experimenter blinded to the treatment group. Immunoreactive cells were counted in lamina I, II of TNC and C1 and C2 of spinal cord within 10 randomly selected sections in both ipsilateral and contralateral sides. For NR1 slides, slides were scanned using Scan Scope and manually counted under LCD monitor using Aperio image scope program (Figure 8). Immunoreactive cells were counted in lamina I, II of TNC and C1 and C2 of spinal cord within 6 randomly selected sections. Then the average number of immunoreactive cells per slide was calculated.

I. Statistical analysis

Data were expressed as an average number of cells per slide. The averages were compared by one-way ANOVA followed by LSD post hoc procedure. Student's two-tailed t-test was used when appropriate for simple comparisons between means. Pearson correlation was used to evaluate the correlation between pNR1 serine-896 and Fos expression. Probability value (*P*-value) of less than .05 was considered to be statistically significant.

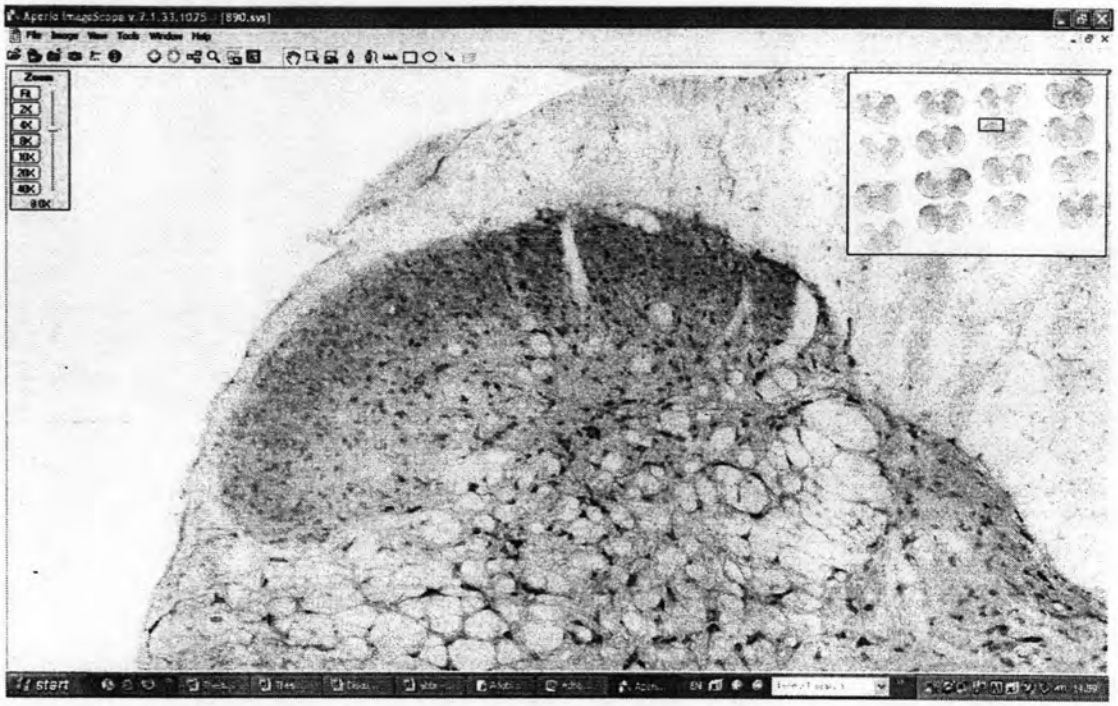

Figure 8 Aperio image scope program. The program was used to view all NR1 slides.