CHAPTER III

MATERIALS AND METHODS

Animals

Adult male wistar rats weighing 200-300 g (National Laboratory Animal Center of Salaya Campus, Mahidol University, Thailand) were housed five per cage in the stainless steel bottom cage and were kept in well-ventilated room which the temperature was 25 ^{0}C with an automatic lighting schedule. All animal were allowed to access food and tap water *ad libitum*. All the protocols in this study were approved by the Ethical Committee of Faculty of Medicine, Chulalongkorn University.

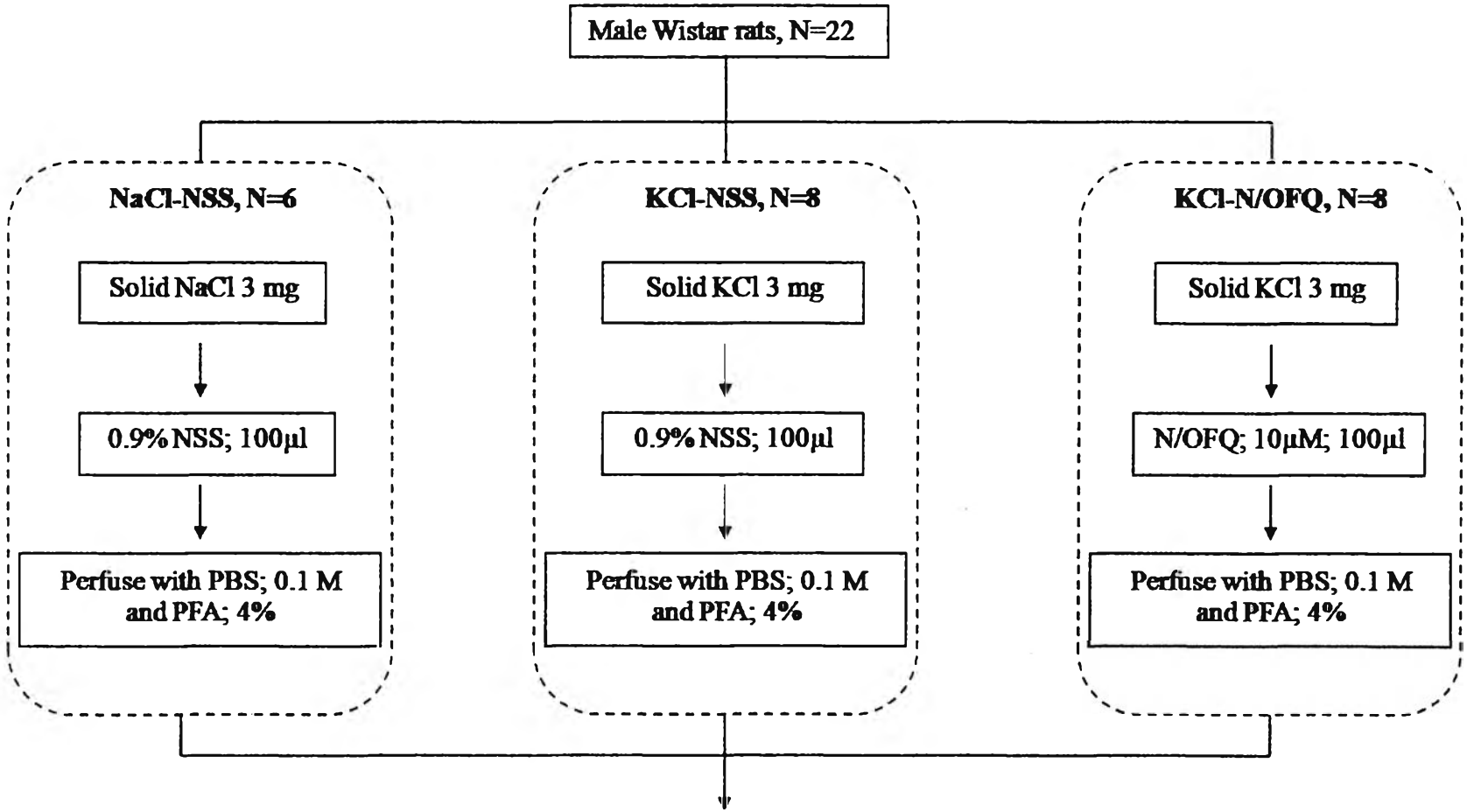
Chemicals

Pentobarbitulate sodium (NEMBUTAL®) was purchased from Sanofi, Thailand. Normal saline was purchased from King Chulalongkorn Memorial Hospital Product Public, Thailand. Purified N/OFQ was purchased from Tocris®, UK. Potassium chloride (KCl), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), sodium carbonate ($NaHCO_3$), hydrogen peroxide (H_2O_2) and ethanol were purchased from Merk®, USA. Liquid DAB+ and Envision+ System-HRP (DAB) for use with Rabbit Primary Antibodies were purchased from Dako, Denmark. 95% Paraformaldehyde was purchased from Sigma, USA.

Rabbit anti-TRPV1 was purchased from Genetex, USA. Rabbit anti-Fos was purchased from Santa Cruz Biotechnology, USA. Normal goat serum was purchased from Dako, Denmark.

Experimental design

The study had two sections. The first experiment was to investigate the effect of N/OFQ on cortical activity by electrocorticographic recording. The second experiment was performed to determine the effect of trigeminal nociceptive system by c-Fos expression and TRPV1 immunoreactivity. In this study, rats were divided into three groups, namely CSD (n=8), N/OFQ-CSD (n=8) and control groups (n=6). In the N/OFQ-CSD and CSD groups, CSD were induced by topical application of 3 mg solid KCl on parietal cortex. Solid NaCl of the same weight was placed on parietal cortex of control group. In the N/OFQ-CSD and control groups, N/OFQ (10 μM/ 100 μl) was intrathecally administered after the third wave of depolarization shift which was induced by 3mg solid KCl application. Saline of the same volume was applied to CSD group.



Outcome measurement

Cortical activity, Fos-IR in TNC, TRPV1-IR in TG

Figure 3.1 Diagram of experimental design

Animal preparation

Rats were anesthetized by intraperitoneal administration of pentobarbital sodium (60 mg/kg). Additional doses (20 mg/kg) were given as required maintain surgical anesthesia based on testing of tail pinch reflex. Tracheotomy was performed to assist ventilation. Left femoral vein cannulation was performed for intravenous administration of anesthetic drug and saline.

After tracheotomy and cannulation, rats were placed on surgical frame and their head were fixed on a stereotaxic frame. The right parietal bone will be exposed by mobilization skin along either side of the midline incision. The anterior craniotomy was performed using saline-cool drill in the frontal bone at 1mm interior and 1mm lateral to bregma. The posterior craniotomy was performed in the parietal bone at 7mm posterior and 1mm lateral to bregma. The anterior and posterior craniotomy was opened for placing the recording electrode and initiating of CSD, respectively. Solid KCl 3 mg was applied to the posterior craniotomy to induce CSD. Application of KCl induced a repetitive pattern of cortical depolarization shifts characterized as the CSD wave (Figure 3.1), whereas NaCl application has no effect on the cortical activity (Figure 3.2).

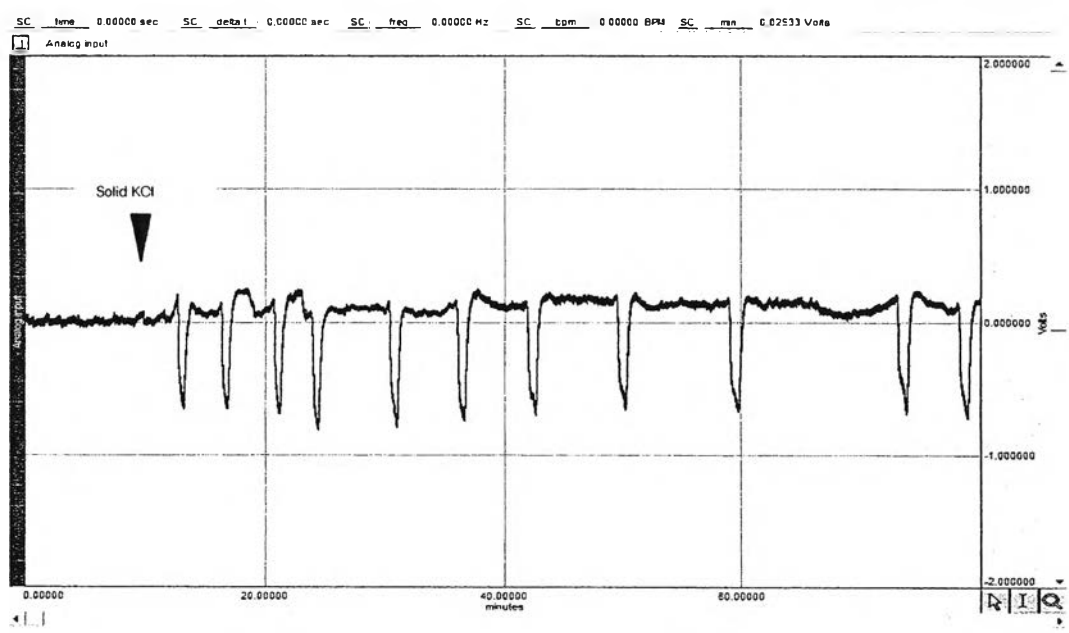


Figure 3.2 KCl application could induce repetitive pattern of cortical depolarization shifts characterized as the CSD wave.

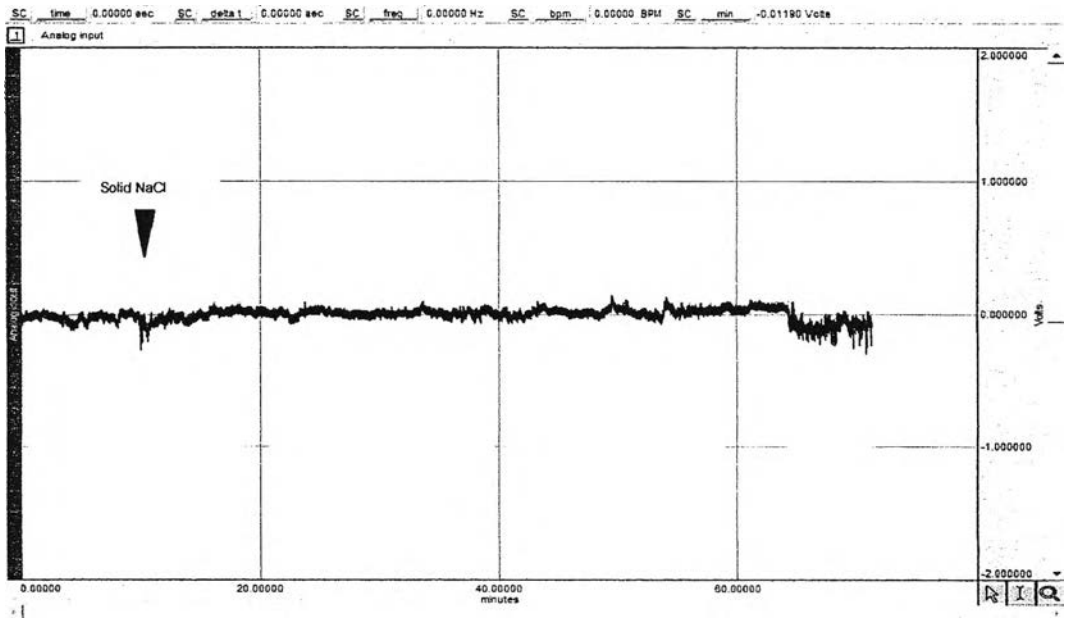


Figure 3.3 NaCl application had no effect on cortical activity.

Electrocorticographic recording

Cortical depolarization was measured by glass microelectrode (internal diameter 500μm) which was prepared from borosilicate glass capillary (Sutter, USA), pulled with microelectrode puller (Sutter, USA). The microelectrode was filled with 4M NaCl solution and then Ag/AgCl wire was inserted.

Completely filled glass microelectrode was inserted perpendicular to cortex to the depth of 500μm from cortical surface using hydraulic micromanipulator (Narishige, Japan). Another Ag/AgCl wire was put on the back which was served as a reference point. The electrical signal was amplified with microelectrode amplifier (Nihon Koden, Japan). The analog data was digitalized by data acquisition system (Biopac, USA) (Figure 3.3).

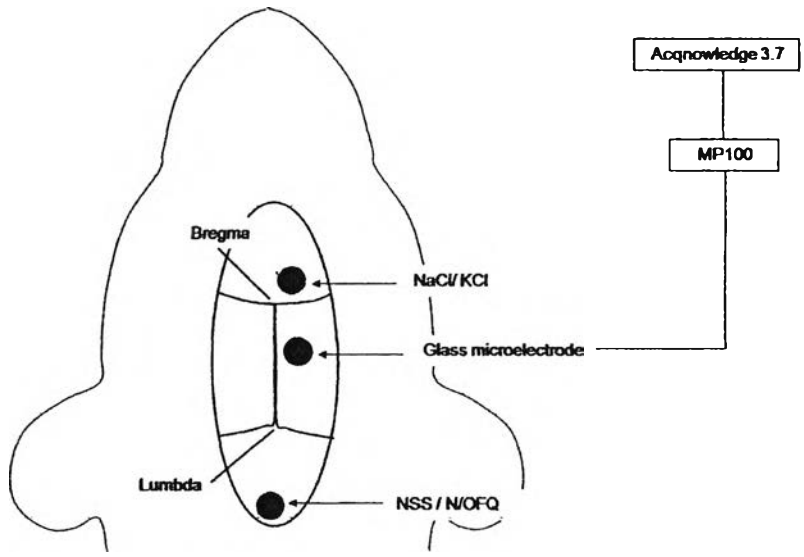


Figure 3.4 Diagram of experimental equipments for recording the CSD wave

To examine the attribute of CSD wave, amplitude, duration, interpeak latency frequency, area under the curve (AUC) of each CSD wave occurring within 1-h period were analyzed after an experiment by computer software AcqKnowlege version 3.7.3 (Biopac, USA). The amplitude was measured as the vertical length from baseline to peak magnitude of each depolarization shift (Figure 3.5). The duration was measured as a horizontal length of temporal difference between a start point and an endpoint of each depolarization shift (Figure 3.6). The interpeak latency was measured as a temporal difference between two peaks of depolarization shift in CSD wave (Figure 3.6). The frequency was counted as a total number depolarization shifts within 1-h after N/OFQ administration. The AUC was a total area limited in the duration of each depolarization shift (Figure 3.7). All of the measured variables were converted to absolute values.

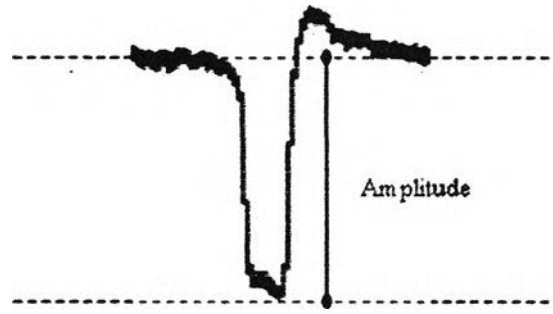


Figure 3.5 Schematic illustration shows the amplitude of a depolarization shift measured from vertical length from baseline to peak magnitude.

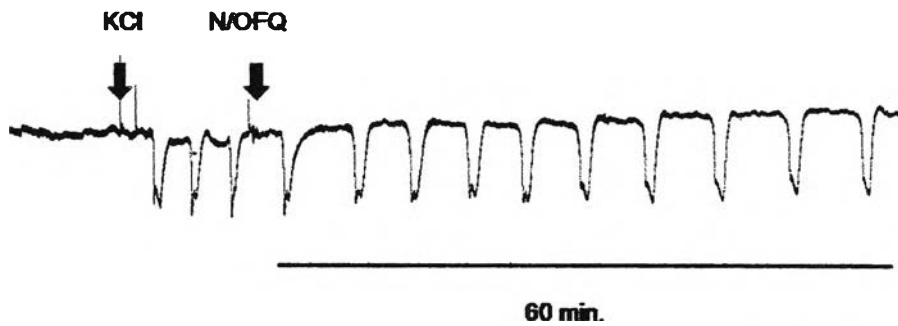


Figure 3.6 Schematic illustration shows the number of a depolarization shift measured after N/OFQ adminitration for 60 min.

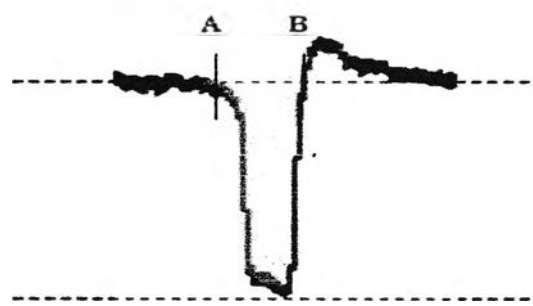


Figure 3.7 Schematic illustration shows the area under the curve (AUC) that was included in the duration of a depolarization.

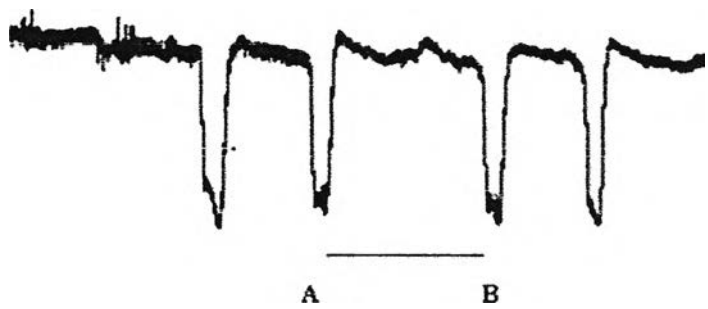


Figure 3.8 Schematic illustration shows the interpeak latency of depolarization shifts measured from horizontal length between earlier peak and latter peak.

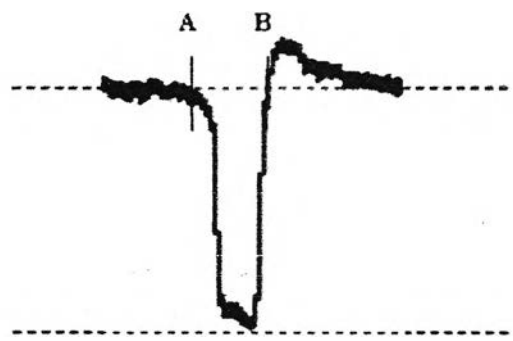


Figure 3.9 Schematic illustration shows the duration of a depolarization shift measured from horizontal length from start point (A) and endpoint (B).

Immunohistochemical study

Perfusion

After completion of electrocorticographic recording, rats were further proceeding for immunohistochemical study. Rats were deeply euthanized by excessive dose of sodium pentobarbital and performed laparotomy and thoracotomy. A cannula was inserted into the apex of the heart and advanced just distally to the aortic arch. Then, rats were perfused transcardially with 300 ml of 0.1 M phosphate-bufferred saline (PBS), following by 300 ml of 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brain, cervical spinal cord and trigeminal ganglia were transferred and stored in 0.01 M PBS at 4° C.

Cryosectioning

After overnight fixation, cervical spinal cord including trigeminal nucleus caudalis, C1 and C2 of spinal cord (approximately -1 to -6 mm from obex) was cut into a 5-mm block, and immersed in a cryoprotectant solution (30% sucrose in 0.01 M PBS, pH 7.4) for 24 hours at 4° C. However, trigeminal ganglia (left and right side) were immersed in a cryoprotectant solution without modification. Then, sample was placed on a stage, and completely covered by optimal cutting temperature (OCT) embedding medium. After freezing of OCT at -20° C, sample was coronally sectioned (20μm thick) by a cryostat. Sections were washed 3 times and kept in cold 0.01 M PBS before continuing the next process.

Immunohistochemical staining

In this study, immunohistochemical studies used free floating technique. All sections were incubated at room temperature during the experiment. First, Sections were rinsed 3 times with washing buffer (0.01 PBS). Then, sections were incubated with 50% ethanol for 30 min and 3% hydrogen peroxide in 50% ethanol for 30 min to minimize endogenous peroxidation. After repeated rinses 3 times with PBS, sections were incubated for 1 hour in PBS containing 3% normal goat serum, 1% bovine serum albumin for 60 min at room temperature and incubated with rabbit anti-TRPV1 polyclonal antibody (Santa Cruz Biotechnology, USA) diluted 1: 500 in PBS rabbit and anti-Fos polyclonal antibody (Santa Cruz Biotechnology, USA) diluted 1:1000 in PBS containing normal goat serum for 20 hours at 4°C, for observation of TRPV1 and c-Fos immunoreactivity, respectively. They were then rinsed 3 times with PBS and incubated for 45 min with envision+HRP anti-rabbit (Dako, Denmark). Bound peroxidase was revealed by incubating section with liquid DAB for 10 min after

rinsing 3 times with PBS. The reaction was terminated by successive rinsing 2 times with distilled water. Sections will be mounted onto gelatin-coated slides, air dried for overnight, and coverslipped with Permount. The c-Fos- and TRPV1-immunoreactive cell was defined with dark brown stain in their nuclei.

Evaluation of the immunoreactivity

TRPV1 immunoreactive (TRPV1-IR) cells were distinguished by their darkly stained cell bodies and processes. Expression of TRPV1 in trigeminal ganglion (TG) cell in each rat was determined by counting small to medium size ($\leq$50μm) of TRPV1-IR cells and all the same size of cells. Data was revealed as the percentage of TRPV1-IR cells per section. The percentage of TRPV1-IR cells were calculated in a TG ipsilateral side of the solid compound (NaCl or KCl) application and a contralateral side for comparative discussion.

The c-Fos immunoreactive cells (Fos-IR cells) were defined as those with a dark brown stain in their nucleus. Only cell profiles with a visible nucleus on the focal plane and located in the lamina I and the lamina II area of dorsal horn were included. A total of Fos-IR cells in 10 sections per rat were averaged. The c-Fos-IR cells were counted in a TG ipsilateral side of the solid compound (NaCl or KCl) application and a contralateral side for comparative discussion.

Statistic analysis

All values were averaged as the mean ± SD. Statistic analysis will be performed using one way analysis of variance (one-way ANOVA) and post-hoc comparisons were used to determine whether there is a statistically significant difference between the means. Probability values of less than 0.05 were considered to be statistically significant.