EFFECT OF PARACETAMOL ON CORTICAL SPREADING DEPRESSION, TRIGEMINAL NOCICEPTION AND 5-HT_{2A} RECEPTOR EXPRESSION

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ผลของพาราเซตามอลต่อปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชั่น ระบบรับความเจ็บปวดไตรเจมินัลและการแสดงออกของตัวรับซีโรโตนินทูเอ

นาย วีระ สุพรศิลป์ชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	Effect of paracetamol on cortical spreading depression, trigeminal nociception and 5-HT_{2A} receptor expression
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วีระ สุพรศิลป์ชัย : ผลของพาราเซตามอลต่อปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชั่นระบบรับความเจ็บปวดไตรเจมินัลและการแสดงออกของตัวรับซีโรโตนินทูเอ. (EFFECT OF PARACETAMOL ON CORTICAL SPREADING DEPRESSION, TRIGEMINAL NOCICEPTION AND 5-HT_{2A} RECEPTOR EXPRESSION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.นพ อนันต์ ศรีเกียรติขจร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.ดร. สุภางค์ มณีศรีเลอกรองด์. 148 หน้า.

การศึกษาครั้งนี้มีจุดประสงค์เพื่อศึกษาผลของการได้รับยาพาราเซตามอลแบบฉับพลันและแบบต่อเนื่องต่อระบบรับความเจ็บปวดไตรเจมินัล โดยแบ่งการทดลองออกเป็นกลุ่มที่ได้รับยาพาราเซตามอล 200 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว ทางช่องท้อง 60 นาที และกลุ่มที่ได้รับยาพาราเซตามอลต่อเนื่องทุกวันเป็นระยะเวลา 30 วัน แบ่งกลุ่มควบคุมออกเป็นกลุ่มที่ได้รับ 12.5% of 1,2-propane-diol ใน 0.9% sterile saline ทางช่องท้อง 60 นาที และกลุ่มที่ได้รับต่อเนื่องทุกวันเป็นระยะเวลา 30 วัน ก่อนการกระตุ้นให้เกิดปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชั่นโดยการวางผลึกโพแทสเซียมคลอไรด์ขนาด 3 มิลลิกรัม ลงบนผิวสมอง สัตว์ทดลองทุกกลุ่มจะถูกนำมาวัดการเปลี่ยนแปลงการทำงานของสมองใหญ่โดยใช้กลาสไมโครอิเลคโทรด การวัดการไหลเวียนเลือดสมองโดยใช้เลเซอร์ดอปเปอร์โฟมิเตอร์ การแสดงออกฟอสโปรตีนและตัวรับซีโรโตนินทูเอโดยใช้ อิมมูโนฮีสโตเคมมิสทรี การเปลี่ยนแปลงของเอ็นโดทีเลียมเซลล์โดยใช้กล้องจุลทรรศน์อิเลคตรอน

ผลการศึกษาพบว่าการวางผลึกโพแทสเซียมคลอไรด์สามารถกระตุ้นให้เกิดการเปลี่ยนแปลงการทำงานของสมองใหญ่โดยทำให้เกิดดีโพลาไรเซชั่นของเซลล์ประสาทสมองใหญ่เป็นช่วงๆ ต่อเนื่อง การเปลี่ยนแปลงการไหลเวียนเลือดสมองและการแสดงออกของฟอสโปรตีนในไตรเจมินัลนิวเคลียสคอดาลลิส ซึ่งการได้รับยาพาราเซตามอลแบบฉับพลันสามารถลดการไหลเวียนเลือดสมองและการแสดงออกของฟอสโปรตีนได้โดยไม่มีผลต่อการเปลี่ยนแปลงความถี่ของการเกิดดีโพลาไรเซชั่นของเซลล์ประสาทสมองใหญ่แต่การได้รับยาพาราเซตามอลแบบต่อเนื่องนั้นสามารถเพิ่มความถี่ของการเกิดดีโพลาไรเซชั่นของเซลล์ประสาทสมองใหญ่และการแสดงออกของฟอสโปรตีนได้ ซึ่งอาจบ่งบอกถึงการเพิ่มความไวในการรับความรู้สึกเจ็บปวดของสมองใหญ่ จากการศึกษาด้วยกล้องจุลทรรศน์อิเลคตรอนพบว่าการได้รับพาราเซตามอลแบบต่อเนื่องนั้นทำให้เอ็นโดทีเลียมเซลล์มีจำนวน pinocytic vesicles และ microvilli เพิ่มขึ้น ซึ่งอาจบ่งบอกถึงกระบวนการควบคุมการผ่านเข้าออกของสารในหลอดเลือดสมองถูกรบกวน ซึ่งจากผลการศึกษาดังกล่าวทำให้ผู้วิจัยศึกษาถึงบทบาทของซีโรโตนินต่อการเปลี่ยนแปลงของระบบรับความเจ็บปวดไตรเจมินัลวาสคูลาในสัตว์ทดลองที่ได้รับยาพาราเซตามอลแบบต่อเนื่องโดยใช้คีแทนเซอรีนซึ่งเป็นสารยับยั้งตัวรับซีโรโตนินชนิด 5-HT_{2A} ผลการทดลองพบว่าการให้คีแทนเซอรีนสามารถลดความถี่ของการเกิดดีโพลาไรเซชั่นของเซลล์ประสาทสมองใหญ่และสามารถยับยั้งการแสดงออกของฟอสโปรตีนได้ทั้งในสมองใหญ่และไตรเจมินัลนิวเคลียสคอดาลลิส

จากผลการศึกษาดังกล่าวแสดงให้เห็นว่าการได้รับยาพาราเซตามอลต่อเนื่องเป็นระยะเวลานานนั้นสามารถเพิ่มความไวของระบบประสาทรับความเจ็บปวด โดยผ่านกลไกการทำงานของระบบซีโรโตนิน ซึ่งผลจากการศึกษาครั้งนี้จะช่วยให้เข้าใจถึงพยาธิกำเนิดของอาการปวดศีรษะจากการได้รับยาแก้ปวดเป็นระยะเวลานานได้

สาขาวิชา สรีรวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2551 ลายมือชื่อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4889674620 : MAJOR PHYSIOLOGY
KEYWORDS: CORTICAL SPREADING DEPRESSION/ TRIGEMINAL NOCICEPTION/5-HT_{2A} RECEPTOR EXPRESSION/MIGRAINE

WEERA SUPRONSILPCHAI: EFFECT OF PARACETAMOL ON CORTICAL SPREADING DEPRESSION, TRIGEMINAL NOCICEPTION AND 5-HT_{2A} RECEPTOR EXPRESSION. ADVISOR: PROF. ANAN SRIKIATKHACHORN, M.D., CO-ADVISOR: ASST. PROF. SUPANG MANEESRI LE GRAND, Ph.D, 148 pp.

The present study aims to investigate the effect of acute and chronic paracetamol administration on trigeminal nociception. The study comprised two experiments based on acute and chronic paracetamol administration. In acute experiment, the rats were divided into paracetamol-treated and control groups (8 rats each). A single dose of paracetamol (200 mg/kg BW, intraperitonealy) was given to the treatment group whereas vehicle (12.5% of 1,2-propane-diol in 0.9% sterile saline) was given to the control group. Trigeminal nociception was evoked by cortical spreading depression (CSD) using topical KCl application. CSD was elicited at 60 minute after paracetamol injection. In chronic paracetamol experiment, the rats were divided into paracetamol treated and control groups. Paracetamol (200 mg/kg BW, intraperitonealy) or vehicle was injected once daily for the period of 30 days. Electrocorticogram was measured continuously for one hour using glass microelectrode. Cortical blood flow was monitored using Doppler flowmetry. Expression of immediate early gene product, Fos, was used as an indicator of neuronal activity. The 5-HT_{2A} receptor and Fos expression were studies by immunohistochemistry. The change of endothelial cell was studied by electron microscopy.

The results showed that cortical application of KCl resulted in a series of depolarization shift which coincided with cortical hyperemia and Fos expression in the TNC. Acute treatment with paracetamol significantly attenuated the CSD-evoked hyperemia and number of Fos-immunoreactive cells in the TNC without changes in the CSD frequency. On the other hand, chronic paracetamol administration substantially increased the CSD frequency, number of Fos-immunoreactive cells in the cortex and TNC, indicating the increase in cortical excitability and trigeminal activation. In addition, the EM studies showed that chronic paracetamol exposure may interfere with the cerebral microvascular permeability as evident by an increase in CSD-evoked pinocytosis and microvillous formation. To investigate the involvement of serotonin system in this process, ketanserin, a 5-HT_{2A} antagonist was given to the rats prior to CSD. We found that pretreatment with ketanserin can decrease the frequency of CSD and can inhibit the expression of Fos both in cortex and the TNC.

The results of the present study indicate that chronic paracetamol exposure can lead to an increase in neuronal excitability in both cerebral cortex and the pain processing pathway. The mechanism of this hyperexcitability may involve the alteration of central serotonin system. These observations provide better understanding regarding the pathogenesis of medication-induced headache and may shed light for future pharmacologic intervention.

Field of Study : Physiology ..... Student's Signature .....

Academic Year : 2008 ..... Advisor's Signature .....

Co-Advisor's Signature .....

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LIST OF ABBREVATIONS

5-HT	= serotonin
5-HTP	= 5- Hydroxytriptophan
5-HIAA	= 5- Hydroxyindoleacetic acid
TG	= trigeminal ganglion
TNC	= trigeminal nucleus caudalis
CSD	= cortical spreading depression
CBF	= cerebral blood flow
DC shift	= depolarization shift
µm	= micrometer
Ca^{2+}	= calcium ion
Na^{+}	= sodium ion
H^{+}	= hydrogen ion
K^{+}	= potassium ion
KCl	= potassium chloride
kg	= kilogram
mV	= millivolt
NaCl	= sodium chloride
PBS	= phosphate buffer saline
mg	= milligram
mm	= millimeter
C1	= cervical spinal cord segment 1
C2	= cervical spinal cord segment 2
DAB	= 3,3-diaminobenzidin
BW	= body weight

CHAPTER I

INTRODUCTION

Analgesic overuse is a common problem associated with chronic daily headache. It is the result of an interaction between chronic consumption of a therapeutic agent and a susceptible patient. The mechanism by which analgesic faster or reinforce chronic daily headache is still unknown. Accumulating evidence show that analgesic overuse may compromise the endogenous 5-HT antinociceptive system.

Paracetamol is one of analgesic drug that most popular and widely used for treatment of pain and headache. Recent studies have indicated that the analgesic effect of paracetamol involve the alterations of the endogenous 5-HT dependent antinociceptive system. Administration of paracetamol led to a rapid increase in 5-HT level in cortex, hypothalamus, striatum, hippocampus, and brainstem (Pini et al., 1996; Courade et al., 2001). Besides altering the 5-HT level, exposure to paracetamol also changes the expression of 5-HT receptors in the central nervous system. In 1996, Pini and coworkers demonstrated that acute administration of aspirin or paracetamol down regulates the expression of 5-HT_{2A} receptors in rat cerebral cortex. This result was later confirmed by Srikiatkhachorn and coworkers in 1999. The pattern of analgesic induced plasticity within the central 5-HT system changes overtime. In rats, a 15 days course of paracetamol led to down regulation of the 5-HT_{2A} receptor and an up regulation of 5-HT transporter in frontal cortex. However, receptor down-regulation and transport up-regulation became less evident following a more prolonged administration of the drug, and these changes coincided with a decrease in the analgesic efficacy of paracetamol. These finding suggest that chronic analgesic use can alter the central 5-HT system (Srikiatkhachron et al., 2000). The alterations in the 5-HT system have been demonstrated in the patient with analgesic induced chronic daily headache. Compared with patients with migraine, patient with chronic daily headache have a lower level of platelet 5-HT and greater density of 5-HT_{2A} receptors (Skikiatkhachorn and Anthony 1996). These changes can be reversed after drug withdrawal, and normalization of platelet 5-HT and its receptor correlate with the improvement of clinical headache (Srikiatkhachorn et al., 1998). The experimental evidence suggest that central, mainly 5-HT dependent

antinociceptive system is impaired in patients with chronic daily headache. Analgesic overuse may further derange this system by inducing a low of 5-HT. Previous experiment have reported the low level of 5-HT subsequently leads to up regulation of the pronociceptive 5-HT_{2A} receptor. Stimulation of these highly expressed receptors and reduction of pain modulation may increase the process of central sensitization. Thus, derangement in central antinociceptive system with up regulation of the 5-HT_{2A} pronociceptive receptor as a result of chronic analgesic use may increase the sensitivity to pain perception and foster or reinforce chronic daily headache involved in trigeminovascular nociceptive system.

This study was designed to determine the effect of acute and chronic treatment with paracetamol on trigeminovascular nociception in cortical spreading depression (CSD) animal model. We chose paracetamol for this study because it is the drug most commonly abuse by patients with headache. In order to study the cortical neurons activity, the depolarization shift (DC shift) was measured by extracellular recording technique. In vascular compartment, the cerebral endothelial cells and cerebral blood flow (CBF) was study by electron microscopy and Laser Doppler flowmetry, respectively. The trigeminal nociception was indicated by the number of fos immunoreactive neurons in cerebral cortex and trigeminal nucleus caudalis (TNC). The expression of 5-HT_{2A} was studied in cerebral cortex and trigeminal ganglion (TG). In addition, the involvement of 5-HT_{2A} receptor in the effect of chronic treatment with paracetamol induced changes in trigeminovascular nociceptive system was observed.

CHAPTER II

REVIEW OF LITERATURE

1. **The neurobiology of vascular headache**
2. **Trigeminovascular system**
3. **Cortical spreading depression (CSD)**
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5. **Association of analgesic and chronic daily headache**
6. **Serotonergic system**
7. **Serotonin/ Analgesic/ Headache**
8. **Propose hypothesis**

1. The neurobiology of vascular headache

The primary headaches including migraine, tension type headache and other headaches (Olsen et al, 2000). Migraine headaches are ascribed as neurovascular disorders which world-wide afflict up to 15-20% of the general population. It is the most common type and characterized by attacks of moderate to severe headache that last for 4-72 hour, often unilateral, pulsating and associated with photophobia, phonophobia, and /or nausea and vomiting (Olsen et al, 2000). Recent data demonstrate that several complex biological pathways can interact at the level of the trigeminal vascular system, suggest that new drugs might be obtained by targeting common pathways associated with the vascular and/or neuronal hypothesis of migraine.

The primary vascular theory of migraine assumes that the major pathophysiological events that initiate the migraine attack occur in the perivascular nerves of the major cerebral vessels. For example, cerebral arterial vasodilatation could activate the perivascular sensory nerve projecting to the brain. It is well known that the meninges and large cerebral vessels are the predominant pain sensing structures in the cranium from the seminal work performed by Wolff and coworker (Graham et al., 1938, Ray et al., 1940). They observed that direct stimulation of the cerebral and meningeal blood vessels produced severe, ipsilateral and penetrating headaches in conscious patients. Intracranial structures that were particularly nociceptive included the dural arteries, the carotid and the vertebral and basilar arteries. The central role of these vessels in migraine aetiology was supported from the clinical observation that balloon distension of major cerebral vessels resulted in severe facial pain, and that pulsation of extracranial vessels was associated with headache in migraineurs. Interestingly, there is evidence to suggest that relief of migraine headache can be obtained by occluding the carotid artery ipsilateral to the headache.

The neurogenic theory was originally proposed by Leao in 1944 after studying cortical spreading depression (CSD). It predicts that alterations in blood flow develop as a consequence of neuronal events. CSD manifests itself as a slowly propagating wave of neuronal burst activity, followed by depression of spontaneous and evoked activity. It has been suggested that this process could either directly or indirectly activate the trigeminal afferents projecting to brain area associated with nociceptive

processing. Many studies review the current literature and highlight the indicate balance and regulation of both cerebral vascular blood flow and afferent nervous system. The hypotheses regarding either a primary vascular or a neuronal event do not reflect the complex array of physiological processes involved in regulation of nociceptive information within the trigeminovascular system. The numerous factors that can precipitate migraine, and the observations that both vascular and neuronal stimuli can induce CSD, strongly suggest that activation of the trigeminovascular nociceptive system represents a key process in the development of migraine.

Theories surrounding the development of migraine headache have focused on either a vascular or neurogenic angle. Recent data show that both vascular and neuronal pathways can produce activation of the trigeminal vascular afferent systems. Indeed, regulation of sensory input is under control of higher brain areas, and mechanisms involving the sensitivity of nociceptive inputs can be controlled entirely within the central nervous system. Migraine is a complex and debilitating disease and it are involved in different patients, and even within the same patient under different environmental conditions. The common pathways and mechanisms, involved in the generation of the clinical phenotype (headache, phono/photo-phobia) are likely to result in effective new therapies. The precise role of CSD in the generation of migraine still remains to be elucidated, but recent exciting data show that this phenomenon can occur in migraine with aura, and might thus link intrinsic brain function with the trigeminal vascular network.

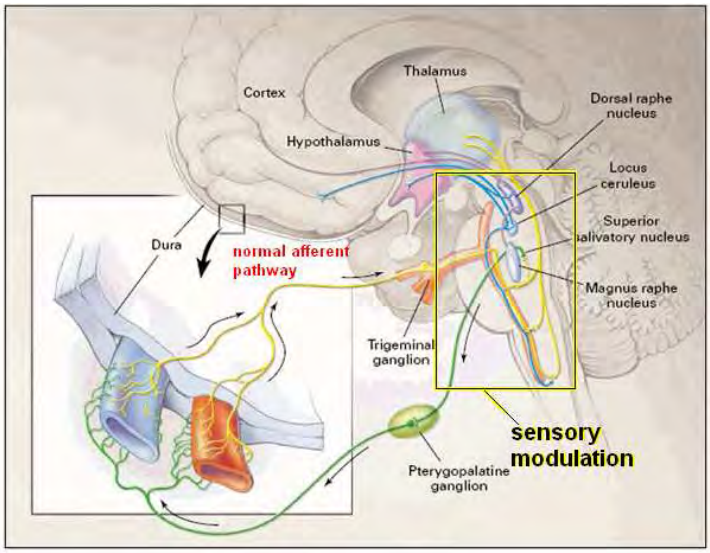

Figure2-1 Trigeminovascular system

2. The trigeminovascular system

Nervous connections between the trigeminal ganglia and cerebral blood vessels have recently been identified in experimental animals and have been termed the trigeminovascular system.

The trigeminovascular system consists of the neurons innervating the cerebral vessels whose cell bodies are located in the trigeminal ganglion. The ganglion contains bipolar cells, the peripheral fiber making a synaptic connection with the vessel, and other cranial structures, particularly the pain-producing large cranial vessels and dura mater (McNaughton, 1938; McNaughton and Feindel, 1977), and the centrally projecting fiber synapsing in the caudal brain stem or high cervical cord (Goadsby and Hoskin, 1997; Kaube et al., 1993). Tracing studies have identified the trigeminal nerve as the major afferent pathway for pain from the vessels and dura mater (Feindel et al., 1960; Huber, 1899; Penfield, 1932; Penfield, 1934; Penfield and McNaughton, 1940). The innervation of the pial vessels from the trigeminal system has been demonstrated using sensitive tracing techniques (Mayberg et al., 1981). These fibers are predominantly found in the first (ophthalmic) division of the trigeminal nerve and have widely ramifying axons that may innervate several vessels ipsilaterally (Liu-Chen et al., 1983a; Liu-Chen et al., 1983b; Mayberg et al., 1984). These systems are, in evolutionary terms, well conserved and may be found in monkey (Ruskell and Simons, 1987), cat (Keller et al., 1985; Liu-Chen et al., 1984), guinea pig (Yamamoto et al., 1983), gerbils (Matsuyama et al., 1985), and rat (Arbab et al., 1986). The trigeminal innervation is predominantly to the forebrain but extends posteriorly to the rostral basilar artery, whereas the more caudal vessels are innervated by the C_2 and C_3 dorsal roots, which also synapse with the central trigeminal neurons (Arbab et al., 1988). Some of the projections involve both cerebral (middle cerebral artery and middle meningeal artery vessels (O'Connor and van der Kooy, 1986).

During the last two decades the trigeminovascular system have been proposed (Moskowitz, 1984) and studies (Saito et al., 1988, Markowitz et al., 1987, Buzzi et al., 1990) to evaluate the peripheral component of the migraine attack and to possibly identify the mechanism of action of migraine aborting drugs, namely ergot derivatives and triptans. The trigeminovascular system has been challenged in the rat with chemical stimulation or unilateral electrical trigeminal ganglion stimulation to induce neurogenic inflammation upon release of vasoactive peptides (CGRP, SP) from nerve

endings (Markowitz et al., 1987, Buzzi et al., 1990). Degranulation of mast cells accompanies the oedema response (Dimitriadou et al., 1991) and a stimulation intensity-dependent increase of CGRP is observed during trigeminal ganglion stimulation in the plasma obtained from the superior sagittal sinus (SSS) (Buzzi et al., 1991).

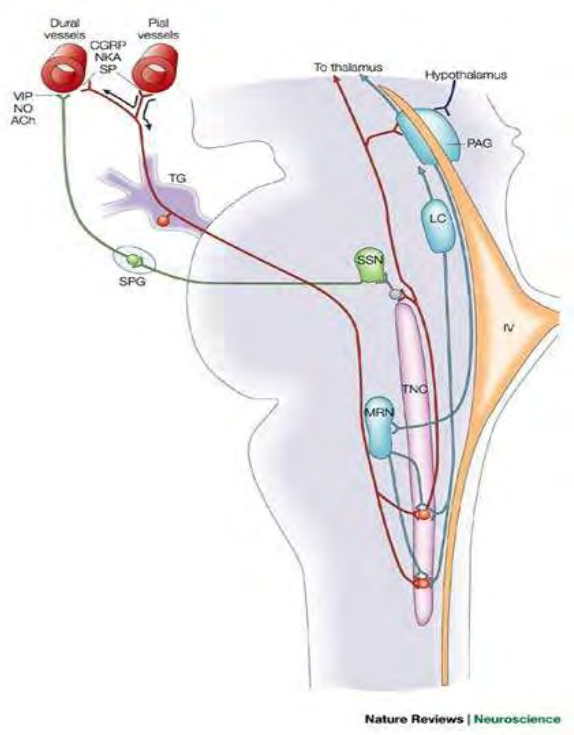

Figure2-2 Neuronal pathway involved in trigeminovascular activation and pain processing (Pietrobon et al., 2003)

3. Cortical spreading depression (CSD)

Despite this increasing attention, little is known about the pathophysiological mechanisms that underlie a migraine attack. Research has, however, advanced several theories concerning the pathophysiology of migraine in general, or the individual aspects of a migraine attack. These theories are:

CSD is a self–propagating front of depolarization associated by a depression of the neuronal bioelectrical activity for a period of minutes. CSD is an experimental reaction induced by local stimulation in most gray matter regions, e.g., in the cortex, the hippocampus and cerebellum of a variety of species (Bures et al., 1974). It appears

first at the stimulated site and spreads out in all directions at the velocity of 2–3 mm/min, so that increasingly distant areas undergo successively a similar temporary depression (Leao et al., 1944). A necessary manifestation of CSD is a propagating extracellular negative potential with an amplitude of 10–30 mV and a duration of more than 0.5 -1 min, which may be preceded or succeeded by a positive deflection of variable amplitude and duration. Underlying this neuroglial depolarization is a dramatic change in the distribution of ions between extra and intracellular spaces. K^+ and H^+ release from the cell, while Na^+, Ca^+ and Cl^- enter together with water (Grafstein et al., 1963, Hamsen et al., 1981, Kraig et al., 1978) causing cells to swell and the volume of the extracellular compartment to be reduced. SD is accompanied by an increase of glucose utilization and O_2 consumption (Bures et al., 1974, Mies et al., 1984). Recovery of CSD depends on energy metabolism (Bures et al., 1974).

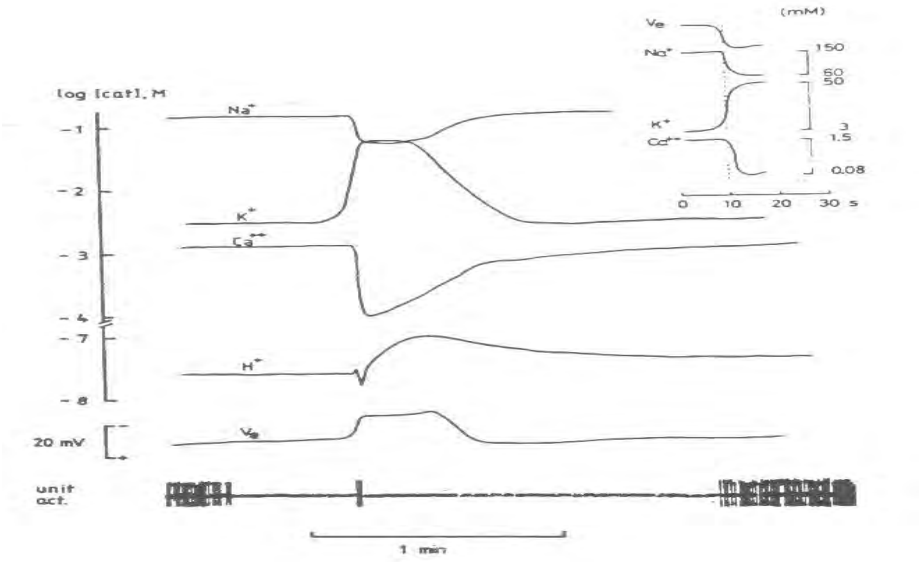

Figure2-3 Electrophysiological changes accompanying CSD in the rat brain. Interstitial ion concentrations of sodium, potassium, calcium and hydrogen were measured by ion-selective electrodes. The extracellular potential (Ve) and the single unit activity were measured by single barreled eletrophysiological changes which were recorded in the parietal cortex (Laurizen, 1994).

Figure2-4 The trace showing change in EEG (upper panel) and cortical DC potential (mV) (lower panel) recorded following the application of KCl to the parietal cortex.

This phenomenon has been studied in vivo in several animal species and in vitro in brain slices and in retinal preparation under various experimental conditions (Bures et al., 1974). It has been also observed in human neocortical tissue in vitro (Avoli et al., 1991, Avoli et al., 1995, Gorji et al., 2001) and in human hippocampus as well as striatum (Sramka et al., 1978) and neocortex (Mayevsky et al., 1996) in vivo. However, there is an in vivo experiment which suggests that CSD is more difficult to elicit in human than rodent cortex and may not occur in man (Leone et al., 1998).

CSD can be regularly initiated if the tissue susceptibility is artificially raised. Hypoglycemia and hypoxia as well as changing the extracellular ionic micromilieu by applying solutions with increased K^+, decreased NaCl or with the Cl^- of the latter replaced by certain other anions lower the threshold. Conversely, the susceptibility of CSD initiation is lowered or the occurrence of CSD is prevented in previously susceptible tissue by solution with increased Mg^{2+} or NaCl, or with the Na^+ replaced by certain other cations. CSD also is triggered by various modes of mechanical, chemical and electrical stimulation (Bures et al., 1974).

No explanation of the propagation of CSD has been suggested that accounts for all the fact presently proven. The hypothesis that gained wide acceptance is that

the spread of CSD probably involves the release and diffusion of the chemical mediators, most likely K^+ and glutamate into interstitial fluid (Vane et al., 1978). Given the widespread potential signaling capacities of Ca^{2+} waves (Parpura et al., 1994) observations of the interactions between astrocytes and neurons in cell culture have suggested that Ca^{2+} waves play a role in CSD initiation and propagation (Kunkler et al., 1998, Nedergaard et al., 1994).

CSD prolongs in the domain of the pathophysiology of the brain, and there are reasons to believe that it is involved in some clinical disorders, including migraine, cerebrovascular diseases, head injury and transient global amnesia. There are a number of reviews of CSD (Bures et al., 1984, Lauritzen et al., 1992) but none of them describes all conceivable projections of CSD research to clinically relevant issues.

The designation migraine with aura denotes the syndrome of headache associated with characteristic sensory, motor, or visual symptoms, usually gradually developed over 5-20 min and lasting less than 60 min. The most common symptoms in aura phases are visual arising from dysfunction of occipital lobe neurons. The positive (stimulative) neurological symptoms, e.g., flashing lights are usually followed by negative (suppressive) ones, e.g., scotoma or hemianopia in this phase. Although a visual aura occurs in about one-third of migraine sufferers, a highly characteristic syndrome occurs in about 10% of migraineurs. Headache usually follows neurological aura symptoms directly or after a free interval of less that an hour but may completely absent (Dalessio, 1980, Pearce, 1985).

Some investigators have described their own migraine aura K.S. Lashley in 1941 speculated that the disturbances started at visual field center and propagated to the temporal parts within 10–15 min, while function returned to normal within another 10 – 15 min, He estimated that a wave front of intense excitation followed by a wave of complete inhibition of activity were spread across the cortex by a velocity of 3 mm/min (Lashley, 1941). In 1945, Leao and Morison hypothesized that slow march of scotoma in the visual or sensory sphere is related to the CSD phenomenon (Leao, 1945). Similar estimations and suggestions were done later by some other investigators (Lord, 1986, Milner, 1958). CSD consists of a wave of neuronal activation followed by a suppression of neuronal activity that spreads slowly across the surface of the brain (Leao, 1944). This spreading process of excitation and

inhibition can explain the stimulative then suppressive characteristics of the neurological symptoms of migraine aura and their propagating quality.

Behavioral changes induced by CSD in animals are consistent with the transient neurological deficits occurred during the migraine aura. Both sensory and motor impairments are caused by CSD in different animal models. Unilateral CSD induces contralateral sensory neglect and motor impairment of the forepaw lasting for 15–30 min in rats (Bures et al., 1974). The degree of disorientation caused by unilateral CSD is well established. Unilaterally depressed rats under free-choice conditions in a T-maze prefer to turn to the side of ipsilateral to the functionally decorticated hemisphere (Koppmann et al., 1966). This result cannot be explained by the contralateral muscular weakness but is evidently due to the inadequate perception of the visual and somesthetic stimuli received by the contralateral side of the body (Bures et al., 1974). In line with this, lateralized somesthetic stimuli applied to the side ipsilateral to CSD evoked more prolonged arousal then contralateral stimuli (Weiss et al., 1959). CSD considerably accelerated habituation of evoked response in the visual cortex, probably by enhancing the depression of synaptic inputs to this region (Koreli et al., 1972). CSD elicited by KCl application on to the occipital cortex of the hemisphere contarlateral to the preferred forelimb suppressed local reaching – for – food reaction for about 40 min (Buresova et al., 1960). The disappearance of cortical postural reflexes on the side of contralateral to CSD is another example of the CSD motor effects (Bures et al., 1960).

In a computational model of CSD, during the cortical activation pattern appearing along the leading edge of the potassium waves, the spatial pattern of neural activity across the simulated cortex broke up into irregular, small spots and roughly parallel lines of highly active cortical elements (Reggia et al., 1996). The corresponding patterns were suggested to resemble the patterns of visual hallucinations observed during migraine aura. The same patterns were reported to be manifested as patterns of visual hallucinations in psychiatric disorders by computational models (Ruppin et al., 1995). Sexual arousal was reported by 10% of migraine sufferers during the attacks (Del et al., 1982, Pearce, 1985). Single waves of unilateral CSD caused by KCl injections into the occipital cortices are followed by frequent penile erection in the rats (Huston, 1971). Yawning and drowsiness are observed both before and after headache in migraine attacks (Blau, 1991, Dalessio,

1980, Pearce, 1985). Repetitive yawning was observed about 5 min after initiation of CSD in rat occipital cortices. Chewing movements preceded a yawning response while drowsiness generally coincided with yawning (Huston et al., 1971). Food craving is reported in some migraineurs (Blau, 1993). CSD was accompanied by activation of consummatory behavior indicating that it may elicit homeostatic hallucination making the animal hungry or cold (Bures, 1993). Aggressive and stereotyped eating and drinking were elicited by unilateral and bilateral CSD. Spreading of depolarization waves from occipital area to frontal cortex caused these behaviors between 4.5 to 6.5 min after KCl injections. In most rats the induced drinking or eating was consistently preceded or accompanied by wall climbing and excited grooming (Huston et al., 1971). Amnesic attacks and anomia are reported during aura period (Dalessio, 1980, Sakai et al., 1978, Santoro et al., 1988). Memory, attention, concentration and retention is usually poor during the migraine attacks (Dalessio, 1980). CSD caused amnesia and the inability to acquire new knowledge in different experimental models (Bures et al., 1974). Nausea and vomiting are relatively common preheadache and postheadache symptoms in migraine with aura (Blau, 1993, Dalessio, 1980). Repetitive CSD in rats shortly after ingestion of saccharin caused subsequent aversion to its taste suggesting that CSD induces nausea or malaise (Winn et al., 1977, Winn et al., 1975). However, it should be noted that some other studies showed that CSD does not elicit conditioned taste–aversion (Davis et al., 1983, Koroleva et al., 1993). Magnetoencephalographic studies in human revealed that the magnetic signals were seen in migraine patients but not in patients suffering from other forms of headache or normal controls. Three distinctive signal patterns; suppression of spontaneous cortical activity, slow field changes and large–amplitude waves, were observed strictly in migraine patients. In some migraineurs, magnetic signals were also recorded between attacks. The same magnetic fields appeared during the propagation of CSD in the cortex of anesthetized animals (Welch et al., 1993).

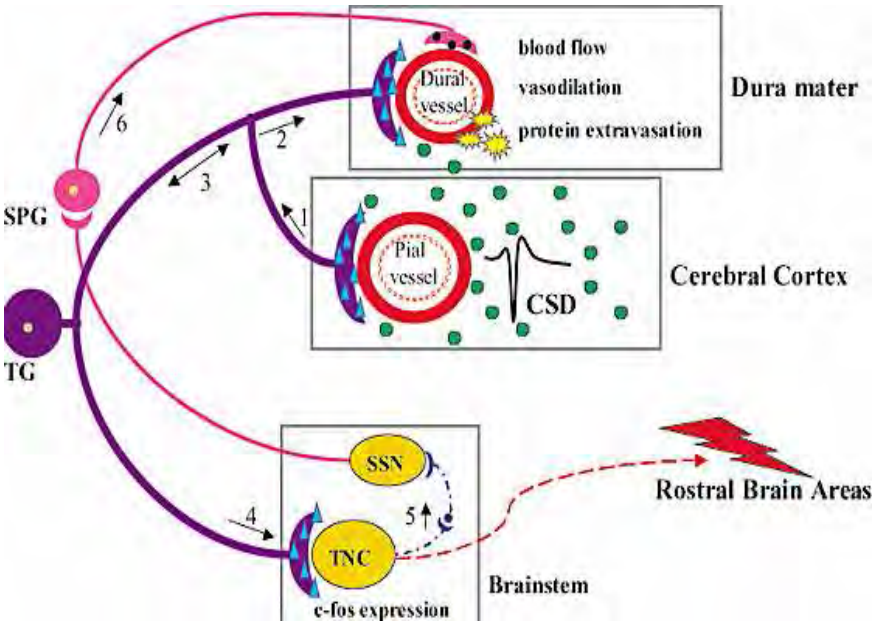

Figure2-5 The mechanism of CSD involved in vascular headache (Bolay et al., 2002).

3.1 CSD and cortical activity

Bures and coworker obtained the first direct evidence that human grey matter in vivo supports CSD (Bures et al., 1974). They showed that micro-injections of potassium chloride into the caudate nucleus or the hippocampus elicited CSD. A large negative change of the DC potential was observed in the hippocampus, spreading at a rate of 3.2 mm/min away from its site of elicitation, consistent with the propagation rate of CSD in rodents. Similar recordings were obtained in the caudate nucleus. One interesting feature of these recordings is that there was no suppression of the spontaneous electrical activity in the caudate nucleus recorded locally while the CSD propagated. Nevertheless, the DC potential changes were unequivocal and should be taken as definite evidence for the occurrence of spreading depression in the human brain. DC changes very similar to CSD have been observed in excised temporal cortical tissue from humans. NMDA receptor antagonists inhibited the reaction. After washing out the blocker CSD could again be elicited (Avoli et al., 1991). Still another line of evidence comes from the work of Mayevsky, who observed repetitive episodes

of CSD in a head-injured patient (in only one of 14 studied) starting approximately 30 hours after the beginning of the monitoring (Mayevsky et al., 1996). The recordings showed characteristic transients of potassium, cerebral blood flow, and cerebral blood volume, oxidation of the cortex and EEG suppression consistent with CSD.

3.2 CSD and cerebral circulation

Olesen and coworker provided the first evidence in human patients during migraine attacks of slowly spreading cortical hypoperfusion incompatible with spasm of a major artery but fully compatible with the occurrence of CSD (Olesen et al., 1981). Several later studies of regional cerebral blood flow also support these observations.

Cerebral blood flow (CBF) undergoes a sequence of changes during attacks of spontaneous migraine that has not been observed in any other patient categories with a neurological disorder (Lauritzen et al., 1994). At the beginning of migraine attacks, CBF decreases in the posterior part of the brain. Subsequently, the low flow region spreads into the parietal and temporal lobes at a rate of 2-3 mm/minute, the maximum decrease of CBF being 30-40%, far above ischemic levels (Lauritzen et al., 1994; Woods et al., 1994). Tests of CBF dynamics have revealed preserved autoregulation in the oligaemic region, while the CO_2 reactivity and the functional coupling between neuronal activity and CBF is attenuated (Lauritzen et al. 1994; Woods et al., 1994; Cutrer et al., 1998).

Two recently published studies using high resolution functional magnetic resonance imaging are particularly interesting (Cao et al., 1999; Hadjikhani et al., 2001). These two studies examined the development of the changes of the blood oxygen level dependent (BOLD) signal during the course of migraine. In one study the onset of a headache or visual symptoms, or both, were preceded by suppression of initial activation. The suppression slowly propagated into the contiguous visual cortex at a rate of 3-6 mm/min, i.e. at the same rate of propagation as CSD (Cao et al., 1999). The second study examined the vascular changes in more detail (Hadjikhani et al., 2001). A focal increase in the BOLD signal, possibly vasodilatation, developed within the extrastriate cortex at the beginning of the attack. This BOLD change propagated at a rate of approximately 3.5 mm/min. over the occipital cortex at the same time as the visual aura developed. The BOLD signal then diminished, possibly reflecting

vasoconstriction. The reactivity to functional activation tests decreased as previously observed with the 133Xenon technique (Olesen et al., 1981; Lauritzen et al., 1994). During periods with no visual stimulation, but while the subject was still having visual symptoms (scintillations), the BOLD signal change followed the retinotopic progression of the visual percept. Thus, the CBF changes in migraine are consistent with a process affecting blood vessels that propagates at a rate of 2-3 mm/min. The vascular changes consist of a vasodilatation that is capricious and precedes the oligaemia and the development of neurological symptoms. The changes of blood flow regulation consist of preserved autoregulation, attenuated CO_2 reactivity, and attenuated reactivity to mental stimuli. This pattern of flow changes was used for comparison with the changes of CBF during CSD.

An initial increase in cerebral blood flow, followed by a long lasting decrease in flow, is observed in animal models after induction of CSD (Duckrow, 1991). These changes in cerebral blood flow are consistent with changes observed in man. In both migraine and CSD, blood flow autoregulation is intact while CO_2 reactivity is impaired, and a reactive hyperaemia is commonly observed in both conditions (Fabricius et al., 1992; Olesen, 1992). In brief the pattern of CBF changes in migraine and CSD are similar, suggesting that CSD is the mechanism of the migraine aura and the related blood flow changes.

Using a 133-Xenon inhalation technique for measuring CBF showed a small increase of 8% during headache, whereas during aura phase it was reduced by 23% (O'Brien, 1967). Improving the techniques, many researchers studied the regional CBF. Series of experiments in migraineurs have been shown that CBF declined during painless aura and increased in the headache phase (Sakai et al., 1978, Skinhoj 1973, Skinhoj et al., 1969). In 1981, Olesen et al. reported the presence of spreading oligemia in the cerebral cortex of migraineurs (Ross 2000, Olesen, 1991). In patients who suffer from migraine with aura, they found that the creeping scotoma is accompanied by formation of a hypoperfusion area in the occipital region. Subsequently, the low flow area moves anteriorly at a rate of 2-3 mm/min for the next 30-60 min. The decrease in blood flow averages 25-30%, persists for 2-6 h, and dose not correspond to the major arteries. The aura symptoms appear during early phase of spreading oligemia; at times, hypoperfusion persists after the aura ceases and pain develops. Eventually, patchy regions of hyperperfusion sometimes appear (Andersen

et al., 1988, Lauritzen 1987). Nearly the same pattern of brain blood flow changes was reported in patients who fulfilled the diagnostic criteria for pseudomigraine with pleocytosis (Fuentes et al., 1998).

Several studies showed that CSD affects the CBF. CSD is accompanied by marked, brief dilatation of pail arterioles (more than 150%) which coincides with the repolarization phase of CSD in experimental animals (Buresova 1957, Hansen et al., 1980, Leao 1944, Shibata et al., 1990). This short preliminary flow rise proceeds by a moderate but prolonged decrease of CBF and terminates by a brief, small hyperperfusion (Duckrow 1991). This pattern compares favorably with the CBF changes in migraineurs and can be considered a reliable sign of CSD involvement in migraine pathogenesis. This interpretation was criticized by Skyhoj Olesen et al. who suggested that CBF reduction is due to increasingly reduced perfusion of a focal area (ischemia) and spreading oligemia castatic an be interpreted as a static area of hypoperfusion complicated by occurrence of a Compton effect (Skyhoj Olsen et al., 1987). However, as it is clear that Compton scatter over stimulate the CBF changes in the low flow area, in most migraine with aura patients the hypoperfusion is moderate (Kronborg et al., 1990) and ischemia during migraine attack is an exception (Lauritzen 1994). Using perfusion and diffusion weighted magnetic resonance imaging (MRI), significant decrease in CBF and cerebral blood volume as well as increase in tissue mean transit time were observed in occipital cortex contralateral to the affected visual hemifield during visual auras in migraineurs while during migraine without aura, no significant hemodynamic changes were observed (Cutrer et al., 1998, Sanchez et al., 1999).

A recent study strongly supports the like between CSD and the aura period in human visual cortex. High-field functional MRI was used to detect blood oxygenation level-dependent (BLOD) changes during visual aura in three migraineurs. A focal increase in BOLD signals developed first in extrastriate cortex and spread at the velocity of 3.5$\pm$1.1 mm/min over occipital cortex. These initial BOLD features were consistent with scintiallations and paralleled by decrease in the stimulus-driven MR oscillations. Increasing in BOLD signals was followed by a decrease in the mean MR signal. This phase appeared to correspond to the localized scotoma and MR stimulus induced response remained suppressed. Within 15$\pm$3 min, both BOLD signals and MR stimulus-induced response recovered. During periods with no visual stimulation,

but while the subject was experiencing scintillations, BOLD signal followed the retinotopic progression of the visual percept. Spreading BOLD signal changes as CSD did not cross prominent sulci (Hadjikhani et al., 2001).

The most common symptoms during the aura phase in migraine are visual. As mentioned, spreading oligemia and excitation wave of aura symptoms start in occipital lobe and propagate anteriorly. Altering the ionic makeup of the extracellular fluid reversibly raises or lowers the susceptibility to CSD. Glial cells act as spatial buffer explicitly for potassium by taking potassium up and carrying it from regions of high concentration to neighboring regions of low concentration (Orkand et al., 1966). In human the lowest glial-neuronal ratio is in the primary visual cortex (Baily et al., 1951).

3.3 CSD and trigeminal activation

The generation of headache from intracranial sources requires stimulation of nociceptive structures with the cranium. Dural stimulation produces headache-like pain in human (Bohdanecky et al., 1963), and a well-known pain pathway exists from the dura to the brain via trigeminal nerve and ganglion (Goadsby et al., 1991). Activation of cells in the TNC in the medulla results in the release of vasoactive neuropeptides at vascular terminations of the trigeminal nerve. It was suggested that the stimulus for the pain may be CSD, which produces an efflux of some mediator into the extracellular space, stimulates the nociceptive receptors and initiates migraine-related pain. The latency period between onset of aura and headache may reflect the time it takes for CSD to propagate from occipital cortex to the pain triggering region. The expression of c-fos has been used to trace nociceptive pathways in CNS (Hunt et al., 1987). The c-fos is an early response proto-oncogene. It is rapidly and transiently expressed in response to noxious inputs in the CNS. The immunocytochemical identification of Fos protein has been used as an indicator of activation of the nociceptive neurons (Colombo et al., 1975). It was shown that drugs which increased the pain threshold in migraine, such as paracetamol, acetylsalicylic acid, methysergide and sumatriptan, decrease the propagation velocity of retinal SD waves.

3.4 CSD and 5-HT receptor

A role for the vasoactive neurotransmitter 5-HT in CSD phenomenon was investigated. It is suggested that serotonin is involved in the modulation of the CBF changes during CSD. 5-$HT_{1D/1B}$ receptor agonist increased the hypoperfusion developed during CSD elicited by KCl injection while 5-$HT_{2A/2C}$ receptor antagonist reduced that and prolonged its duration in rats (Gold et al., 1998). Furthermore, sumatriptan reversibly reduces the propagation speed of retinal SD deflections, accelerates the recovery of the optical and electrical signal and decreases the amplitude of the negative potential shift, associated with the SD in the chick retina (Maranhao-Filho et al., 1997, Wiedemann et al., 1996). Since the chick retina us a blood vessel-free tissue, this indicates that serotonin effect must be related to the nervous tissue. In line with these finding, D-fenfluramine, a serotonin reuptake blocker, significantly decreased CSD rate in the cortical slices (Cabral-Filho et al., 1995). Cerebral 5-HT_{1A} hypersensitivity was suggested in migraineus as they had a greater prolectin response to m-chlorophenylpiperazine, a selective 5-HT_{1A}, 5-$HT_{2A/C}$ receptor agonist, and greater anxiety than control (Leone et al., 1998). Several antimigraine agents, such as sumatriptan, propranolol and methysergide, display marked 5-HT_{1A} receptor activity (Newman-Tancredi et al., 1997). Effect of the 5-HT_{1A} agonist 8-OH-DPAT on the properties of a KCl-induced CSD was evaluated in neocortical tissues of adult rats. It was pointed out that activation of 5-HT_{1A} receptor significantly attenuates the duration of CSD (Kruger et al., 1999).

4. Analgesic effect of paracetamol

Paracetamol (Acetaminophen, N-acetyl-para-aminophenol) was first synthesized in 1888. Even through it was an intermediate in the synthesis of phenacetin, did not test this compound as a drug, because phenols were generally regarded as too toxic for medicinal use. In 1889, the Swedish physiological chemistry discovered that paracetamol is a urinary metabolite of phenacetin. In 1893, the German physiologist tested paracetamol and showed that it was also an analgesic and antipyretic.

4.1 Pharmacology of paracetamol

Paracetamol is the metabolite of two previously used drugs, phenacetin and acetanilide which have the chemical structures as shown in Figure1 Paracetamol possesses analgesic and antipyretic effects which resemble those of aspirin, but is devoid of anti-inflammation activity. However, it is usually classified with the NSAIDs as it possesses a similar profile of activity in relief non-specific pain. Alone or in combination with other drugs, it is found in more than 200 formulations promoted for symptomatic relief of pain, cough and colds (Weiss, 1973).

Like aspirin, paracetamol is widely used in the treatment of pain of moderate intensity such as headache, toothache, dysmenorrheal and pains of musculo-skeletal origins. Its popular use is partly due to the low incidence of adverse effects relative to aspirin (Miller, 1976). At therapeutic doses, adverse effects rarely occur with paracetamol. Because paracetamol is widely available and forcefully promoted as a “safe” aspirin substitute, there is a need to re-evaluate its status as an analgesic antipyretic agent in clinical medicine.

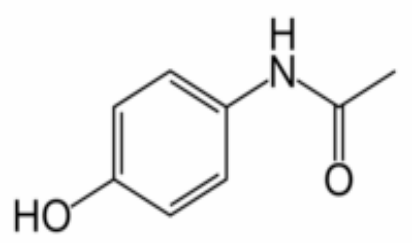

Figure2-6 Structure of paracetamol

4.2 Pharmacokinetics of paracetamol

Paracetamol is a weak acid with a pKa of 9.5. The drug is rapidly absorbed from the gastrointestinal tract, reaching peak plasma levels within 40 to 60 minutes of ingestion (Dordoni et al., 1973). Binding to plasma proteins is variable but considerably less extensive than that of aspirin (Gazzard et al., 1973).

Paracetamol is rapidly and uniformly distributed throughout body tissues; it achieves a tissue: plasma concentrations of less that 60 mg/L. Paracetamol do not apparently bind to plasma proteins; at 90 mg/L protein binding was less than 5%; and after toxic doses (plasma paracetamol concentrations of up to 280 mg/L) protein binding varied from 8 to 43% with no correlation between binding and plasma paracetamol concentration (Gazzard et al., 1973).

4.3 Metabolism of paracetamol

Liver is typically and by far the most involved organ in paracetamol acute toxicity. Once absorbed, approximately 90% of paracetamol normally undergoes hepatic glucuronide (40–67%) and sulphate (20–46%) conjugation to form inactive, harmless metabolites, which are eliminated in urine. A small fraction of unchanged paracetamol (<5%) and other minor metabolites reach the urine, but are not thought to be clinically relevant. The remaining fraction, usually ranging from 5 to 15%, is oxidized by the CYP2E1, CYP1A2, CYP3A4, and CYP2A6 subfamilies of the P450 mixed-function oxidase system, resulting in the formation of N-acetyl-p-benzoquinoneimine (NAPQI) (Chen et al., 1998, Dahlin et al., 1984, Patten et al., 1993, Thummel et al., 1993). Glutathione quickly combines with NAPQI; the resulting complex is then converted to non-toxic cysteine or mercaptate conjugates, which are eliminated in urine. After appropriate paracetamol dosing, glutathione supply far exceeds that which is required to detoxify NAPQI. After overdose, the rate and quantity of NAPQI formation may outstrip glutathione supply and regeneration. When glutathione stores are depleted below a critical value (about 30% of normal stores) free NAPQI rapidly and covalently binds and arylates critical cell proteins, inducing a series of events that may result in cell death (Mitchell et al., 1973). Critical, possibly irreversible, events in cell death include oxidation of enzymes, DNA fragmentation, and mitochondrial injury. A massively increased production of nitrogen oxygen species may also be important in paracetamol-induced acute toxicity (James et al., 2003) as well as several components of liver innate immune system (natural killer cells, natural killer T cells, Kupfer cells macrophages, neutrophils) (Liu et al., 2006). It is now clear that this process can be not only prevented or interrupted, but even reversed after NAPQI binding has occurred. Factors that may predispose to hepatotoxicity include increased frequency of paracetamol dosing, prolonged duration of excessive dosing, increased capacity for P450 activation to NAPQI (as in patients chronically treated with agents that induce hepatic microsomal enzymes, like anticonvulsants, isoniazid, etc.), decreased glutathione availability, or decreased capacity for glucuronidation and sulfation.

In the liver, most oxidative drug metabolism is concentrated in the centrilobular zone (zone III), and this zone is first and most profoundly affected by paracetamol toxicity, due to the local formation of NAPQI. In more severe cases,

necrosis may extend into zones I and II to destroy the entire liver parenchyma. Fulminant hepatic failure may develop in severely poisoned patients from the third to the sixth day. It is characterized by deepening jaundice, encephalopathy, increased intracranial pressure, grossly disordered homeostasis with disseminated intravascular coagulation and hemorrhage, hyperventilation, acidosis, hypoglycemia and renal failure. These patients are candidates for as early as possible liver transplantation (O' Grady et al., 1991). The bioartificial liver is used as supportive care in patients awaiting transplant, and as primary therapy in patients who have contraindication that precludes transplantation (Detry et al., 1999). Kidney is the second target organ of paracetamol toxicity: renal dysfunction occurs in about 25% of cases with significant hepatotoxicity (Davenport et al., 1988) and in more than 50% of those with hepatic failure (Makin et al., 1994, Wilkinson et al., 1977). Overt renal failure necessitating hemodialysis occurs nearly always among patients with marked hepatic injury (Campbell et al., 1992). Renal abnormalities are more common after sustained repeated excessive dosing (Prescott et al., 1996). However, renal impairment after acute paracetamol overdose may also occur in the absence of hepatotoxicity (Campbell et al., 1992, Prescott et al., 1982). Also the pathophysiology of renal dysfunction after acute paracetamol overdose is mainly the result of the local formation of NAPQI that causes tubular necrosis (Breen et al., 1982, Emeigh-Hart et al., 1994, Hoivik et al., 1995). However, several other nephrotoxic mechanisms have been proposed (Hart et al., 1994, McCrae et al., 1989), also because acute renal failure has been reported despite adequate treatment with N-acetylcysteine (Daveport et al., 1988). In addition, volume depletion and hepatorenal syndrome are often cofactors. While the peak disturbance in liver function occurs 2 to 4 days after a paracetamol overdose, renal impairment, if it develops, becomes more evident after 1 week and returns to normal about 2 to 3 weeks after ingestion (Cobden et al., 1982, Curry et al., 1982). Renal damage is also produced by chronic use of paracetamol; in a case-control study involving 1,077 subjects who frequently took paracetamol, a dose-dependent relationship between heavier paracetamol use and an increased risk of end-stage renal disease was demonstrated (Perneger et al., 1994). Injury to other organs is rarely reported. The mechanism causing myocardial damage, reported in some patients with paracetamol-induced fulminant hepatic failure, is thought to be part of multi system organ failure rather than being paracetamol specific (Brent et al., 1996,

Lip et al., 1996). Pancreatic toxicity is extremely rare (Gilmore et al., 1977, Mofenson et al., 1991). Early recognition and treatment of patients with paracetamol poisoning are essential in order to minimize morbidity and mortality. This task is made difficult by the lack of predictive clinical findings early in the course of paracetamol poisoning, and clinicians should not feel reassured by a patient's lack of symptoms soon after ingestion. The first symptoms after paracetamol overdose may be those of hepatic injury, which develops many hours after the ingestion, when antidotal therapy is already less effective (Bizovi et al., 2002).

4.4 Mechanism of action of paracetamol

The basic pharmacological mechanism of action of paracetamol has not received the scientific attention accorded to the salicylates and consequently, many explanations for its activity appear somewhat speculative. It has analgesic and antipyretic properties which do not differ significantly from those of aspirin. However, paracetamol lacks the potent anti-inflammatory actions of aspirin.

Paracetamol, an effective analgesic but only a weak anti-inflammation agent has not been satisfactorily established, although most recent explanations involve a selective inhibition of some facet of prostaglandin biosynthesis (Flower et al., 1980; Jackson et al., 1984; Meredith and Goulding, 1980; Ramwell, 1981). Some evidence suggests that paracetamol has a weak inhibitory influence on peripheral prostaglandin biosynthesis (which would account for its lack of substantial anti-inflammatory activity), but that it is a potent inhibitory of prostaglandin production within the central nervous system (presumably accounting for its analgesic and antipyretic properties). Like the salicylates, paracetamol possesses analgesic activity which is effective against pain of mild to moderate severity. However, unlike the salicylates, which act mainly peripherally against pain associated with inflammation, paracetamol has few or no anti-inflammatory properties and apparently exert its analgesic effects via central actions (Bowman and Rand, 1980; Flower et al., 1980; Jackson et al., 1984). This is supported by the work of Ferreira et al., 1978 which suggests that release of prostaglandins within the central nervous system, involving pain circuits, as well as sensitization of peripheral pain receptors by locally released prostaglandins both contribute to inflammatory hyperalgesia, However, Guzman and Lim (1976)

demonstrated that paracetamol relieved pain by blocking impulse generation at bradykinin sensitive chemoreceptors which evoke pain a peripheral mechanism.

- A central serotonergic mechanism

Central serotonergic systems may be involved in paracetamol mediated antinociception (Pelissier et al., 1994; Tjolsen et al., 1991). The analgesic activity of paracetamol was altered in rats in which the serotonergic pathway were lesion with intrathecal 5,6-dihydroxytryptamine (Tjolsen et al., 1991). It would be hazardous to infer from these observations that paracetamol interact directly with 5-HT receptors in the CNS. The following data should be considered. Firstly, 5-HT released antinociception is mediated by a 5-HT induced release of noradrenaline in the spinal cord (Sawynock and Reid, 1992). Accordingly in vitro studies failed to show any binding of paracetamol to 5-HT_3 receptors or any inhibitory effect of this drug on 5-HT reuptake (Pelissier et al., 1994). Secondly, mutual interactions between 5-HT or catecholamine transmitters and opiate neurons have been reported (Groppetti et al., 1988; and Taiwo and Levine, 1988). It is therefore possible that the central antinociceptive properties of paracetamol could be mediated indirectly by opioid mechanisms. Interestingly, naloxone did not abolish the antinociceptive activity effects of Ketorolac, aspirin and paracetamol (Carlsson et al., 1988; Groppetti et al., 1988; Uphouse et al., 1993).

Paracetamol concentrations in the cerebrospinal fluid mirror response to fever (Anderson et al., 1998) and pain (Anderson et al., 2001) to a greater extent than plasma concentrations. Paracetamol is effective in rat pain models after central administration (Pelissier et al., 1996). Animal data supports the contention that spinal 5-hydroxytryptamine type 3 (5-HT_3) receptors are involved in the antinociceptive effect of paracetamol (Tjolsen et al., 1991, Alloui et al., 2002) and that paracetamol interferes with serotonergic descending pain pathways. Support for these data in humans comes from the demonstration that coadministration of tropisetron or granisetron (5-HT_3 receptor antagonists) with paracetamol completely blocked the analgesic effect of acetaminophen in volunteers (rapid metabolizers of tropisetron) when assessed by pain induced from electrical stimulation of the median nerve. Volunteers given granisetron, a more specific antagonist, had greater pain (measured as area under the time-pain curve) than those given tropisetron (Pickering et al.,

2006). It is believed that paracetamol reinforces descending inhibitory pain pathways (Pickering et al., 2008). Data supporting the central effect of paracetamol through activation of descending serotonergic pathways do not refute arguments that its primary site of action may still be inhibition of PG synthesis, as for the NSAIDs (Graham et al., 2005). For example, the expression of a PGE_2 receptor (EP_3) by most of the serotonergic, noradrenergic, and adrenergic cell groups suggests that PGE_2 modulates many physiologic processes. It may modulate nociceptive and autonomic processes by affecting the descending serotonergic pathway from the raphe magnus nucleus to the spinal cord (Nakamura et al., 2001). Serotonergic cell bodies in the raphe magnus nucleus provide dense projections to the dorsal horn of the spinal cord, and this descending pathway has been shown to mediate the antinociceptive action of morphine (Fields et al., 1991, Tork, 1990).

- Prostaglandin inhibition of paracetamol

Despite their common mechanism of action, paracetamol and other NSAIDs differ pharmacologically. At usual doses, floctafenin appears as a simple analgesic whereas paracetamol is devoid of any significant anti-inflammatory effect in humans (Bannwarth et al., 1992). This observation lends clinical support to the assumption that PG independent mechanisms are involved in the properties of NSAIDs, particularly in their anti-inflammatory effects (Abramson and Weissman, 1989). Alternatively, the dissociation of antipyretic, analgesic and anti-inflammatory properties may arise from the heterogeneity in the cyclo-oxygenase enzymes (Flower and Vane, 1974). Thus it is possible that different isoenzymes differ with respect to their susceptibility to drugs (Laburn et al., 1980; Neuman et al., 1987; Weksler et al., 1983). Whether paracetamol selectivity impedes cyclo-oxygenase activity in the CNS is however debated (Tolman et al., 1983; Vane, 1987).

Systemically administered paracetamol as well as various NSAIDs were shown to be capable of inhibiting ischemia induced PGE_2 formation in mouse brain (Ferrari et al., 1990). The degree of inhibition of brain cyclooxygenase correlated well with the antinociceptive potencies of these drugs in the acetylcholine induced constriction test (Ferrari et al., 1990). Nevertheless, non acetylated salicylates are generally considered as poor PG biosynthesis inhibitors on the basis of in vitro data (Abramson and Weissmann, 1989). Since numerous variables may influence these

investigations, their results must be considered cautioned (Neuman et al., 1987; Tolman et al., 1983). Concurrently, significant inhibition of PG production was observed on the brain after analgesic doses of R-flurbiprofen (Brune et al., 1992). Thus, it was hypothesized that inhibition of PG synthesis within the CNS may account for the central analgesic effect of paracetamol.

- COX-3

The ability of paracetamol to effectively inhibit CNS PGE_2 production during fever and pain led to the hypothesis that an exquisitely paracetamol sensitive variant of PGHS exists with the CNS or vascular endothelium. In 2002 Chandraselharan et al reported to have found such an enzyme within the canine cerebral cortex, which they desingted “COX-3”. This enzyme is the product of an alternatively spliced messenger ribonucleic acid (mRNA) of the COX-1 gene that is identical to COX-1 mRNA except that intron 1 is retained. Because it is a product of alterative splicing of PGHS-1 and not a genetically distinct the name COX-3 has been rejected by many authors.

- Cannabinoid receptor activity

Two research groups (Hogestatt et al., 2005 and Ottani et al., 2006) have demonstrated an activity metabolite of paracetamol (the fatty acid amide N-arachidonoylphenolamine; AM404); a compound that shares the ability of cannabinoid receptors to display analgesic activity and to lower body temperature (Bisogno, 2008 and Guhring et al., 2002). Paracetamol is mostly cleared by the liver through glucoronide and sulfate conjugation. However, it is de-acetylated in the mouse brain and spinal cord to p-aminophenol. This primary amine is then conjugated with arachidonic acid by fatty acid amide hydrolase to AM404. AM404 dose not work directly on cannabinoid receptors (Kelley et al., 2004). It is a potent activator of the vanilloid subtype 1 receptor (TRPV1) (Zygmunt et al., 2000), which is a ligand at cannabinoid 1 receptors and an inhibitor of cellular anandamide uptake resulting in increased levels of endogenous cannabinoid receptors. Anandamide uptake would result in the activation of nociceptors. AM404may also work through PGHS, particularly in areas of the brain with high concentrations of fatty acid amid hydrolase (mesencephalic trigeminal nucleus, primary sensory neurons), although this argument remains speculative.

5. Association of analgesic and chronic daily headache

Chronic Daily Headache (CDH) is characterized by the daily or almost daily occurrence of headache. Its pattern is usually episodic in the beginning, either in the form of migraine or tension type headache. According to the temporal courses and patterns, this syndrome can be classified into four categories, including transformed migraine, chronic tension type headache, new daily persistent headache, and hemicranial continua.

No clear explanation exists regarding the pathogenesis of headache transformation. Despite its clinical varieties, the development of daily headache from an initial episodic character, either migraine or tension type headache, may share some common mechanisms. The overuses of analgesic have been proposed to be accounted for this process.

Chronic daily headaches are frequently associated with rebound headaches after ergotamine, barbiturate, caffeine, and analgesic overuse. The analgesic overuse headaches are among the most common and challenging of the chronic daily headache disorders. The definition of overuse of analgesic is:

- Regular overuse of a headache medication for >3 month
- Use of ergotamine, triptans, opioids, and combination analgesics >10 days per month
- Use of simple analgesics ≥15 days per month
- Total use of all headache medications ≥15 days per month

Headache present at least 15 days per month characterized by the development or marked worsening of pain during analgesic overuse and resolution of pain and reversion to previous episodic pattern (<15 days per month) within 2 month after discontinuation of analgesic (IHS 2004).

Most patients with transformed migraine and analgesic-overuse headache are women and have a history of episodic migraine that dates back to adolescence or early adulthood (Mathew et al., 1982, Saper et al., 1990). Patients often report a period of transformation that occurs over months or years in which headaches become more frequent, until a pattern of daily or near-daily headaches develops that clinically resembles a mixture of tension-type headache and migraine. This clinical phenotype explains why labels such as “mixed headache” and “tension–vascular headache” have been informally applied to this group of patients. The overuse of acute-headache

analgesic by patients with frequent headache may lead to analgesic-overuse headache, a syndrome of daily headaches that is induced and maintained by the very analgesic used to relieve the pain (Mathew et al., 1990, Saper et al., 1987). The prevalence in the population of chronic daily headache associated with the overuse of acute-headache analgesic was recently estimated to be 1.4 percent overall and was particularly high among women (2.6 percent), especially those over the age of 50 years (5 percent) (Lanteri-minet 2003). Overuse of acute-headache analgesic is reported by approximately 80 percent of the patients with transformed migraine who are seen in headache clinics (Bigal et al., 2002) but by less than a third of those with transformed migraine in the general population (Scher et al., 1998). Furthermore, in a substantial proportion of patients, daily headache may continue once they stop overusing acute-headache analgesics. Therefore, the overuse of acute-headache analgesics is neither necessary nor sufficient to cause transformed migraine. Patients with transformed migraine most often overuse acute-headache analgesics such as analgesics that combine aspirin, acetaminophen, and caffeine and those that contain butalbital, opioids, ergotamine, or triptans or a combination of these analgesics. The interval from the frequent intake of these analgesics to the development of analgesic overuse headache has been reported to be shortest for triptans (1.7 years), longer for ergots (2.7 years), and longest for analgesics (4.8 years). It is unclear whether this observation relates to the pharmacologic characteristics of the medications (Katsarava et al., 2001). Although it is often difficult to be certain whether the overuse of acute-headache analgesic is the cause or the consequence of the daily headaches, accurate diagnosis and management require the withdrawal of such analgesic in all patients, especially in the light of the observation that their overuse may preclude the efficacy of preventive analgesic. If a pattern of episodic headaches (fewer than 15 days per month) recurs within 2 months after drug withdrawal, analgesic-overuse headache is diagnosed (IHS 2004). If headache continues to occur on at least 15 days per month despite the withdrawal of acute-headache analgesic, a diagnosis of transformed migraine is often made. Although this distinction is obviously arbitrary, the reduction in the frequency of headache after the withdrawal of analgesic is often dramatic in patients who have true analgesic-overuse headache.

6. Serotonergic system

5-HT is a major neurotransmitter involved in a large number of physiological processes including the regulation of mood, arousal, aggression, sleep, learning, and pain modulation. Alterations of 5-HT have been shown to occur in many psychiatric diseases including depression, anxiety, eating disorders, schizophrenia, migraine etc. (see review of Jocobs and Azmita, 1990).

- Biosynthesis and metabolism of 5-HT

5-HT is synthesized from the amino acid precursor tryptophan which is derived from dietary sources. The dietary amino acid tryptophan is converted to 5-hydroxy-tryptophan (5-HTP) by the enzyme tryptophan hydroxylase (the rate limiting enzyme). This enzyme is found only in cells that synthesize 5-HT. 5-HTP decarboxylase then converted this intermediate amino acid to 5-HT. 5-HT is metabolized both pre and postsynaptically by the enzyme monoamine oxidase (MAO), which produces the inactive metabolite 5-hydroxyindoleacetic acid (5-HIAA).

- Physiological pathway of 5-HT

After synthesis, 5-HT is stored in synaptic vesicles via an ATP dependent, low affinity transport system. Depolarization of the presynaptic endplate membrane induces Ca^{2+} influx, which in turn triggers the release of 5-HT, possibly by activation of intracellular microtubules (Mulder et al., 1975). The excreted 5-HT diffuses to the postsynaptic membrane and binds to and activates postsynaptic receptors. Concomitantly, 5-HT is taken up into the presynaptic neuron to terminate the activation of postsynaptic receptors and relieve 5-HT synthesis. The relevance of reuptake for homeostasis of 5-HT can be derived from the high efficacy of 5-HT recycling. 5-HT is translocated into the presynaptic neuron via an active Na^+ dependent transport system. Following reuptake it is stored in synaptic vesicles by a reserpine-sensitive, H^+ dependent, active transport system and it available for release again (Rudnick, 1986). It has been postulated that the Na^+ dependent 5-HT reuptake system is functionally associated with the hypothetical presynaptic 5-HT autoreceptor (Bonnano and Raiteri, 1987; Galsin et al., 1985) and, as such, is involved in feedback processes regulated 5-HT synthesis and release.

- Anatomical distribution of 5-HT

The 5-HT cell bodies in the brain are located in the brain stem. Their axons, however, innervate nearly every area of the central nervous systems (Fig9). In 1964, Dahlstrom and Fuxe observed that the majority of serotonergic soma was found in cell body groups previously designated by Taber, Bradal and Walberg as the raphe nuclei. This earlier description of the raphe nuclei was based on cytoarchitectural criteria, i.e., on cell body structural characteristics and organization. Dahlstrom and Fuxe described nine groups of 5-HT containing cell bodies, which they designated B1 through B9, and which correspond for the most part with the raphe nuclei. Some serotonergic neuronal cell bodies, however, are found outside the raphe nuclei are serotonergic.

The large group of serotonergic cells is group B7 of Dahlstrom and Fuxe. Group B7 is contiguous with a smaller group of serotonergic cells, B6. Groups B6 and B7 are often considered together as the dorsal raphe (DR) nucleus, with B6 being its caudal extension. Another prominent serotonergic cell body group is B8, which corresponds to the median raphe (MR) nucleus, also termed the nucleus centralis superior. Group B9, part of the ventrolateral tegmentum of the pons and midbrain, forms a lateral extension of the median raphe and therefore is not considered one of the midline raphe nuclei. Ascending serotonergic projections innervating the cerebral cortex and other regions of the forebrain come from the dorsal raphe, median raphe, and B9 cell group. The other raphe nuclei, B1 to B5, are more caudally situated (mid pons to caudal medulla) and contain a small number of serotonergic cells. These cell body groups give rise to serotonergic axons that project within the brain stem and to the spinal cord.

The highest percentage of 5-HT is located in the DR (79%) and the bulk of the remainder produced by the MR. Thus the main source for limbic and neocortical 5-HT is DR and MR nuclei (Azamita, 1978; Azamita and Gannon, 1986; Wilson and Molliver, 1991a,b). Specifically, the amygdale, hypothalamus, basal ganglia, primary and association receiving area, and frontal lobe are innervated by the DR, whereas the hippocampus, cingulated gyrus, and septum receive their 5-HT from the MR nuclei (Azamita, 1978; Azamita and Gannon, 1986; Wilson and Molliver, 1991 a,b)

The MR, however, is diffusely organized and appears to exert a nonspecific and global influence on arousal and excitability (Wilson and Molliver, 1991 a,b). The

DR is much more discretely organized, can exert highly selective inhibitory or excitatory influences, and plays a role in the coordination of excitation in multiple functionally related areas, including the frontal lobes and amygdale (Wilson and Mollove, 1991 a,b). Because of the manner in which they are organized, the DR and MR can exert select inhibitory influences so as to engage in perceptual filtering in one or a variety of areas.

In summary, it can be said that the number of 5-HT cell bodies in the brain are remarkably few, with the over whelming majority being in the raphe and reticular systems, Newer techniques, especially the immunocytochemical technique for 5-HT, have confirmed the main outlines of the original distribution of 5-HT neurons described by Dahlstrom and Fuxe with a number of significant additions, particularly in terms of the spread of the brain stem 5-HT groups laterally from the midline confines of the raphe nuclei. Some species differences may exist, which account for minor discrepancies reported among the rat, cat, and monkey, such as the area postrema.

- 5-HT receptor

5-HT exerts its effect on 5-HT receptors which are classified into many classes including 5-HT_1 to 5-HT_7 receptors. All of 5-HT receptors, except 5-HT_3 receptor, are member of the G-protein coupled receptor superfamily. The 5-HT_3 receptor belongs to ligand gated ion channel superfamily. Specific receptor subtypes have recently been associated with either the pathogenesis or the treatment of migraine headache.

Many types of analgesic drugs have been proposed to act through an increase in brain 5-HT levels. The multiple 5-HT subtypes have been identified with the CNS and 5-HT_1, 5-HT_2 and 5-HT_3 receptor are now thought to be involved in the 5-HT mediated antinociceptive mechanism (Sufka et al., 1992). The bulk of the data suggests that stimulation of 5-HT_1 receptor reduces nociceptive sensitivity, whereas activation of 5-HT_2 receptors increases nociceptive responsiveness (Eide and Hole, 1993).

- The 5-HT_2 receptor family

The 5-HT_2 receptor class now comprises three distinct receptor subtypes, namely 5HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}. Each is a seven transmembrane, G-protein

linked receptor similar in molecular size and displaying about 60% sequence homology. In contrast to 5- HT_1 receptors, 5-HT_2 receptor genes possess introns and exons and are coupled preferentially to phospholipase C. Receptor activation therefore leads to stimulation of phosphatidyl inositol metabolism and an increase in inositol triphosphate (IP_3) production. The 5-HT_2 receptor has been identified in a variety of tissues, both in the peripheral and central nervous systems. In the periphery, this receptor has been localized to several smooth muscle types including the guinea pig trachea and rat uterus. In these tissues stimulation with 5-HT_2 receptor agonists causes a contraction of the smooth muscle (Heller and Baraban, 1987; Cohen et al., 1985). In the central nervous system, the distribution of 5-HT_2 receptor has been extensively mapped, initially with radioligand receptor autoradiography and RNA blot analysis and, more recently, with immunohistochemistry.

- 5-HT_{2A} receptor

The newly named 5-HT_{2A} subtype refers to the 'classical' 5-HT_2 receptor widely described in the peripheral tissues and the CNS. In the periphery, activation of the receptor leads to contraction of vascular and non-vascular smooth muscle, platelet aggregation and increase in capillary permeability. Available evidence also implies a role in modulating the release of other neurotransmitters and hormones, including acetylcholine (Muranmatsu et al., 1988a), adrenaline (Feniuk et al., 1981), dopamine (Muranmatsu et al., 1988b), excitatory amino acids (Maura et al., 1988), and vasopressin (Rittenhouse et al., 1990). In the CNS, autoradiographic studies reveal highest receptor concentrations in the cortex and to a lesser extent in hippocampus and caudate nuclei (Hoyer et al., 1986; Pazos et al., 1987). Precise roles in the CNS remain unclear, but in rodents agonists acting at $5HT_{2A}$ receptor evoke a stereotypical syndrome comprising head twitch and wet-dog shakes, implying a possible involvement of the receptors in motor behaviors. An important role in regulating sleep has been suggested (Sharpley et al., 1990). Furthermore, in peripheral (e.g. rat facial and spinal motoneurons: Connell and Wallis, 1989) as well as central (e.g. nucleus accumbens: North and Uchimura, 1989) neurons, 5-HT_{2A} receptors mediate neuron excitation and have been associated with the transmission of nociceptive stimuli.

A wide range of potent 5-HT_{2A} receptor antagonists are available and several are useful radiolabelled probes, e.g. spiperone, ketanserin, mesulergine, mianserin, lysergic acid diethylamide DOB and more recently (Mylecharance, 1990; Malgouris et al., 1993). Unfortunately, all display poor selectively with regard to the three 5-HT_2 receptor subtypes, and none alone can be relied upon to define the 5-HT_{2A} receptor unequivocally. Among the most useful antagonists are ketanserine and RP62203, both of which display selectivity over 5-HT_{2C} receptors.

Several specific antagonists exist to separate 5-HT_2 effects from other 5-HT receptors. Spiperone, for example, can distinguish between the 5-HT_{1C} receptors and the 5-HT_2 receptors with 1000-fold difference in affinity. But it has a fairly high affinity for the 5-HT_{1A} receptor, and an even higher affinity for dopamine D_2 receptors. Ketanserin has approximately 100-fold difference in affinity for the 5-HT_2 receptor than for any of the other 5-HT receptors, but it has appreciable affinity for both histamine H_1 receptors and α1-adrenergic receptors. DOB and DOI have been used as selective agonists for 5-HT_2 receptors, though both compounds have some affinity for the 5-HT_{1C} receptors and some caution must be used in studies using these compounds (Leysen, 1989). There are no subtype selective 5-HT_2 receptor agonists. α-Methyl-5HT and the phenylalkylamines (DOI, DOB) are most often used in this regard, but are equally active at 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors and, in addition, are potent agonists at an orphan 5-HT receptor mediating endothelium-dependent vasorelaxation (Martin, 1994).

It is important to recognize that antagonist affinity estimates at the 5-HT_{2A} receptor may vary considerably, but there is presently no evidence to suggest that this reflects the existence of 5-HT_{2A} subtypes. Rather, it appears to result from species differences in receptor primary structure. However, binding data obtained using DOB and ketanserin reveal intriguing differences indicating that either the receptor exists in two non-interconvertible states or that post-translational modification have yielded two closely similar forms of the receptor (Branchek et al., 1990).

- Transductional characteristics

Receptor coupling to IP_3 production has been demonstrated in numerous studies using either native or recombinant 5-HT_{2A} receptors. However, Felder et al. (1990) have shown that 5-HT can stimulate phospholipase A_2 and subsequent release

or arachidonate in hippocampal and cortical neurons via 5-HT_2 receptors presumed to be 5-$HT_{2A.}$

Unlike the 5-HT_1 receptors, agonist activation of the 5-HT_2 receptor results in phospholipids hydrolysis and production of the second messenger phosphoinositide. Conn and Sanders-Bush (1985) demonstrated a 5-HT mediated increase in phosphoinositide turnover in rat cortical slices that could be blocked by ketanserin. This effect is diminished in cortical slices from animals chronically treated with imipramine and iprindole (Kendall and Nohorski, 1985). As would be expected of a receptor linked to phosphoinositide hydrolysis, stimulation of the 5-HT_2 receptor cause translocation of protein kinase C from the cytosol to the membrane as part of the second messenger cascade (Wang and Friedman, 1989).

7. Serotonin/ Analgesic/ Headache

It has been known that 5-HT plays an important role in migraine pathogenesis (Srikiatkhachorn et al., 2000). In 1989 Ferrari and coworker demonstrated that attacks of migraine coincided with a fall in platelet 5-HT as well as increase in plasma 5-HT. Lowered levels of platelet 5-HT is thought to reflect monoamine depletion in brainstem nuclei. In 1971 Sicuteri and coworker demonstrated that p-chlorophenylalanine (PCPA), a 5-HT depletory, provoked systemic pain after 20 to 40 days in 4 of 18 migraineurs. The administration of 5-HT or 5-HT agonists (mainly 5-$HT_{1B/1D}$ agonists) can abort the attack of migraine. It may hypothesized that the descending inhibitory serotonergic pathway involved in the control pain is defective in migraine sufferer, and that central 5-HT depletion accentuates the perception of pain by reducing the efficacy of the endogenous pain control system and induces migraine attacks (Sicuteri et al., 1990, Sicuteri et al., 1972). In 2000, Srikiatkhachorn and coworker found the role of 5-HT depletion in the modulation of a cranial vascular response to nitric oxide. They investigated that 5-HT depleted animal enhanced response of meningeal and cerebral microvessels to NO. They also found that low 5-HT produced more considerably changes in cerebral microvessel, characterized by focal ballooning of endothelial cells, increased microvillous formation, and increased endothelial cells than control animals. These finding imply a relationship between 5-HT depletion and the development of migraine attack.

The experimental and clinical studies suggest a primary role of central sensitization in the pathogenesis of CDH. Suppression of the endogenous pain control system can facilitate the process of CDH includes decrease platelet 5-HT and up regulation of 5-HT_{2A} receptors. Alterations in 5-HT system have been demonstrated in patient with analgesic induced headache (Srikiatkhachorn et al., 1996, Srikiatkhachorn et al., 1998). Chronic analgesic exposure can alter endogenous 5-HT dependent system of pain modulation by inducing a low 5-HT state. The low level of 5-HT subsequently leads to up regulation of the pronociceptive 5-HT_{2A} receptor. Stimulation of these highly expressed receptors and reduction of pain modulation may increase the process of central sensitization. Activation of 5-HT_{2A} receptor may potentiate nociception by enhancing the release of substance P from the primary afferent (Eide et al., 1991). In 2002, Srikiatkhachorn et al investigated that 5-HT_{2A} agonist, DOI treatment led to shortening of tail flick latency and to an increase in the number of Fos-ir neurons in TNC. These results suggest that activation of 5-HT_{2A} receptor leads to an increase activity in trigeminal nociceptive system. Up regulation of this receptor can increase headache attacks and contribute to development of CDH. In 1999, Srikiatkhachorn et al demonstrated that the antinociceptive effect of acute and chronic paracetamol treatment on plasticity of 5-HT_{2A} receptor at frontal cortex and brainstem membrane. The results show that decreasing the maximum number of 5-HT_{2A} binding site founded in all treatment with paracetamol 300 and 400 mg/kg on frontal cortex membrane. These results suggest that down regulation of 5-HT_{2A} receptor in response to 5-HT release involved in the mechanism of antinociceptive effect of paracetamol.

8. Propose hypothesis

Based on this review, the pathophysiological basis of analgesic induced headache is still controversy. The relationship between an excessive use of analgesic and deterioration of primary headaches, especially migraine, is an issue of interest.

There are several hypotheses have been proposed to explain the pathogenesis of analgesic induced headaches including changes in the central 5-HT system, central pain modulating system. 5-HT is major neurotransmitter involved in pain modulation. Recent evidence has shown that thee analgesic efficacy of various pharmacological and non-phramacological pain control measures may be mediated via this

neurotransmitter. A central mechanism of action for paracetamol has proposed (Piletta et al., 1991, Bannwarth et al., 1992). Paracetamol is effective in rat pain models after central administration (Pelissier et al., 1996). Data supporting the central effect of paracetamol through activation of descending serotonergic pathways do not refute arguments that its primary site of action may still be inhibition of PG synthesis, as for the NSAIDs (Graham et al., 2005). It may modulate nociceptive and autonomic processes by affecting the descending serotonergic pathway from the raphe magnus nucleus to the spinal cord (Nakamura et al., 2001).

In order to investigate the effect of commonly used analgesic on trigeminovascular nociception, the deterioration of the trigeminovascular nociceptive responses induced by CSD in animal with chronic consumption of paracetamol were measured. Both neuronal and vascular changes were monitored in acute and chronic paracetamol treated rats. In order to study the cortical neurons activity, the DC shift was measured by extracellular recording technique. In vascular compartment, the cerebral endothelial cells and CBF was study by electron microscopy and Laser Doppler flowmetry, respectively. The trigeminal nociception was indicated by the number of fos immunoreactive neurons in cerebral cortex and TNC. The expression of 5-HT_{2A} was studied in cerebral cortex and TG. In addition, involvement of 5-HT_{2A} receptor in the effect of chronic treatment with paracetamol induced changes in trigeminovascular nociceptive system was observed.

The objectives of this study were:

I: To study the effect of acute treatment with paracetamol on the cortical activity, trigeminal nociception, 5-HT_{2A} receptor expression, CBF, and ultrastructural changes induced by CSD

II: To study the effect of chronic treatment with paracetamol on the cortical activity, trigeminal nociception, 5-HT_{2A} receptor expression, CBF, and ultrastructural changes induced by CSD

III: To study the effect of 5-HT_{2A} receptor antagonist (ketanserin 1 and 10 mg/kg) on the cortical activity, trigeminal nociception, 5-HT_{2A} receptor expression, CBF, and ultrastructural changes induced by CSD in chronic treatment with paracetamol

CHAPTER III

MATERIALS AND METHODS

1. Chemicals and Biochemicals

- Pentobarbital sodium (Nembutal®) (Sanofi)
- Potassium chloride (KCl) (Merck)
- Sodium chloride (NaCl) (Merck)
- Sodium dihydrogen phosphate ($NaH2PO_4.H_2O$) (Merck)
- Normal horse serum (Gibco)
- Sucrose (Sigma)
- Heparin (Leo)
- Absolute ethanol (Merck)
- Hydrogen peroxide (H_2O_2) (Merck)
- Paraformaldehyde (Sigma)
- Rabbit anti 5-HT_{2A} (Santa Cruz)
- Rabbit anti c-fos (Santa Cruz)
- Envision+HRP anti rabbit (Dako)
- Liquid DAB substrate kit (Dako)

2. Animals

Adult male Wistar rats weighing 200-300 grams at the same age were purchased from the National Laboratory Animal Center of Salaya Campus, Mahidol University. The animals were housed five animals per cage in stainless-steel bottom cages and were kept in a well-ventilated room in which the temperature was $25\pm2^{\circ}C$ with an automatic lighting schedule. All animals were allowed to access food and tap water *ad libitum*. To limit the effect of nonspecific stress, all animals were accustomed to daily handing for at least one week before experimentation. All procedures were done with the approval of the Animal Use Committee, Faculty of Medicine, Chulalongkorn University.

Testing of CSD model

In order to test that the application of KCl on the brain surface used in this study could induce the CSD via the release of glutamate.

The effect of NMDA receptor antagonist (MK-801) on DC shift, CBF and Fos expression evoked by CSD were observed. The rats were injected with MK-801 1 mg/kg of body weight in 0.9% sterile saline intraperitoneally after first peak of DC shift and CBF induced by KCl application.

The results demonstrated that administration of MK-801 after the first peak DC shift and hyperemic cycle inhibited the DC shift and CBF evoked by CSD. The repetitive DC shift and hyperemic cycle evoked by KCl application was significantly inhibiting as compare with those of control CSD group (Figure 3-1 and Figure 3-2). The Fos expression after CSD induction for 2 hour was mainly distributed in lamina I and II of the TNC section and lamina I-V of cerebral cortex on the side ipsilateral to the KCl application. Pretreatment with MK-801 reduced Fos expression in cerebral cortex and TNC.

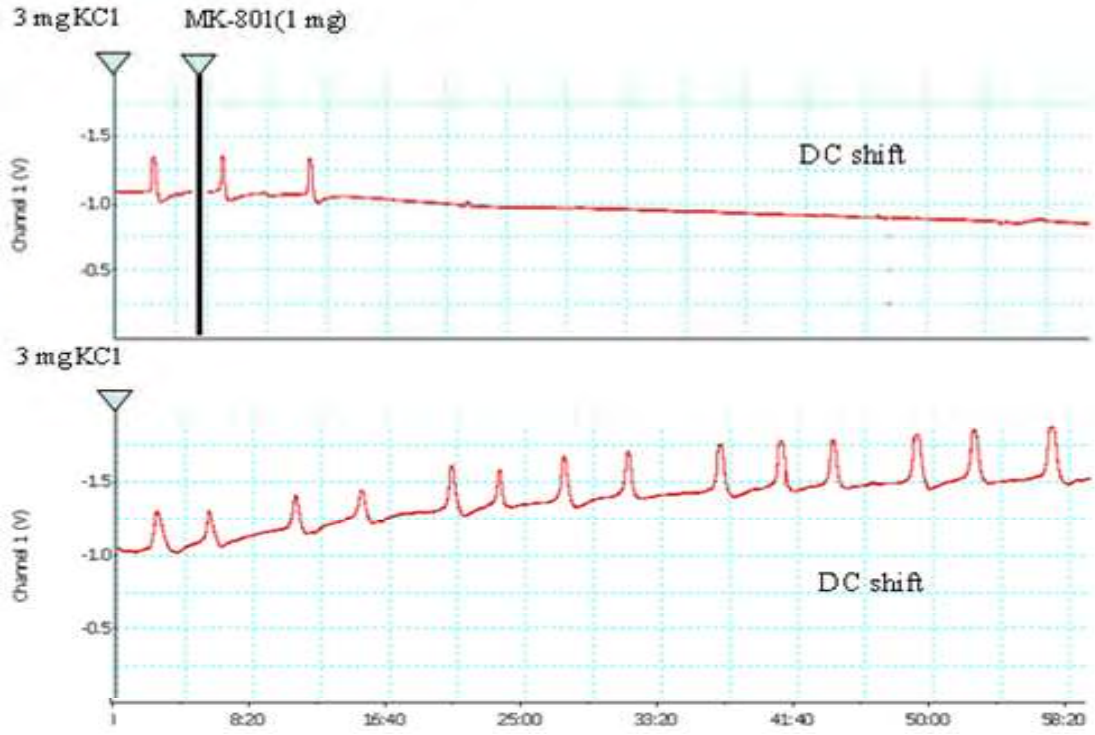

Figure3-1 The tracing showing the DC shift evoked by 3 mg KCl after MK-801 (1 mg/kg i.p.) (upper panel) and control KCl (lower panel).

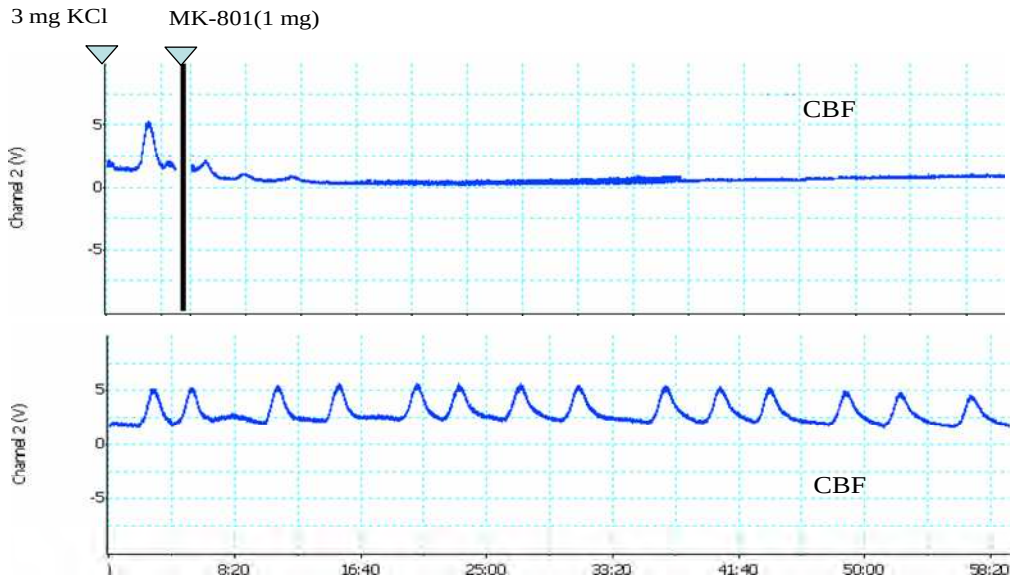

Figure3-2 The tracing showing the CBF evoked by 3 mg KCl after MK-801 (1 mg/kg i.p.) (upper panel) and control KCl (lower panel).

3. Experimental design

This study was divided into three experiments as follows:

Experiment I: The study of the effect of acute treatment with paracetamol on the trigeminovascular nociception induced by CSD

The effect of acute treatment with paracetamol on trigeminovascular nociception induced by CSD was studied by measuring the alteration of cortical neurons activity, trigeminal neurons activity and cerebral circulation.

- In order to measure the changes of cortical neurons activity, three parameters were observed as follows:
  - Depolarization shift
  - Fos expression in cerebral cortex
  - 5-HT_{2A} receptor expression in cerebral cortex
- In order to measure the changes of trigeminal neurons activity, two parameters were observed as follows:
  - Fos expression in TNC
  - 5-HT_{2A} receptor expression in TG
- In order to measure the changes of cerebral circulation, two parameters were observed as follows:
  - Cerebral blood flow
  - Ultrastructural of cerebral microvesssels

Experiment II: The study of the effect of chronic treatment with paracetamol on the trigeminovascular nociception induced by CSD

The effect of chronic treatment with paracetamol on trigeminovascular nociception induced by CSD was studied by measuring the alteration of cortical neurons activity, trigeminal neurons activity and cerebral circulation.

- In order to measure the changes of cortical neurons activity, three parameters were observed as follows:
  - Depolarization shift
  - Fos expression in cerebral cortex
  - 5-HT_{2A} receptor expression in cerebral cortex

- In order to measure the changes of trigeminal neurons activity, two parameters were observed as follows:
    - Fos expression in TNC
    - 5-HT_{2A} receptor expression in TG
- In order to measure the changes of cerebral circulation, two parameters were observed as follows:
    - Cerebral blood flow
    - Ultrastructural of cerebral microvessels

Experiment III: The study of the effect of 5-HT_{2A} receptor antagonist (ketanserin 1 and 10 mg/kg) on the trigeminovascular nociception induced by CSD in chronic treatment with paracetamol

The effect of 5-HT_{2A} receptor antagonist on trigeminovascular nociception induced by CSD in chronic treatment with paracetamol was studied by measuring the alteration of cortical neurons activity, trigeminal neurons activity and cerebral circulation.

- In order to measure the changes of cortical neurons activity, three parameters were observed as follows:
    - Depolarization shift
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    - 5-HT_{2A} receptor expression in cerebral cortex
- In order to measure the changes of trigeminal neurons activity, two parameters were observed as follows:
    - Fos expression in TNC
    - 5-HT_{2A} receptor expression in TG
- In order to measure the changes of cerebral circulation, two parameters were observed as follows:
    - Cerebral blood flow
    - Ultrastructural of cerebral microvessels

Experiment I: The study of the effect of acute treatment with paracetamol on the trigeminovascular nociception induced by CSD

The rats were divided into 2 main groups as follows:

Acute control group: The rats were injected with 12.5% of 1,2-propane-diol in 0.9% sterile saline intraperitoneally in a volume of 10 ml/kg 60 minute before KCl or NaCl application. In this group, the rats were further divided into 2 subgroups as follows:

- KCl group (n=8): CSD were induced in rat brain by topical application of 3 mg of solid KCl on the surface of parietal cortex.

- NaCl group (n=8): Solid of NaCl 3 mg were placed on the surface of rat parietal cortex instead of KCl.

Acute paracetamol treated group: The rats were injected with paracetamol 200 mg/kg BW dissolved in 12.5% of 1,2-propane-diol in 0.9% sterile saline intraperitoneally in the same 60 minute before KCl or NaCl application. In this group, the rats were further divided into 2 subgroups as follows:

- KCl group (n=8): CSD were induced in rat brain by topical application of 3 mg of solid KCl on the surface of parietal cortex.

- NaCl group (n=8): Solid of NaCl 3 mg were placed on the surface of rat parietal cortex instead of KCl.

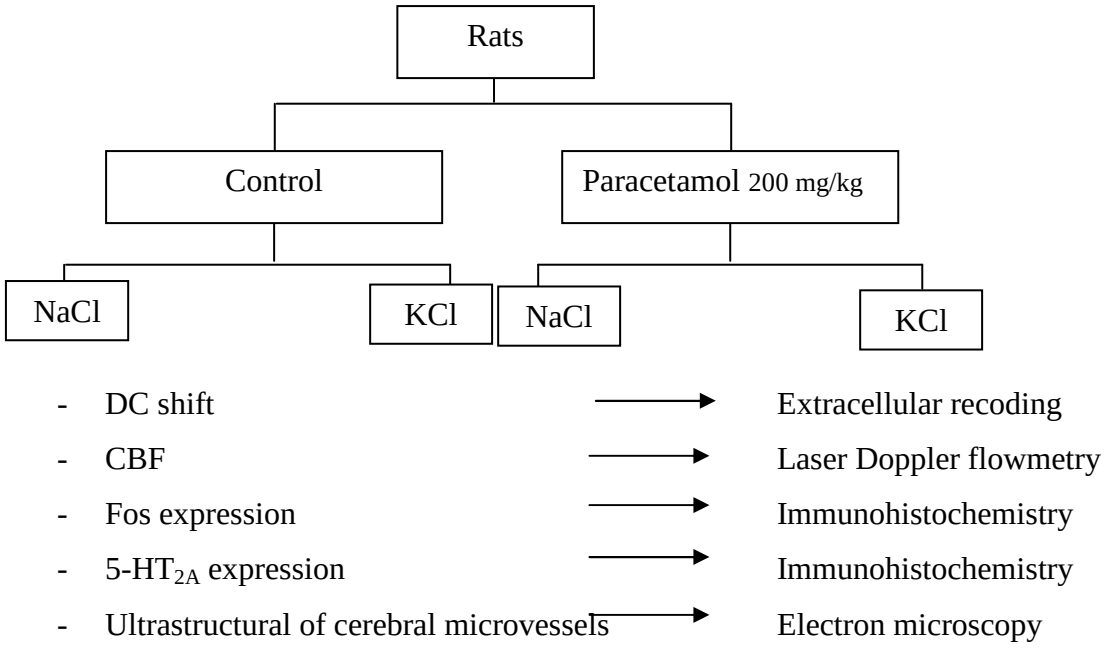


Figure3-3 Diagram of experiment I

Experiment II: The study of the effect of chronic treatment with paracetamol on the trigeminovascular nociception induced by CSD

The rats were divided into 2 main groups as follows:

Chronic control group: The rats were injected with 12.5% of 1,2-propane-diol in 0.9% sterile saline intraperitoneally in a volume of 10 ml/kg 30 days before KCl or NaCl application. In this group, the rats were further divided into 2 subgroups as follows:

- KCl group (n=8): CSD were induced in rat brain by topical application of 3 mg of solid KCl on the surface of parietal cortex.

- NaCl group (n=8): Solid of NaCl 3 mg were placed on the surface of rat parietal cortex instead of KCl.

Chronic paracetamol treated group: The rats were injected with paracetamol 200 mg/kg BW dissolved in 12.5% of 1,2-propane-diol in 0.9% sterile saline intraperitoneally in the same volume 30 days before KCl or NaCl application. In this group, the rats were further divided into 2 subgroups as follows:

- KCl group (n=8): CSD were induced in rat brain by topical application of 3 mg of solid KCl on the surface of parietal cortex.

- NaCl group (n=8): Solid of NaCl 3 mg were placed on the surface of rat parietal cortex instead of KCl.

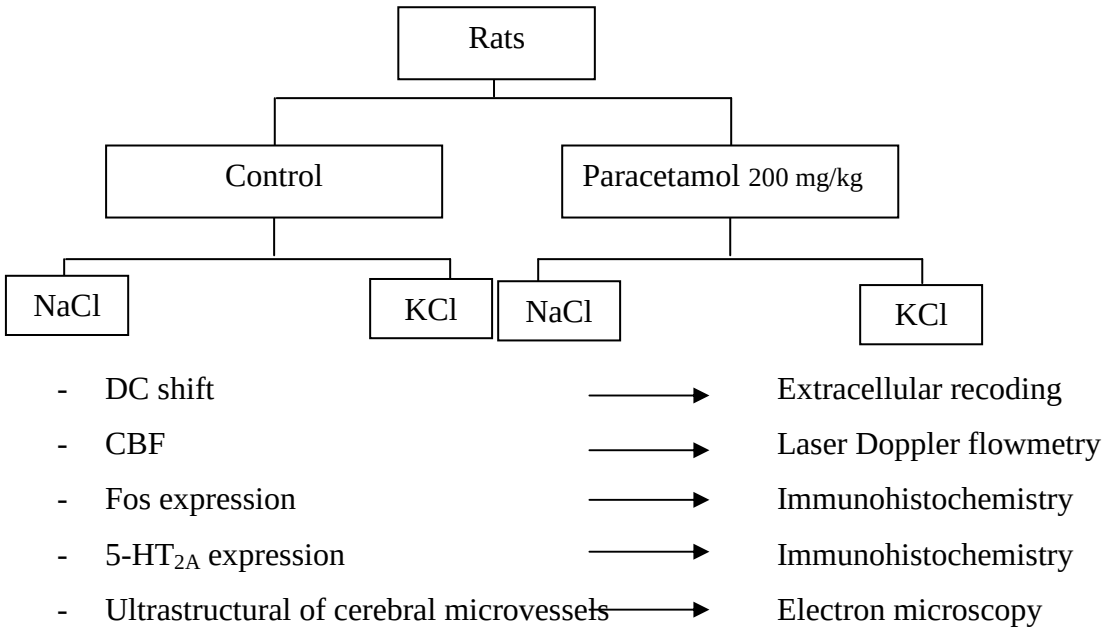


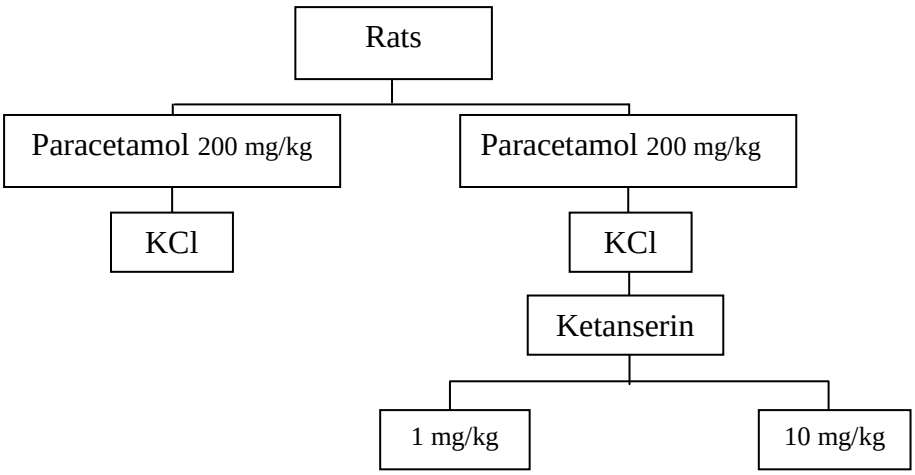
Figure3-4 Diagram of experiment II

Experiment III: The study of the effect of 5-HT_{2A} receptor antagonist (ketanserin 1 and 10 mg/kg) on the trigeminovascular nociception induced by CSD in chronic treatment with paracetamol

The rats were divided into 2 main groups as follows:

Chronic paracetamol treated group (n=8): The rats were injected with paracetamol 200 mg/kg BW dissolved in 12.5% of 1,2-propane-diol in 0.9% sterile saline intraperitoneally in a volume of 10 ml/kg 30 days before KCl application

Chronic paracetamol with ketanserin treated group: The rats were injected with paracetamol 200 mg/kg BW dissolved in 12.5% of 1,2-propane-diol in 0.9% sterile saline intraperitoneally in the same volume 30 days before KCl application. The rats in this group were further divided into 2 subgroups of 8 each rats. They were injected intraperitoneally with various dosages of ketanserin (1, 10 mg/kg body weight) 30 minutes before KCl application.



- DC shift → Extracellular recoding
- CBF → Laser Doppler flowmetry
- Fos expression → Immunohistochemistry
- 5-HT_{2A} expression → Immunohistochemistry
- Ultrastructural of cerebral microvessels → Electron microscopy

Figure3-5 Diagram of experiment III

4. Methods

4.1 Animal Preparation

The rats were anesthetized for the duration of the experiments by intraperitoneal administration of 60 mg/kg of sodium pentobarbital. Additional doses of anesthetics were given as required to maintain surgical anesthesia based on testing of corneal reflex and response to tail pinch. After tracheostomy, the ventilation was assured by using positive pressure ventilator, and the rats were mounted in a stereotaxic apparatus. Femoral artery and vein were canulated to record blood pressure and for intravenous infusion of drug, respectively. Blood pressures were monitored throughout experiments with pressure transducer, which recorded on PowerLab (PowerLab/4SP Queensland, Australia).

4.2 The cortical spreading depression model (CSD)

After tracheostomy and canulation, the rats were placed on surgical frame and its head were fixed on a stereotaxic frame. The right parietal bones were exposed by mobilization of skin either side of the midline incision. The anterior craniotomy (diameter about 7 mm.) was performed using saline-cool drill in the frontal bone at 1mm anteriorly and laterally from bregma. The posterior craniotomy (diameter about 2 mm.) were performed in the parietal bone at 7 mm posteriorly and 1 mm laterally from bregma. The anterior and posterior craniotomy openings were used for placing of the electrode and for initiation of CSD, respectively. The dura were opened by using microneedle. An artificial cerebrospinal fluid was infused in the intracranial space. Solid KCl 3 mg was applied to the parietal craniotomy opening to induce CSD. Application of KCl induced a repeated pattern of cortical depolarization characterizing the CSD.

4.3 Perfusion and tissue preparation

After two hours, the experimental rats were deeply anesthetized with sodium pentobarbital. Laparotomy and thoracotomy were done. A cannula was inserted into the apex of the heart and was advanced just distally to the aortic arch. Then, the vasculature was flushed transcardially with 250 ml PBS, followed by 250 ml of 4% paraformaldehyde in a 0.1 M phosphate buffer, pH 7.4. Brain, TG and the cervical spinal cord were removed and were immediately immersed in 4% paraformaldehyde in 0.1 M PBS for 60 minutes. Then the tissue was stored in a 30% sucrose solution in a phosphate

buffer overnight for cryoprotection. The brain, TG and cervical spinal cord (C1-C2) were cut in a horizontal plane by a cryostat microtome (Microm HM 50N) at 30 µm thickness and collected in a series of two in four sections.

5. Measurement of the DC shift

DC shift were measured by cortical electrode. Glass microelectrodes (internal diameter 5 µm) were prepared from bromosilicate, pulled with microelectrode puller (Narishige PP-88, Scientific Instrument Lab, Tokyo, Japan). Microelectrode was filled with NaCl 4 M and then an Ag/AgCl wire was inserted (Figure 3-6).

Rats were fixed to stereotaxic frame. Completely filled glass microelectrode were inserted perpendicular to cortex to depth 500 µm from cortical surface using hydraulic micromanipulator (Narishige, Scientific Instrument Lab, Tokyo, Japan). Another Ag/AgCl electrode was fixed to the back which was served as a reference point. The obtained electrical signal was amplified using microelectrode amplifier (Grass, P16B, and U.S.A). Analog data were converted to digital from using data acquisition system. All tracing were analyzed using computer software. Such waveform was continuously monitored for one hour.

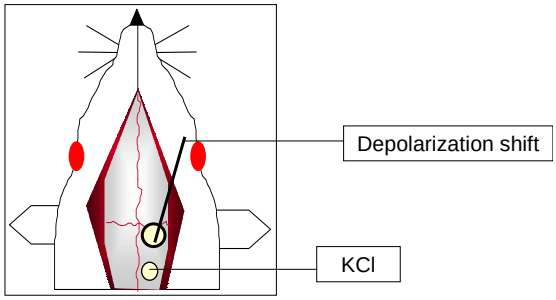


Figure3-6 Diagram of experimental animal groups: measurement of DC shift

- Data collection of DC shift

Tracings of DC shift were recorded with PowerLab for one hour after CSD induction. Measured variables included A. Number of peak (number of peak in one hour) (peak in first hour) (Figure 3-7) B. Amplitude (Maximal peak amplitude relative to baseline) (mV) C. Area under the curve (Area between the waveform and baseline) (mV.s) D. Duration (Width of the peak at the baseline) (second) (Figure 3-8).

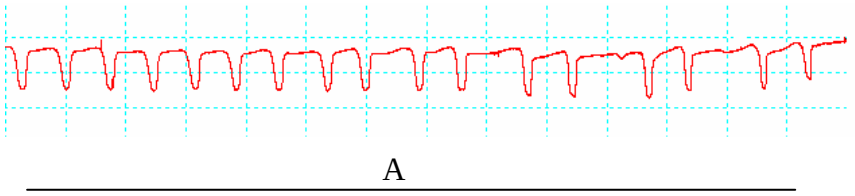


Figure3-7 Measurements of DC shift in one hour. A) Number of peak of DC shift in one hour

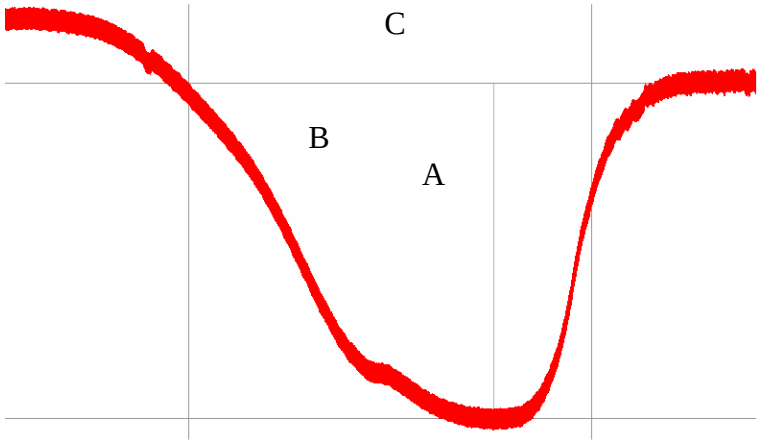


Figure3-8 Individual measurements of DC shift A) Amplitude of DC shift B) Area under the curve of DC shift C) Duration of the peak of DC shift.

6. Measurement of the CBF

CBF were measured using laser Doppler flowmetry. After tracheotomy and cannulation, the rat was placed on a surgical frame and the head was fixed on an adjustable head holder. The incision was made in the skin to expose the skull. The skin edges were retracted with retractors. The craniotomy were performed by drilling with a dental drill in the frontal bone at 1 mm anteriorly and laterally from bregma and its diameter were about 7 mm. Care was taken not to damage the dura and excess heating were prevent by cooling the drill with saline. An artificial cerebrospinal fluid was infused into the intracranial space. The fiber optic needle probe of the laser Doppler flowmeter (wavelength 780 nm) (MoorLAB, Moor Instruments Ltd., Millwey, England) were placed perpendicularly to the cortical surface via the craniotomy opening (Figure3-9). The probe was fixed 1-2 mm above the cortical surface of the brain. The results of CBF were recorded on the PowerLab/4SP.

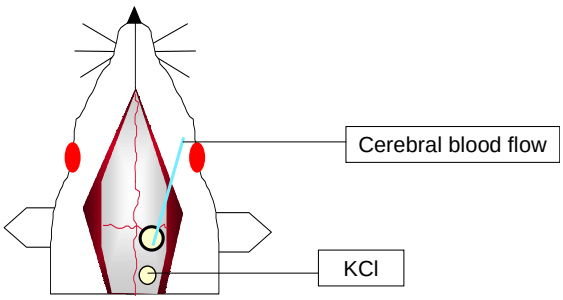


Figure3-9 Diagram of experimental animal groups: measurement of CBF

- Data collection of CBF

The CBF were measured until a steady state was reached. The CBF were continuously recorded for one hour. The results were expressed as percentage change in the CBF from the baseline value (Figure 3-11). After finishing the recording the rats were deeply anesthetized and perfuse with 4% paraformaldehyde for further immunohistochemical and ultrastructural studies.

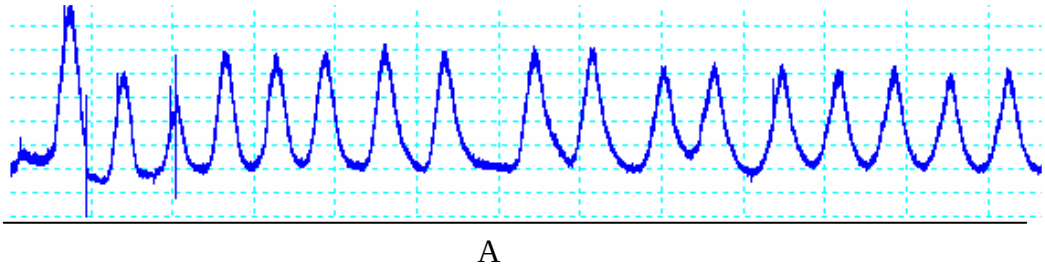


Figure3-10 Measurements of CBF in one hour. A) Number of peak hyperemia of CBF in one hour

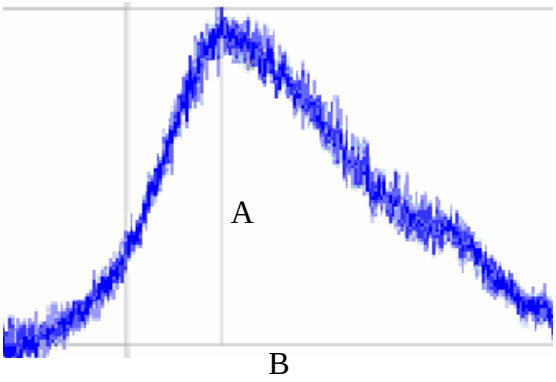


Figure3-11 Individual measurements of CBF A) Percent change from baseline B) Duration of the peak hyperemia.

7. Fos immunohistochemitry

The sections were rinsed in three changes of PBS. To block endogenous peroxidase, the sections were incubated with 50% ethanol for 30 minute and 3% hydrogen peroxide in 50% ethanol for 30 minutes. After repeated rinses in PBS, the non-specific binding of the antibody were blocked by incubating the tissues with a 3% normal horse serum (NHS), 1% bovine serum albumin (BSA) in PBS for 60 minutes at room temperature. After three rinses in PBS, the sections were incubated with rabbit anti c-fos (Santa Cruz Biotechnology) at a dilution of 1:500 in the same solution at 4 ºC for 24 hours. After overnight incubation, the sections were then rinsed in PBS, incubated for 30 minutes with biotinylated mouse anti rabbit (Dako LSAB 2 system, Denmark). This was followed by rinses in PBS and bound peroxidase was revealed by incubation of all sections in a solution containing DAB, 0.01% hydrogen peroxide for 10 minutes. The reaction was stopped by repeated rinses in PBS. Following this reaction the tissue sections were washed and mounted onto gelatinized glass slides.

- Data collection of Fos immunostaining

Cell counts were carried out in sections processed for the c-fos immunohistochemistry. A total of 10 sections per animal were used. Only cell profiles with a visible nucleus on the focal plane were analyzed by using image analysis software (Image Pro®Plus 5.1.1., Media Cybernetics Inc, USA). The c-fos immunoreactive cells (Fos-IR cells) were defined as those with a dark brown stain in their nucleus. The Fos-IR neurons in the cerebral cortex and in the lamina I and the lamina II of cervical spinal dorsal horn area of each section were counted. To determine the number of Fos-IR cells in cerebral cortex, a 250 x 250 µm square was drawn in layer III-V and Fos-IR cells confined in the square were counted. Data from ten sampling areas from each rat were averaged and was expressed as number per 6.25×10^4 μm^2. The Fos-IR cells in ipsilateral and contralateral side to KCl induction were counted separately. Expression of Fos in TNC was determined by counting Fos-IR cells in lamina I and II of TNC. The data were averaged and expressed as number of cells per slide. All counting procedures were performed manually by one of the authors (W.S.) who was blinded to the treatment groups.

8. 5-HT_{2A} Immunohistochemistry

The sections were rinsed in three changes of PBS and, incubated with 50% ethanol for 30 minutes. To block endogenous peroxidase, the sections were incubated with 3% hydrogen peroxide in 50% ethanol for 30 minutes. After repeated rinses in PBS, the non-specific binding of the antibody were blocked by incubating the tissues with a 3% normal horse serum (NHS), 1% bovine serum albumin (BSA) in PBS for 60 minutes at room temperature. After three rinses in PBS, the sections were incubated in the anti 5-HT_{2A} antiserum at a dilution of 1:200 in PBS at 4 ºC for overnight. After incubation, the sections were rinsed 3×10 min with PBS and were then incubated with biotinylated immunoglobulin at a dilution of 1:400 in PBS at 4 ºC for 24 hours. After overnight incubation, the sections were rinsed 3×10 min with PBS, and incubated for 30 minute with ABC-horseradish peroxidase complex. After incubation, the sections were then again rinsed 3×10 min in PBS. Finally, they were reacted for peroxidatic activity in a solution containing diaminobenzidine (DAB) and 0.01% hydrogen peroxide for 10 minutes. Then, tissue sections were washed 2×5 min with PBS, mounted onto gelatinized glass slides, and cover slipped the slides with permount.

- Data collection of 5-HT_{2A} immunostaining

For all experimental rats, the 5-HT_{2A}-IR cells were counted and reported as the number of immunoreactive cells in cerebral cortex and in TG ipsilateral to the KCl application and contralateral side by using image analysis software (Image Pro®Plus 5.1.1., Media Cybernetics Inc, USA). 5-HT_{2A}-IR cells were distinguished by their darkly stained cell bodies and processes. Expression of 5-HT_{2A} in cerebral cortex and in TG in each rat were determined by counting the 5-HT_{2A}-IR cells from 10 randomize selected section per animal. In TG, total 100 cells were count per section. Data were expressed as percent of 5-HT_{2A}-IR cells per rat. To determine the number of 5-HT_{2A}-IR cells in cerebral cortex, 1000 μm^2 square was drawn in layer III-V and 5-HT_{2A}-IR cells confined in the square were counted. The 5-HT_{2A}-IR cells were counted and reported as the number of immunoreactive cells in the ipsilateral to the KCl (CSD group) application and contralateral side. The 5-HT_{2A} positive cells in ipsilateral / contralateral counted separately. All counting procedures were performed manually by one of the authors (W.S.) who was blinded to the treatment groups.

9. Ultrastructural study of endothelial cell change

For transmission electron microscopic study, 1 mm^3 pieces of specimen were post-fixed in 2% osmium tetroxide for 1 hour. Next, the specimens were dehydrated in graded series of ethanol alcohol, transferred to propylene oxide and embedded in Epon 812. Semithin (500 nm) and ultrathin sections (60-90 nm) were cut with an ultramicrotome. The semithin plastic sections were stained with toluidine blue in order to select randomly the suitable area for the transmission electron microscopic study. The ultrathin sections were stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (JEOL, JEM 1210).

Morphological changes in the ultrastructure of endothelial cells of cerebral microvessels were quantified. To define possible differences between ultrastructural changes in capillaries and arterioles, the pial microvessels studies were divided into two groups on the basis of their diameter. Capillaries were defined as such if their diameter was rang between 5-10 µm. Vessels with a diameter about 15-20 µm were defined as arterioles. The morphometric parameter studied were the number of microvilli, the number of intracytoplasmic pinocytic vesicles.

- Data collection the number of microvilli

The number of endothelium microvilli was randomly counted from 5 vessels of arterioles (15-20 µm in diameter) and 10 vessels of capillaries in every experimental rat. The data were reported as the number of microvilli per vessel.

- Data collection the density of pinocytic vesicles

The density of pinocytic vesicles were investigated in both the capillaries and the arterioles. The number of pinocytic vesicles was counted from an electron micrograph taken from randomly selected capillaries and arterioles. The 0.04 μm^2 square grids were fixed to the micrographs. The number of pinocytic vesicles was counted in at least 25 squares of the random area of the endothelial cell for quantitative analysis. The density of pinocytic vesicles were expressed as the number of pinocytic vesicle per square micrometer.

10. Data analysis

All values were presented as mean ± standard deviation (SD). The differences among groups were tested using ANOVA. Significant differences between means were determined using the Bonferroni test. Non-parametric statistics were applied where appropriated. Probability values of less than 0.05 were considered to be statistically significant. All statistical analyses were performed using computer software version 16 for windows (SPSS, Chicago, IL, USA).

CHAPTER IV

RESULTS

The results of this study were divided into three experiments on these following topics:

Experiment I: The study of the effect of acute treatment with paracetamol on the trigeminovascular nociception induced by CSD

The effect of acute treatment with paracetamol on trigeminovascular nociception induced by CSD was studied by measuring the alteration of cortical neurons activity, trigeminal neurons activity and cerebral circulation.

- In order to measure the changes of cortical neurons activity, three parameters were observed as follows:
  - Depolarization shift
  - Fos expression in cerebral cortex
  - 5-HT_{2A} receptor expression in cerebral cortex
- In order to measure the changes of trigeminal neurons activity, two parameters were observed as follows:
  - Fos expression in TNC
  - 5-HT_{2A} receptor expression in TG
- In order to measure the changes of cerebral circulation, two parameters were observed as follows:
  - Cerebral blood flow
  - Ultrastructural of cerebral microvessels

Experiment II: The study of the effect of chronic treatment with paracetamol on the trigeminovascular nociception induced by CSD

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- In order to measure the changes of cerebral circulation, two parameters were observed as follows:
  - Cerebral blood flow
  - Ultrastructural of cerebral microvessels

Experiment III: The study of the effect of 5-HT_{2A} receptor antagonist (ketanserin 1 and 10 mg/kg) on the trigeminovascular nociception induced by CSD in chronic treatment with paracetamol

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  - Cerebral blood flow
  - Ultrastructural of cerebral microvessels

Experiment I: The study of the effect of acute treatment with paracetamol on the trigeminovascular nociception induced by CSD

In the rat with acute treatment with paracetamol, body weight and arterial blood pressure were not statistically different from control group. The data are shown in Table 4-1. The body weight in acute control and acute paracetamol treated rat were 281± 10.69 grams and 291±9.27 grams, respectively. The arterial blood pressure in acute control and acute paracetamol treated rat were 109.12±11.82 mmHg and 107.96±10.11 mmHg, respectively.

Table4-1 The mean value ± SD of body weight and arterial blood pressure of rats obtained from acute control and acute paracetamol treated group.

Group	Acute control	Acute paracetamol	p-value
Body weight (g)	281±10.69	291±9.27	0.88
Blood pressure (mmHg)	109.12±11.82	107.96±10.11	0.38

- The effect of acute treatment with paracetamol on cortical neurons activity induced by CSD

- The effect of acute treatment with paracetamol on depolarization shift

In order to evaluate the effect of acute control and acute treatment with paracetamol on development of CSD variable including number of peak, peak amplitude, duration, and area under the curve (AUC) were studied.

Application of KCl induced a repeated pattern of cortical depolarization characterizing the CSD (Figure4-1). In acute control group, duration of these CSD waves and mean number of CSD wave occurring within one hour were 41.08±8.46 seconds and 13.14±1.2 waves, respectively (Table 4-2). The duration and number of peak were 44.07±9.57 second and 13.75±1.50 waves, respectively. No significant difference was observed when duration of CSD waves and averaged number of CSD wave were compared with the acute control group. The averaged AUC and the peak amplitude were 73.40±18.58 mV-seconds and 28.10± 3.31 mV, respectively (Table 4-2). Comparing with the acute paracetamol-treated group, we found that the averaged AUC of CSD waves and peak amplitude were increased significantly (93.84±24.72 mV-seconds and 31.47±2.33 mV). The DC shift was not changes when the NaCl topical application in all group (Figure4-1).

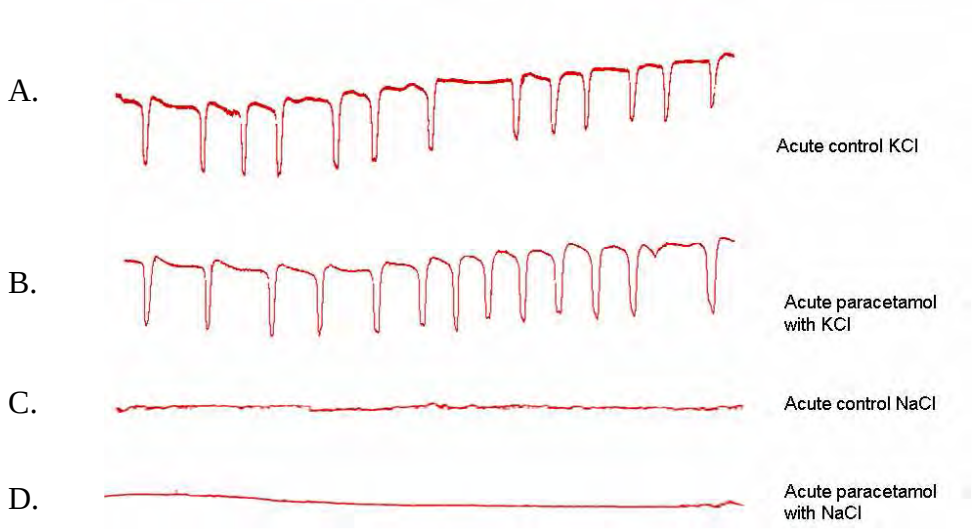

Figure4-1 The tracing showing the DC shift obtained from A. acute control with KCl application B. acute paracetamol with KCl application C. acute control with NaCl application D. acute paracetamol with NaCl application.

Table4-2 Comparing the electrophysiology variables related to CSD between acute control and acute paracetamol treated groups.

Measure variables	Acute control	Acute paracetamol	p-value
Number of peak (peak per hour)	13.00 ± 1.15	13.75 ± 1.50	0.332
AUC (mV.s)	73.40 ± 18.58	93.84 ± 24.72	0.006
Amplitude (mV)	28.10 ± 3.31	31.47± 2.33	0.001
Duration (s)	41.08 ± 8.46	44.07 ± 9.57	0.362

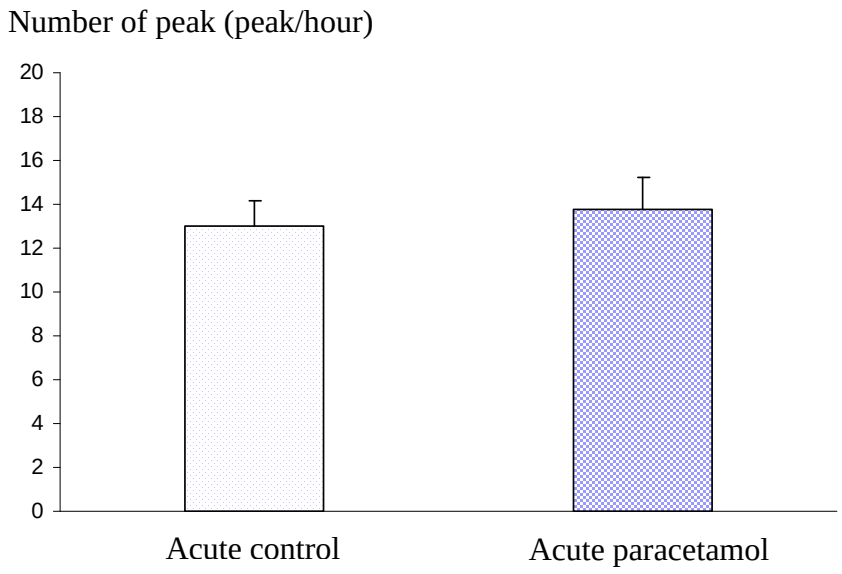

Figure4-2 Bar graphs showing the mean value ± SD of number of peak of DC shift obtained from acute control and acute paracetamol treated group.

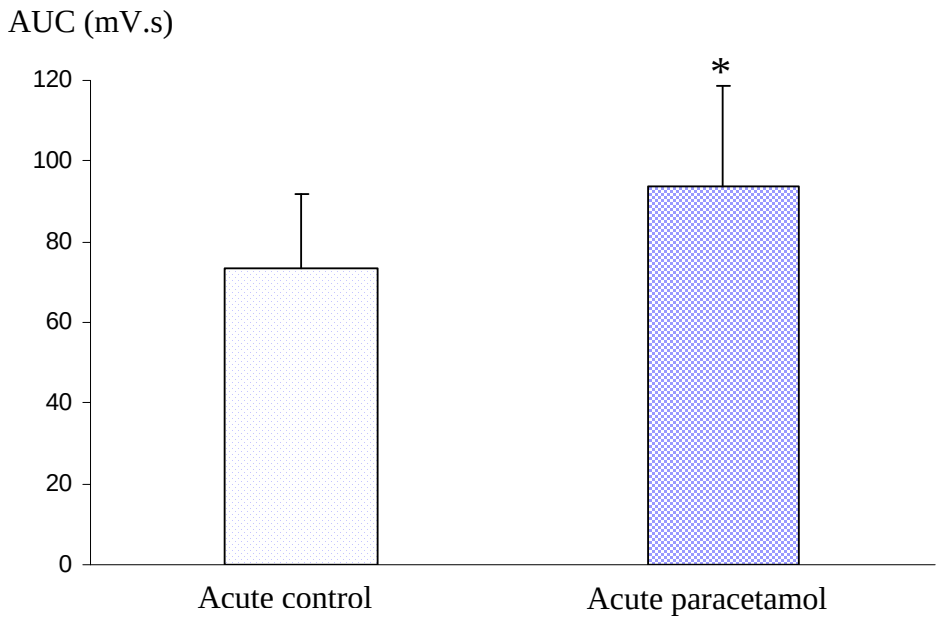

Figure4-3 Bar graphs showing the mean value ± SD of area under the curve of DC shift obtained from acute control and acute paracetamol treated group. Significant difference was assessed with Student t-test. * p< 0.05 compared with acute control group.

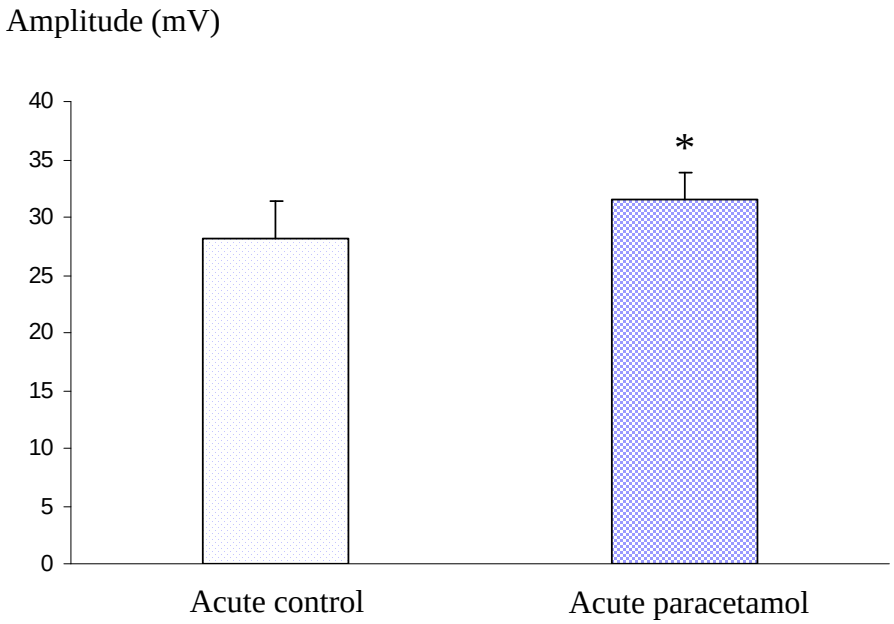

Figure4-4 Bar graphs showing the mean value ± SD of amplitude of DC shift obtained from acute control and acute paracetamol treated group. Significant difference was assessed with Student t-test. * p< 0.05 compared with acute control group.

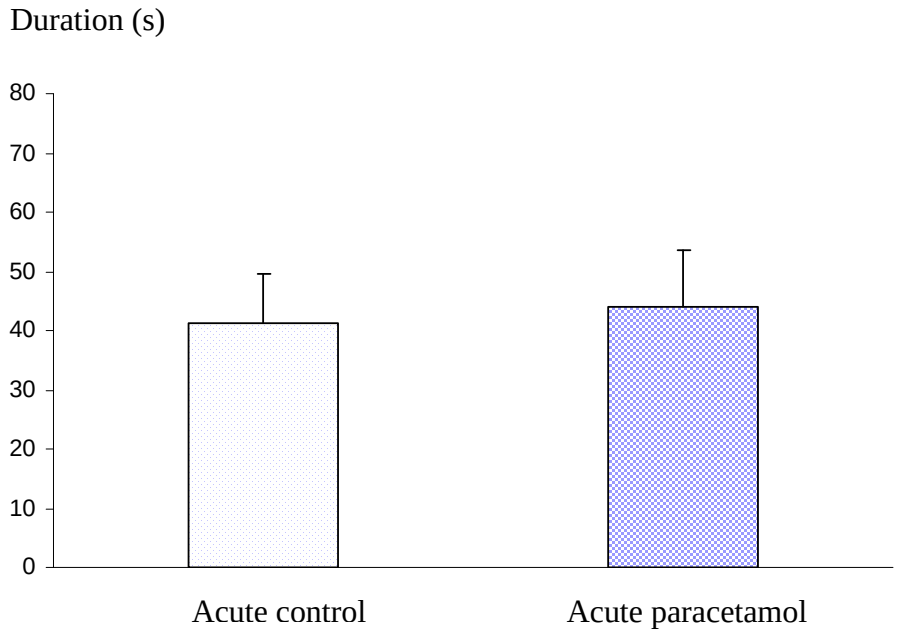

Figure4-5 Bar graphs showing the mean value ± SD of duration of DC shift obtained from acute control and acute paracetamol treated group.

- The effect of acute treatment with paracetamol on Fos expression in cerebral cortex

In order to evaluate the effect of acute treatment with paracetamol on the activation of neuron in cerebral cortex, the fos expression was measured in cerebral cortex obtained from control and paracetamol treated groups.

Our immunohistochemical study showed that the induction of CSD evoked Fos expression in cerebral cortex in both group. Fos-ir cells were distributed mainly in the ipsilateral cerebral cortex, primarily in layer I, II, III and IV. They appeared as dark, round or ovoid structures with variable staining intensity. Only few Fos-ir cells were observed on the contralateral side to KCl application. Acute treatment with paracetamol substantially reduced the number of CSD-induced Fos expression in layer III and IV ipsilateral side of the cerebral cortex compared with acute control group (Figure4-6). Fos-ir cells in acute control and acute paracetamol treated rat were 75.08$\pm$18.70 cells per 6.25 x 10^4 μm^2 and 54.20$\pm$16.10 cells per 6.25 x 10^4 μm^2, respectively (Table4-3). Statistical analysis demonstrated significantly difference between two groups. Fos-ir cells were also presented bilateral in cerebral cortex to NaCl application but less than KCl application (10.32$\pm$4.53 cells per 6.25 x 10^4 μm^2 and 8.15$\pm$3.33 cells per 6.25 x 10^4 μm^2, in acute control with NaCl and acute paracetamol with NaCl treated groups, respectively) (Figure4-6 and Table4-3).

Table4-3 The mean value $\pm$ SD of the number of Fos-ir cells in cerebral cortex sections obtained from acute control with KCl application, acute paracetamol with KCl application, acute control with NaCl application and acute paracetamol with NaCl application.

	Acute control		Acute paracetamol	
	KCl	NaCl	KCl	NaCl
Ipsilateral side	75.08$\pm$18.70*	10.32$\pm$4.53	54.20$\pm$16.10*#	8.15$\pm$3.33

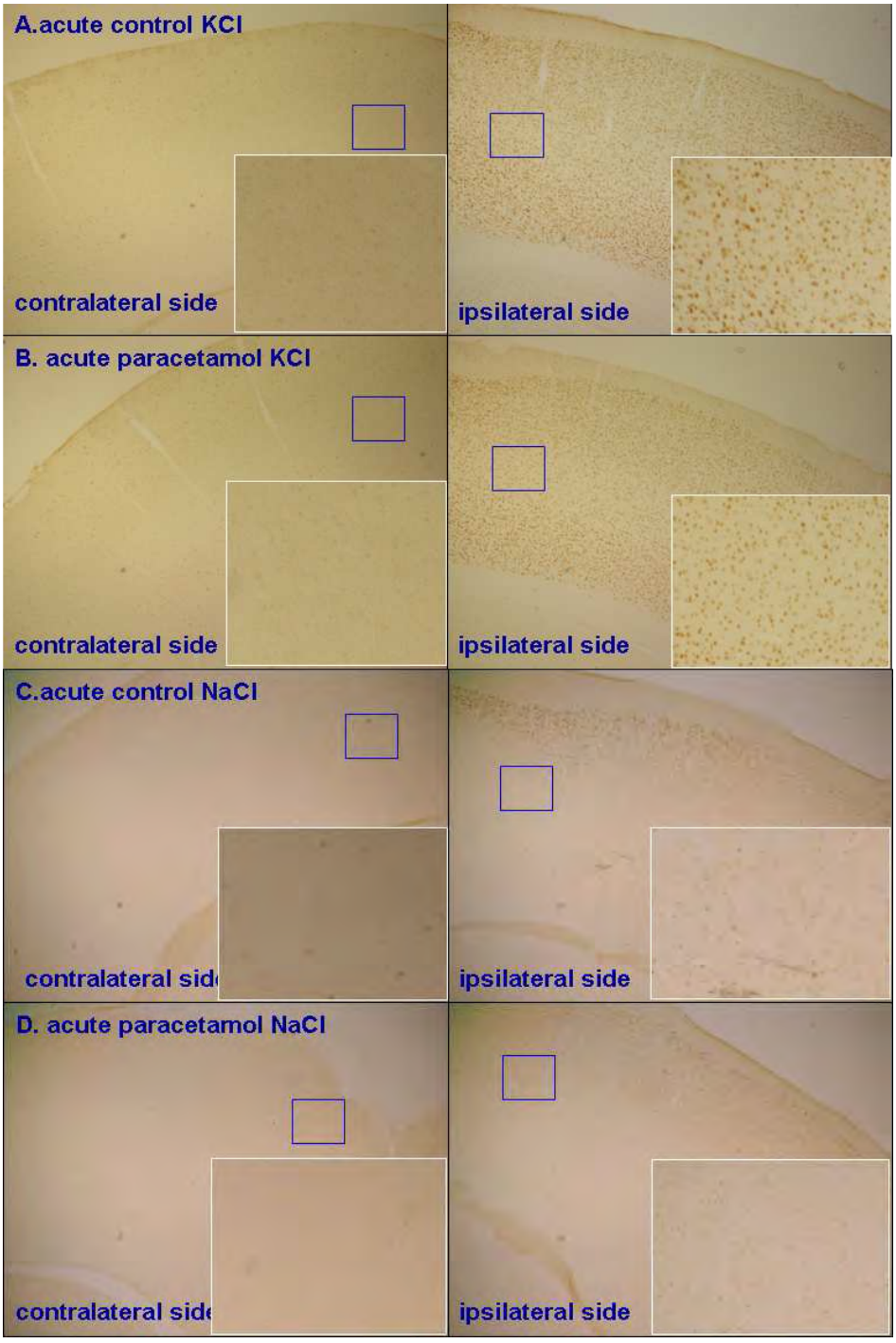

Figure4-6 The photomicrograph showing the Fos-ir cells in the brain sections in ipsilateral side and contralateral side to KCl and NaCl application obtained from A. acute control with KCl application B. acute paracetamol with KCl application C. acute control with NaCl application D. acute paracetamol with NaCl application. Fos positive cells are easily visible and well differentiated from the background.

- The effect of acute treatment with paracetamol on 5-HT_{2A} receptor expression in cerebral cortex

In this experiment, 10 sections of brain were collected from each rat. The sections were studied under the light microscope and neurons were classified as immunoreactive or non-reactive based on the immunostaining feature. The 5-HT_{2A}-ir cells were defined as those with dark-brown stained in their cytoplasm. We found that the CSD induced an expression of 5-HT_{2A}-ir cells in the cortical layers III-VI of the cerebral cortex was the same pattern in all experimental groups. CSD induced expression of 5-HT_{2A} in the cerebral cortex. 5-HT_{2A}-ir cells were confined in cerebral cortex and were more prevalent on the side ipsilateral to the operation (Figure4-7). In acute control group, the numbers of 5-HT_{2A}-ir cells in the ipsilateral were 11$\pm$2.65 cells per μm^2. The numbers of 5-HT_{2A}-ir cells in acute paracetamol-treated group was 13$\pm$1.73 cells per μm^2 for ipsilateral side. The data are shown in Table4-4. The difference in the number of 5-HT_{2A}-ir cells between acute paracetamol treated group and acute control groups were not statistically significant.

In acute control NaCl group, the numbers of 5-HT_{2A}-ir cells in the ipsilateral sides was 5.67$\pm$1.53 cells per μm^2. The numbers of 5-HT_{2A}-ir cells in acute paracetamol-treated group was 5.33$\pm$1.53 cells per μm^2 for ipsilateral side. The data are shown in Table 4-4. The difference in the number of 5-HT_{2A}-ir cells between acute paracetamol treated group and acute control groups were not statistically significant (Table4-4).

Table4-4 The mean value $\pm$ SD of the number of 5-HT_{2A}-ir cells in cerebral cortex sections obtained from acute control with KCl application, acute paracetamol with KCl application, acute control with NaCl application, and acute paracetamol with NaCl application

	Acute control		Acute paracetamol	
	KCl	NaCl	KCl	NaCl
Ipsilateral	11$\pm$2.65*	5.67$\pm$1.53	13$\pm$1.73*	5.33$\pm$1.53

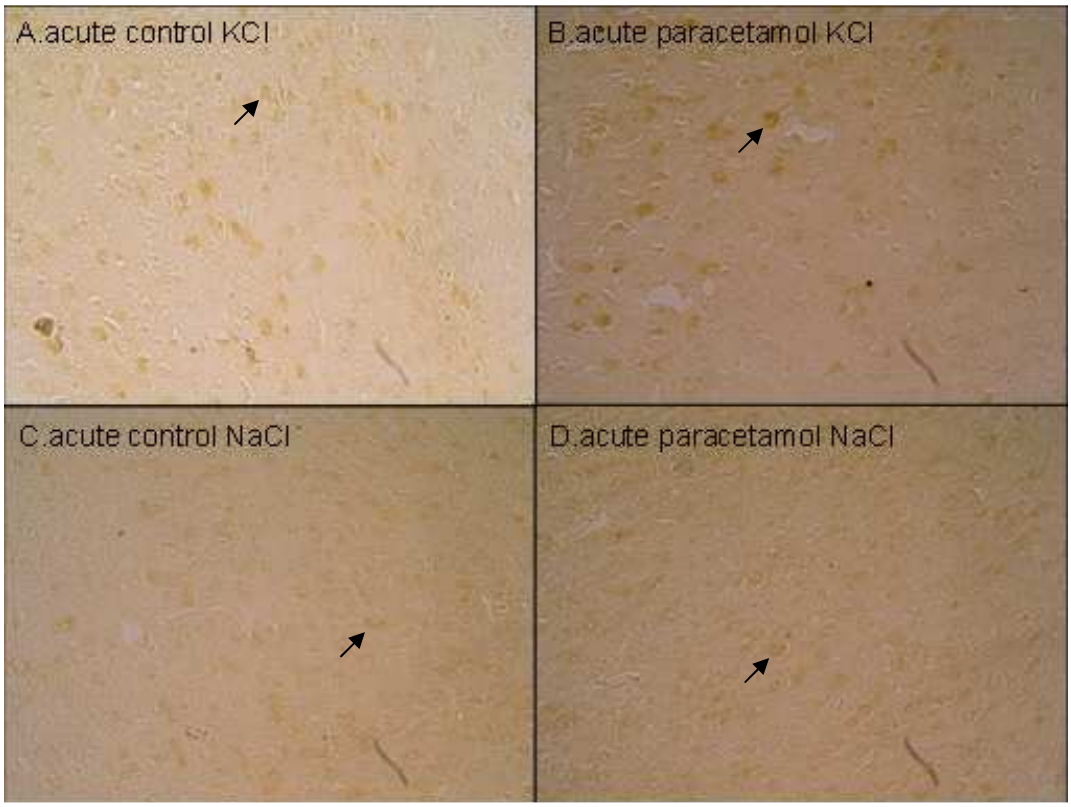

Figure4-7 The photomicrograph showing the 5-HT_{2A}-ir cells (arrow) in the brain sections ipsilateral side to KCl and NaCl application obtained from A. acute control with KCl application B. acute paracetamol with KCl application C. acute control with NaCl application D. acute paracetamol with NaCl application.

- Effect of acute treatment with paracetamol on trigeminal neurons activity induced by CSD

- The effect of acute treatment with paracetamol on Fos expression in TNC

After induction of CSD for two hours, the Fos expression was mainly observed in the lamina I and II of the cervical spinal cord sections on the side ipsilateral to the KCl application. In acute control KCl group, the numbers of Fos-ir cells in ipsilateral and contralateral sides were 10.51±2.26 cells per slide and 4.93±2.10 cells per slide, respectively. Pretreatment with acute paracetamol decreased the response of trigeminal nociception to CSD. The numbers of Fos-ir cells in paracetamol-treated group were 5.00±2.36 cells per slide and 2.41±1.05 cells per slide for ipsilateral and contralateral sides, respectively. The data are shown in Table4-5. The difference in the number of Fos-ir cells between acute control and acute paracetamol treated groups were statistically significant (Figure4-8, Figure4-9 and Table 4-5).

In acute control NaCl group, the numbers of Fos-ir cells in ipsilateral and contralateral sides were 5.10±2.99 cells per slide and 2.95±1.98 cells per slide, respectively. Pretreatment with acute paracetamol did not change the response of trigeminal nociception to NaCl application. The numbers of Fos-ir cells in acute paracetamol-treated group were 4.84±3.16 cells per slide and 2.21±1.05 cells per slide for ipsilateral and contralateral sides, respectively. The data are shown in Table4-5. The difference in the number of Fos-ir cells between acute control and acute paracetamol treated groups were not statistically different (Figure4-8, Figure4-9 and Table 4-5).

Table4-5 The mean value ± SD of the number of Fos-ir cells in the C1 and C2 cervical spinal cord sections obtained from acute control with KCl application, acute paracetamol with KCl application, acute control with NaCl application, and acute paracetamol with NaCl application

	Acute control		Acute paracetamol	
	KCl	NaCl	KCl	NaCl
Ipsilateral	10.51±2.26*	5.10±2.99	5.00±2.36#	4.84±3.16
Contralateral	4.93±2.10	2.95±1.98	2.41±1.05	2.21±1.05

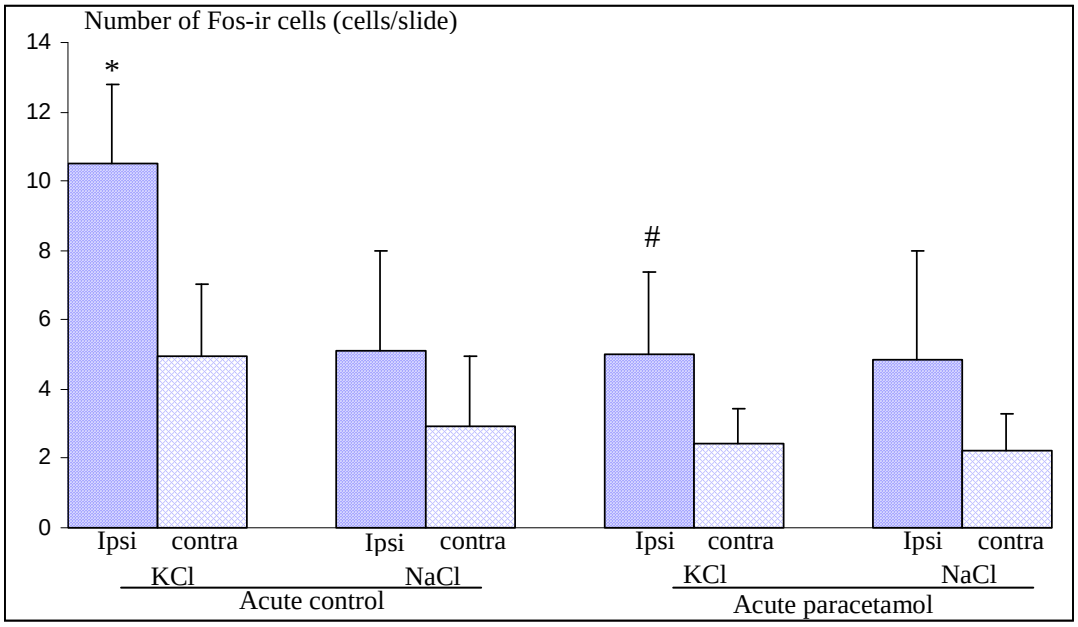

Figure4-8 Bar graph showing the mean value ± SD of the number of Fos-ir cells in the C1 and C2 cervical spinal cord sections obtained form acute control with KCl application, acute paracetamol with KCl application, acute control with NaCl application, and acute paracetamol with NaCl application. Significant difference was assesses with ANOVA with the Bonferroni test. *p<0.05 compared with acute control KCl group. #p<0.05 compared with contralateral side.

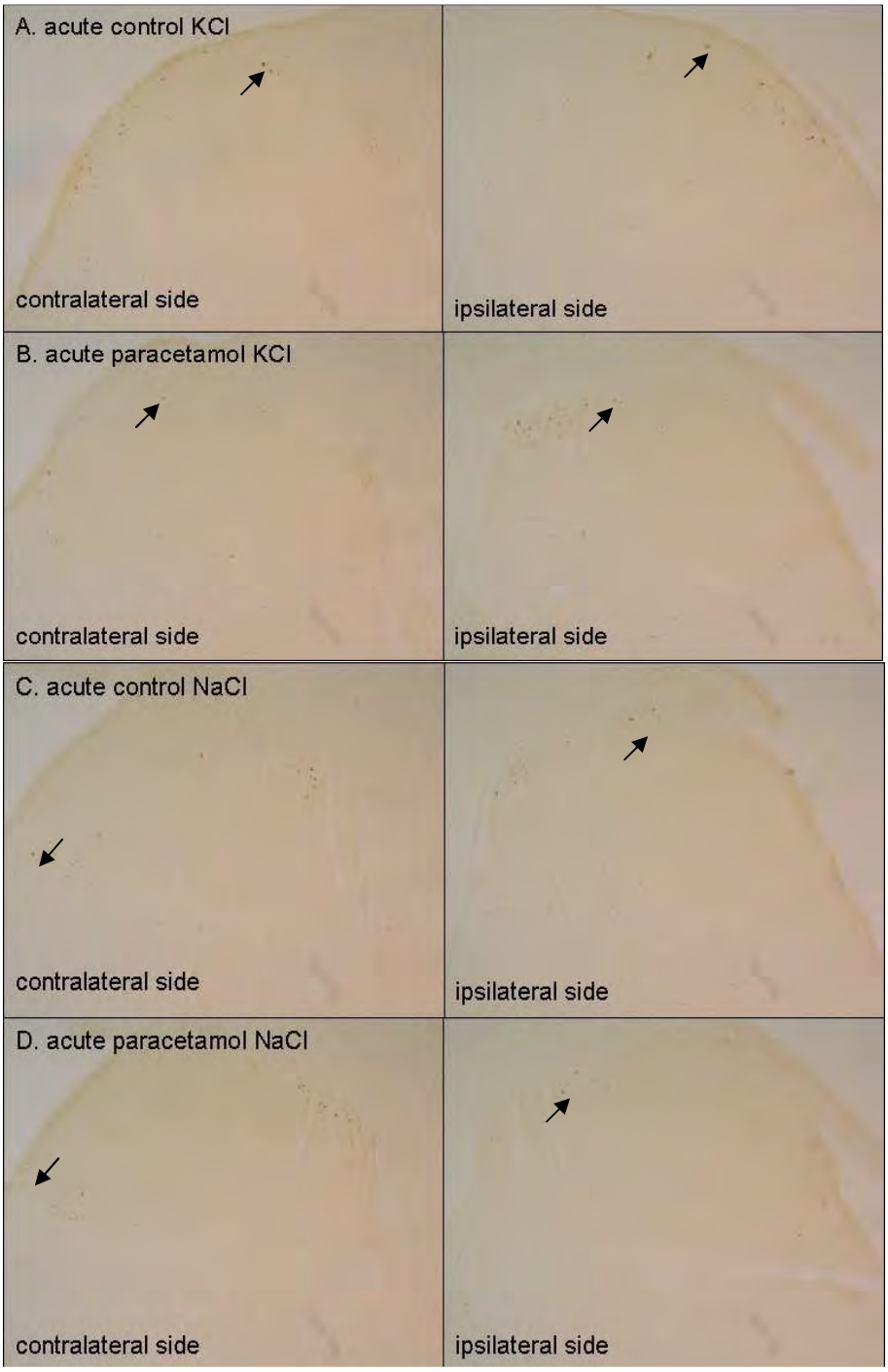

Figure4-9 The photomicrograph showing the Fos-ir cells (arrow) in the C1-C2 cervical spinal cord sections ipsilateral side and contralateral side to KCl and NaCl application obtained from A. acute control with KCl application B. acute paracetamol with KCl application C. acute control with NaCl application D. acute paracetamol with NaCl application. Fos positive cell are easily visible and well differentiated from the background.

- The effect of acute treatment paracetamol on 5-HT_{2A} receptor expression in TG

In this experiment, 10 sections of TG were collected from each rat. The sections were studied under the light microscope and neurons were classified as immunoreactive or non-reactive based on the immunostaining feature. The 5-HT_{2A}-ir cells were defined as those with dark-brown stained in their cytoplasm. It was shown that the 5-HT_{2A}-ir cells comprised small to medium sized neurons. The large-diameter neurons were usually 5-HT_{2A}-negative. The total of 100 cells was counted from each slide.

CSD induced expression of 5-HT_{2A} in the TG. 5-HT_{2A}-ir cells were confined in TG and were more prevalent on the side ipsilateral to the operation (Figure4-10). In acute control group, the numbers of 5-HT_{2A}-ir cells in the ipsilateral and contralateral sides were 28.5$\pm$13.3 cells per slide and 25.7$\pm$10.4 cells per slide, respectively. The numbers of 5-HT_{2A}-ir cells in acute paracetamol-treated group were 29.3$\pm$9.4 cells per slide and 21.2$\pm$13.7 cells per slide for ipsilateral and contralateral sides, respectively. The data are shown in Table4-6. The difference in the number of 5-HT_{2A}-ir cells between acute paracetamol treated group and acute control groups were not statistically significant.

In acute control NaCl group, the numbers of 5-HT_{2A}-ir cells in the ipsilateral and contralateral sides were 23.8$\pm$12.3 cells per slide and 21.2$\pm$13.7 cells per slide, respectively. Pretreatment with acute paracetamol did not inhibit the response of trigeminal nociceptive to NaCl application. The numbers of 5-HT_{2A}-ir cells in acute paracetamol-treated group were 21.4$\pm$11.2 cells per slide and 20.1$\pm$10.3 cells per slide for ipsilateral and contralateral sides, respectively. The data are shown in Table 4-6. The difference in the number of 5-HT_{2A}-ir cells between acute paracetamol treated group and acute control groups were not statistically significant (Table4-6).

Table4-6 The mean value ± SD of the number of 5-HT_{2A}-ir cells in the TG sections obtained from acute control with KCl application, acute paracetamol with KCl application, acute control with NaCl application, and acute paracetamol with NaCl application.

	Acute control		Acute paracetamol	
	KCl	NaCl	KCl	NaCl
Ipsilateral	28.5±13.3	23.8±12.3	29.3±9.4	21.4±11.2
Contralateral	25.7±10.4	21.2±13.7	26.4±12.2	20.1±10.3

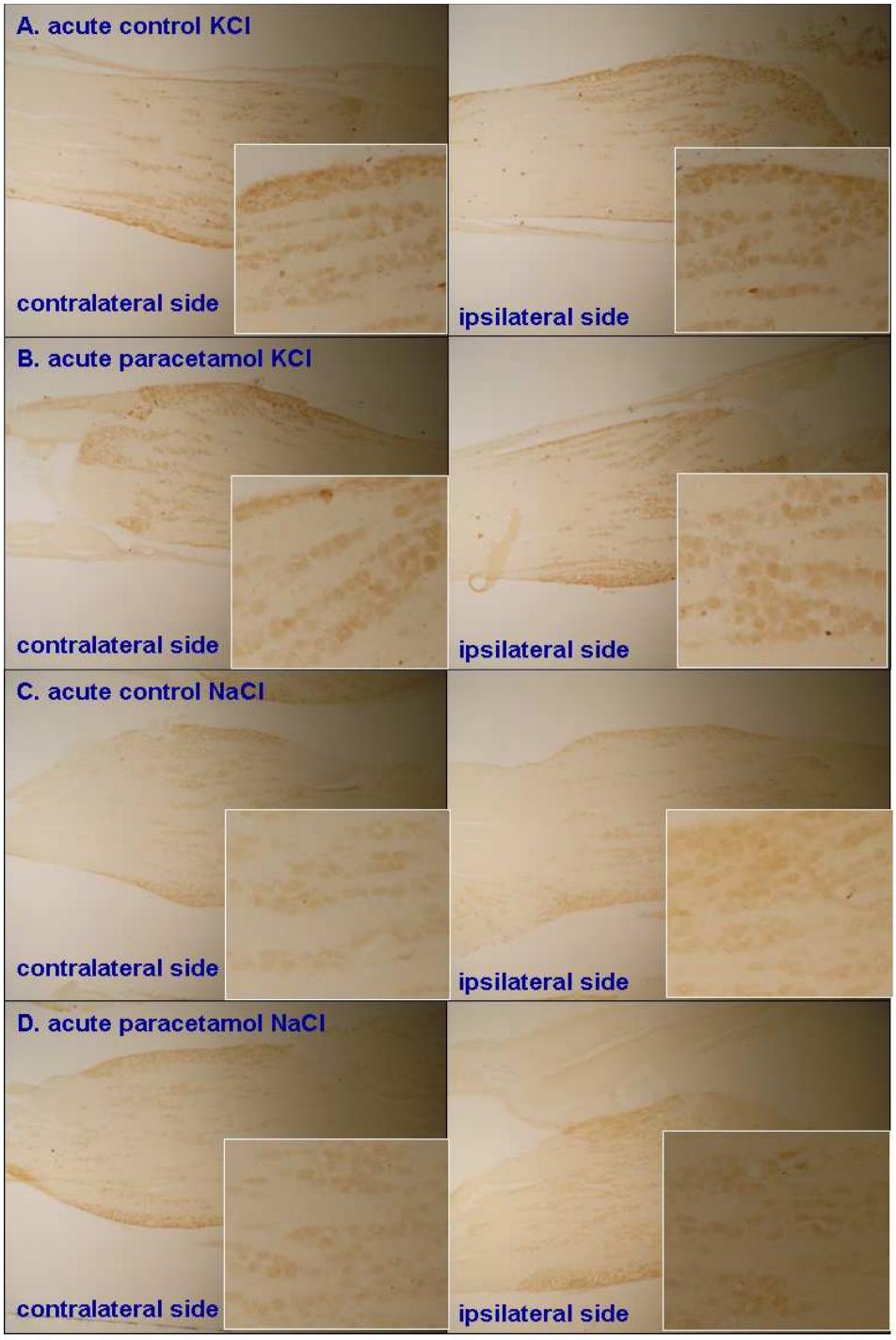

Figure4-10 The photomicrograph showing the 5-HT_{2A}-ir cells in the TG sections ipsilateral side and contralateral side to KCl and NaCl application obtained from A. acute control with KCl application B. acute paracetamol with KCl application C. acute control with NaCl application D. acute paracetamol with NaCl application

- Effect of acute treatment with paracetamol on cerebral circulation induced by CSD

- The effect of acute treatment with paracetamol on cortical blood flow

Application of KCl induced the repeated pattern of cerebral hyperemia. In acute control group, the first hyperemia peak developed within 2.16±0.32 minutes and 2.51±0.69 minutes in acute paracetamol treated groups after KCl application.

The amplitude from each peak was calculated as percent changes from the resting stage. The percent changes from base line were 222.74 ± 62.77 and 185.77 ± 37.23 in acute control and acute paracetamol treated group, respectively. Statistical analysis showed significant difference between acute paracetamol and acute control groups (Table4-7). The average number of hyperemia cycles within one hour were 13.25 ± 0.95 and 14.00 ± 1.73 peak per hour in acute control and acute paracetamol treated groups, respectively. The average duration of these cycles were 136.65 ± 33.50 and 135.53 ± 21.88 second in acute control and acute paracetamol treated groups, respectively. Statistical analysis showed no significant difference between these two groups (Table 4-7). There were no changes of CBF in the NaCl application group (Figure 4-11).

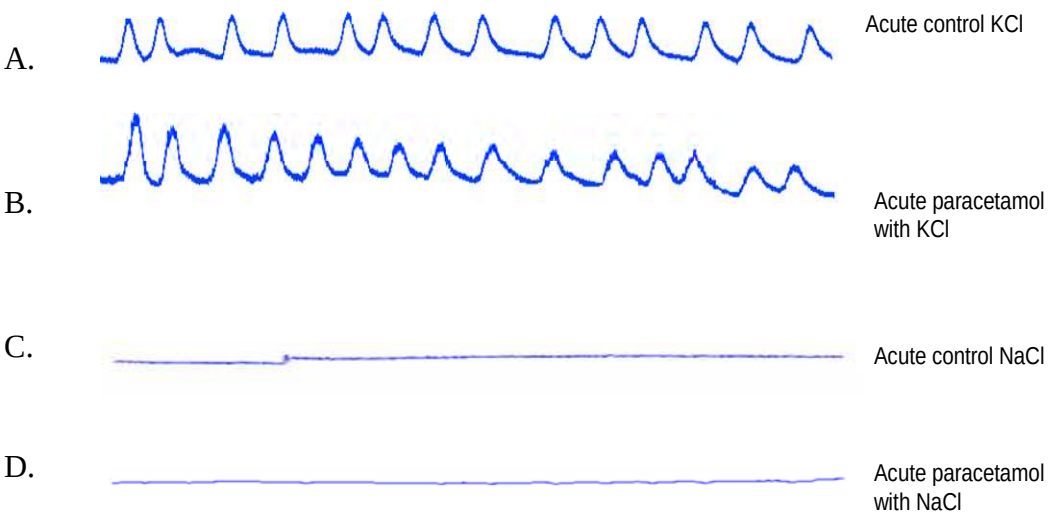

Figure4-11 The tracing showing the CBF changes obtained from A. acute control with KCl application B. acute paracetamol with KCl application C. acute control with NaCl application D. acute paracetamol with NaCl application

Table4-7 The average number of peak hyperemia, percent changes from base line and duration of CBF between acute control and acute paracetamol treated groups.

	Acute control	Acute paracetamol	p-value
Number of peak (peak per hour)	13.25 ± 0.95	14.00 ± 1.73	0.67
%Changes	222.74 ± 62.77	185.77 ± 37.23	0.001
Duration (s)	136.65 ± 33.50	135.53 ± 21.88	0.92

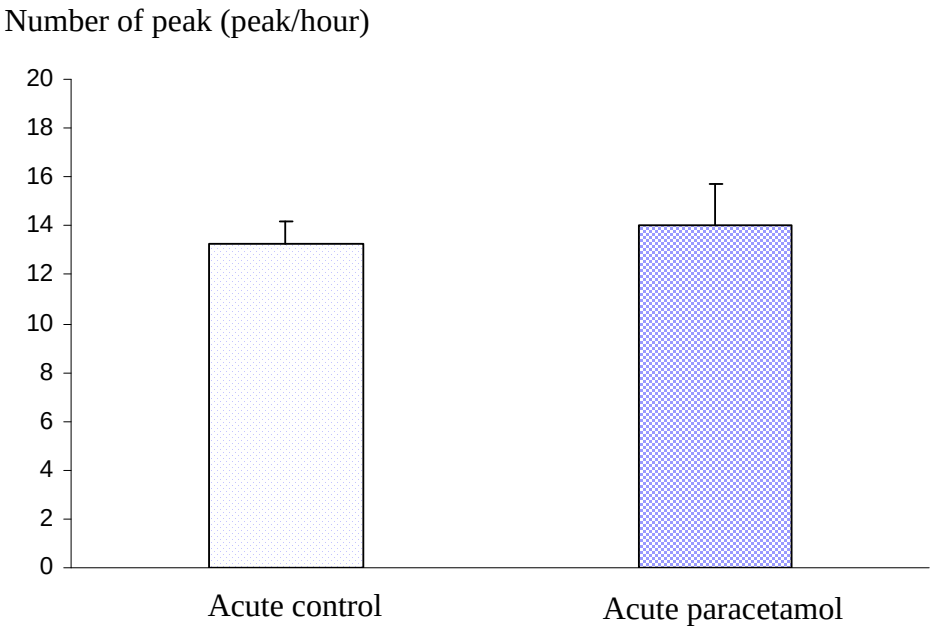

Figure4-12 Bar graphs showing the mean value ± SD of number of peak of CBF obtained from acute control and acute paracetamol treated group.

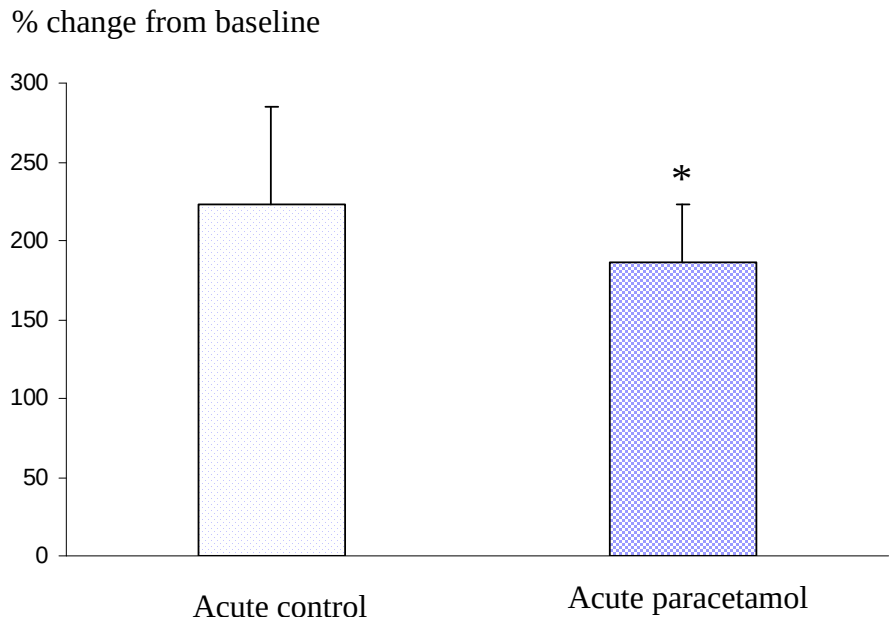

Figure4-13 Bar graphs showing the mean value ± SD of percent change from baseline of CBF obtained from acute control and acute paracetamol treated group. Significant difference was assessed with Student t-test. * p< 0.05 compared with acute control group.

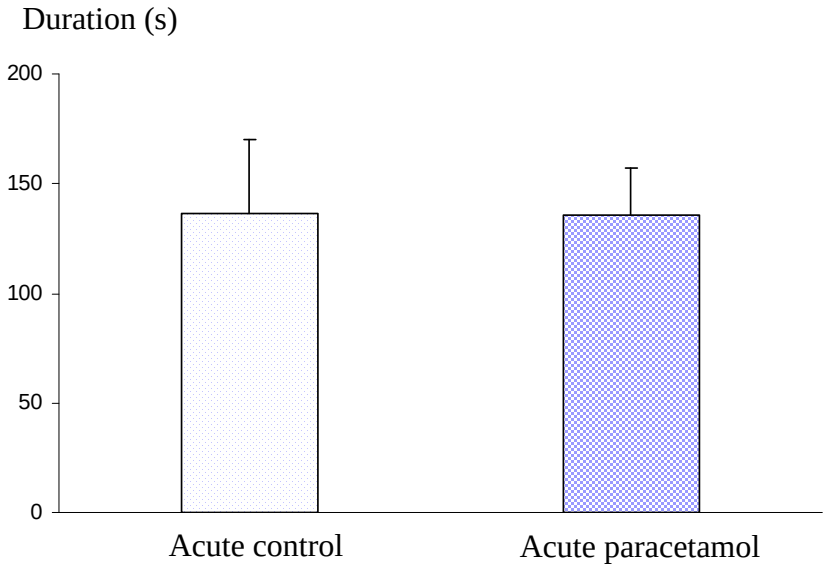

Figure4-14 Bar graphs showing the mean value ± SD of the duration of CBF obtained from acute control and acute paracetamol treated group.

- The effect of acute treatment with paracetamol on ultrastructural of cerebral microvessels

Analysis of 50 randomly selected capillaries and 25 arterioles obtained from the acute control and acute paracetamol treated group showed mean values of microvilli per vessel from capillary and arteriole, respectively. The average density of pinocytic vesicle was demonstrated in vesicles per μm^2 for capillary and arteriole, respectively.

The number of microvilli in capillaries and arteriole were 1.38±0.59 and 15.13±6.98 microvilli per vessel in the acute control group and 1.68±0.86 and 14.13±9.13 microvilli per vessel in the acute paracetamol treated group, respectively. However, the number of microvilli in capillary and arteriole obtained from the acute control and acute paracetamol treated group was not significant different. The data are shown in Table4-8 and Figure4-15.

The average density of pinocytic vesicles in capillaries and arterioles were 33±15 and 43±10 vesicle per μm^2 in the acute control group and 28±11 and 41±13 vesicle per μm^2 in acute paracetamol treated group, respectively. The difference in the number of pinocytic vesicles between acute paracetamol treated group and acute control groups were not statistically different. The data are shown in Table4-8 and Figure4-15.

- The effect of acute treatment with paracetamol on ultrastructural of endothelial cell change evoked by NaCl application

The number of microvilli in capillaries and arteriole were 1.20±0.37 and 11.31±4.62 microvilli per vessel in the acute control group and 1.98±1.10 and 10.25±4.11 microvilli per vessel in the acute paracetamol treated group, respectively. However the number of microvilli in capillary and arteriole obtained from the acute control and acute paracetamol treated group was not significant different. The data are shown in Table4-8 and Figure4-16.

The average density of pinocytic vesicles in capillaries and arterioles were 16±7 and 18±7 vesicle per μm^2 in the acute control group and 12±6 and 22±12 vesicle per μm^2 in acute paracetamol treated group, respectively. The difference in the number of pinocytic vesicles between acute paracetamol treated group and acute control groups were statistically significant. The data are shown in Table 4-8 and Figure 4-16.

Table4-8 The mean value ± SD of the number of microvilli per vessel and the number of pinocytic vesicle/µm^2 in the endothelial cell of cerebral capillaries and arterioles obtained from acute control with KCl application, acute paracetamol with KCl application, acute control with NaCl application and acute paracetamol with NaCl application.

Group	Control with KCl	Paracetamol treated with KCl	Control with NaCl	Paracetmol treated with NaCl
Microvilli (number of microvilli/ vessel)				
Capillary	1.38±0.59	1.68±0.86	1.20±0.37	1.98±1.10
Arteriole	15.13±6.98	14.13±9.13	11.31±4.62	10.25±4.11
Pinocytic vesicles (number of pinocytic vesicles/µm^2)				
Capillary	33±15*	28±11*	16±7	12±6
Arteriole	43±10*	41±13*	18±7	22±12

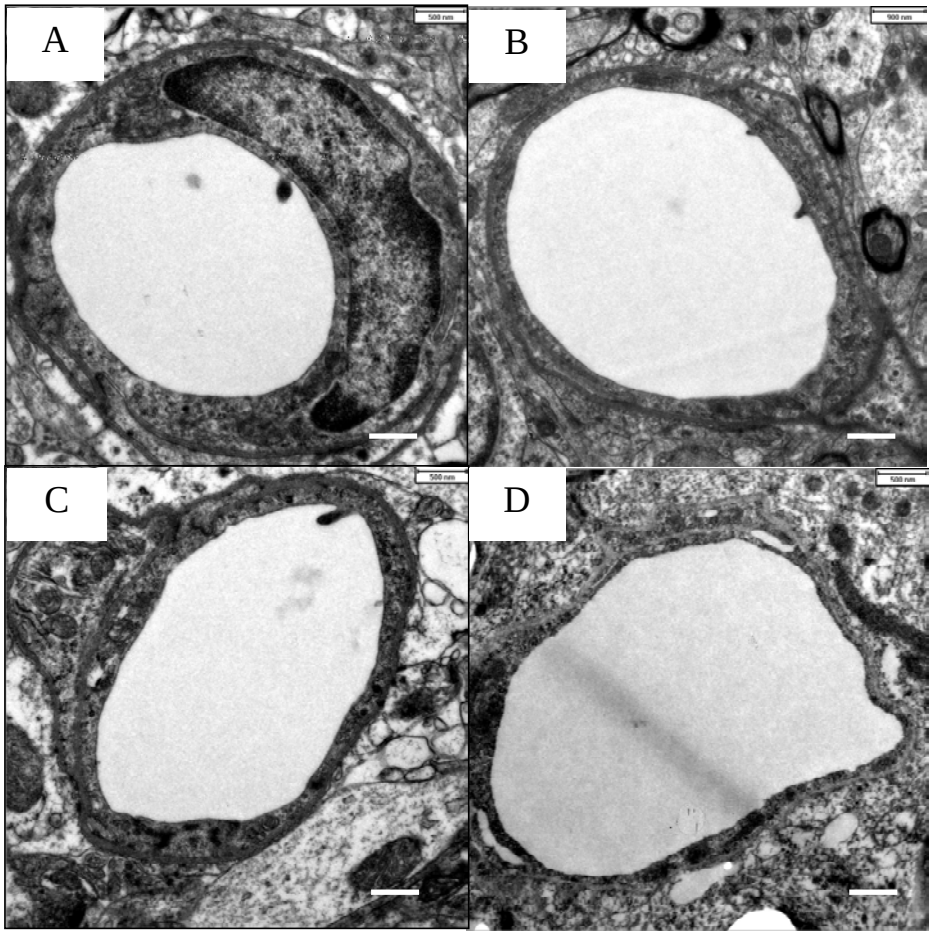

Figure4-15 Electron micrograph of endothelial cell obtained from A. acute control with KCl application B. acute paracetamol with KCl application C. acute control with NaCl application D. acute paracetamol with NaCl application. (x5000) Bar=500 nm.

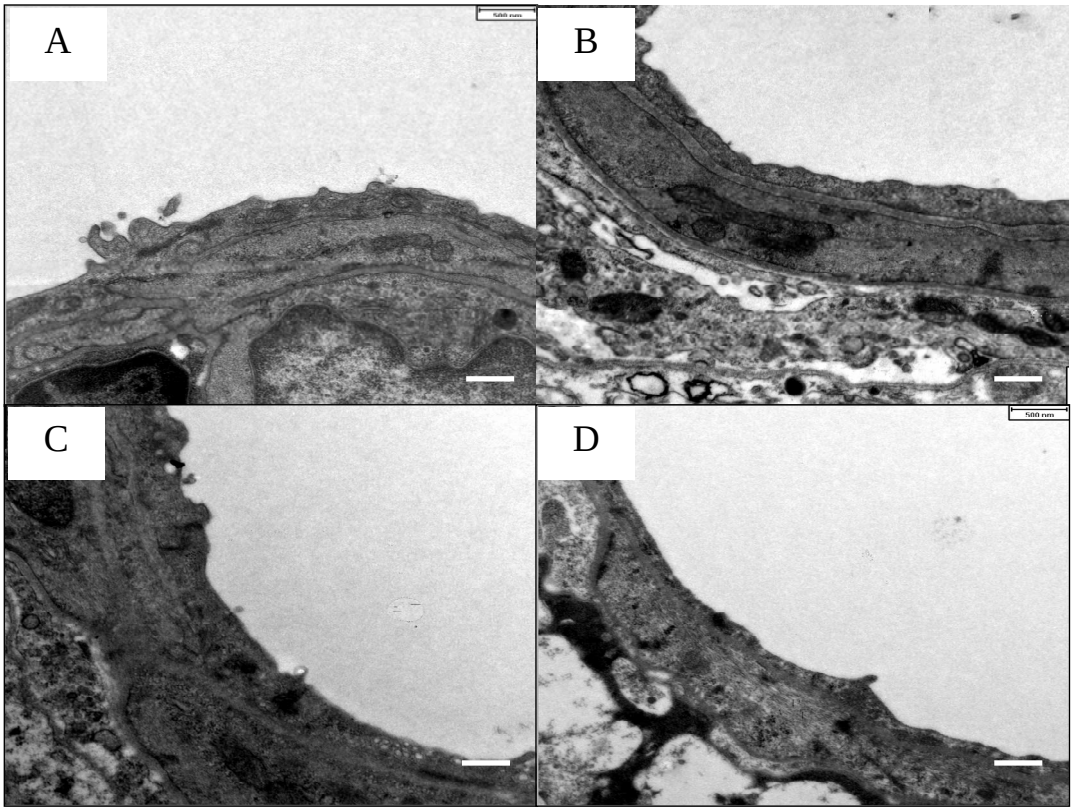

Figure4-16 Electron micrograph of an endothelial cell obtained from A. acute control with KCl application B. acute paracetamol with KCl application C. acute control with NaCl application D. acute paracetamol with NaCl application. (x5000) Bar=500 nm.

Experiment II: The study of the effect of chronic treatment with paracetamol on the trigeminovascular nociception induced by CSD

In the rat with chronic treatment with paracetamol, body weight and arterial blood pressure were not statistically different from control group. The data are shown in Table 4-9. The body weight in chronic control and chronic paracetamol treated rat were 392± 29.36 grams and 410±20 grams, respectively. The arterial blood pressure in chronic control and chronic paracetamol treated rat were 113.12±23.44 and 96.90±19.23 mmHg, respectively.

Table4-9 The mean value ± SD of body weight and arterial blood pressure of rats obtained from chronic control and chronic paracetamol treated group.

Group	Chronic control	Chronic paracetamol	p-value
Body weight (g)	392± 29.36	410±20	0.06
Blood pressure (mmHg)	113.56±23.44	96.90±19.23	0.17

- Effect of chronic treatment with paracetamol on cortical neurons activity induced by CSD

- The effect of chronic treatment with paracetamol on depolarization shift

In order to evaluate the effect of chronic paracetamol treatment on development of CSD variable including number of peak, peak amplitude, duration, area under the curve (AUC) were observed.

In chronic paracetamol administration, the average amplitude, duration and AUC of those CSD waves were 29.76±3.38 mV, 33.54 ± 7.32 and 65.38±16.77 mV-seconds, respectively while those of chronic control group were 27.30±2.98 mV, 36.76 ± 6.19 and 70.01±19.84 mV-seconds, respectively. Statistical analysis revealed no significant difference between the two groups. Despite no difference in peak amplitude, duration and AUC, the CSD frequency was significantly increase in chronic paracetamol treated group as compare to that of chronic control group (Figure4-17). The number of CSD peak in one hour from chronic paracetamol treated group was significantly higher than those observed in chronic control group (15.75±1.50 and 12.50±1.29 peak per hour, respectively) (Table4-10 and Figure4-17).

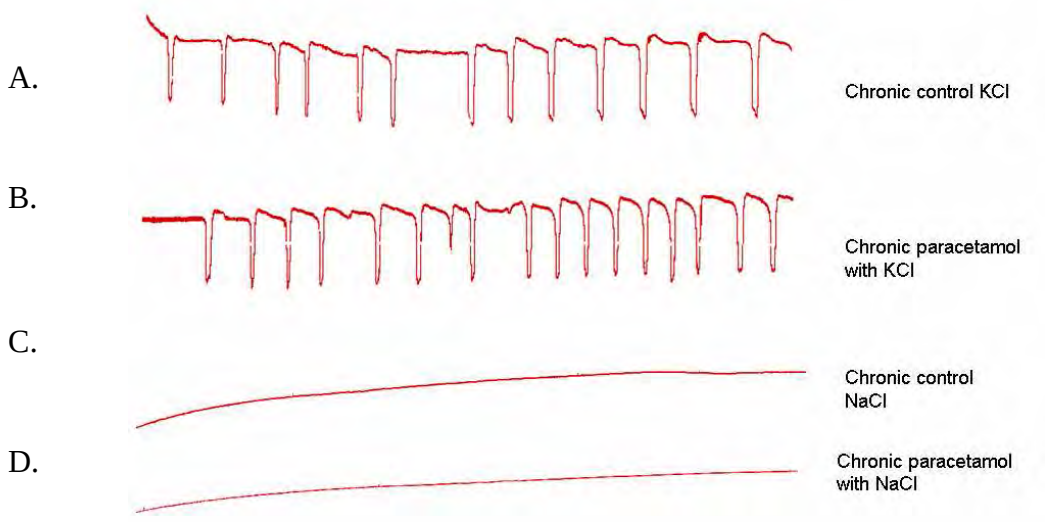

Figure4-17 The tracing showing the DC shift obtained from A. chronic control with KCl application B. chronic paracetamol with KCl application C. chronic control with NaCl application D. chronic paracetamol with NaCl application

Table4-10 Comparing the electrophysiology variables related to CSD between chronic control and chronic paracetamol treated groups.

	Chronic control	Chronic paracetamol	p-value
Number of peak	12.50 ± 1.29	15.75 ± 1.50	0.03
AUC (mV.s)	70.01 ± 19.84	65.38 ± 16.77	0.35
Amplitude (mV)	27.30 ± 2.98	29.76 ± 3.38	0.10
Duration (s)	36.76 ± 6.19	33.54 ± 7.32	0.06

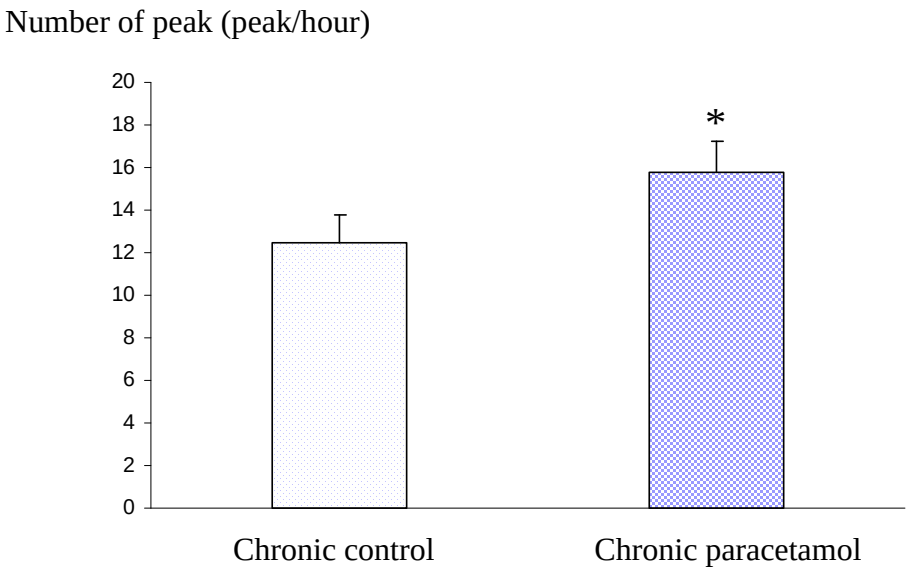

Figure4-18 Bar graphs showing the mean value ± SD of number of peak of DC shift obtained from chronic control and chronic paracetamol treated group. Significant difference was assessed with Student t-test. * p< 0.05 compared with chronic control group.

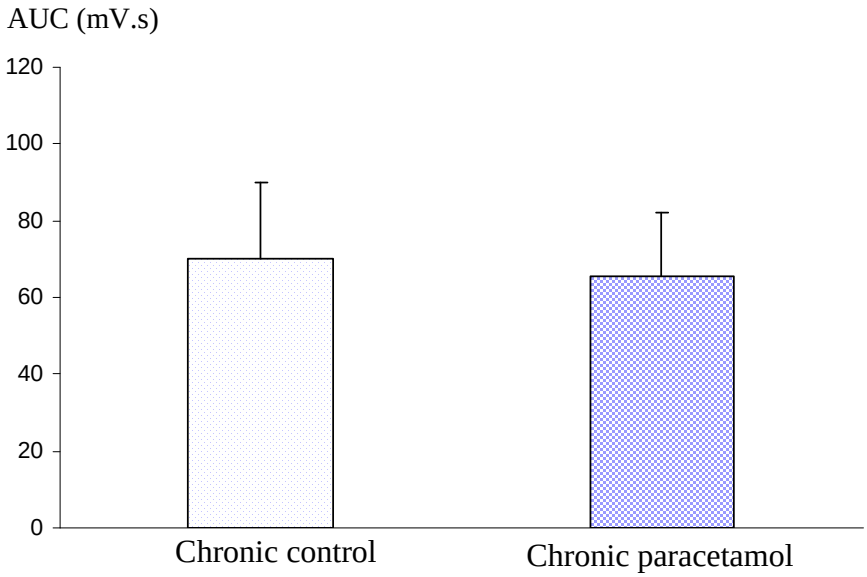

Figure4-19 Bar graphs showing the mean value ± SD of area under the curve of DC shift obtained from chronic control and chronic paracetamol treated group.

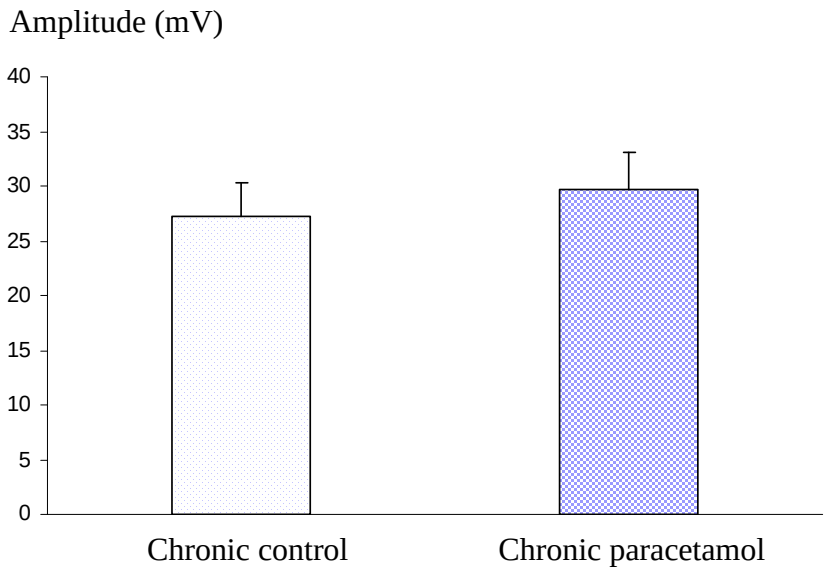

Figure4-20 Bar graphs showing the mean value ± SD of amplitude of DC shift obtained from chronic control and chronic paracetamol treated group.

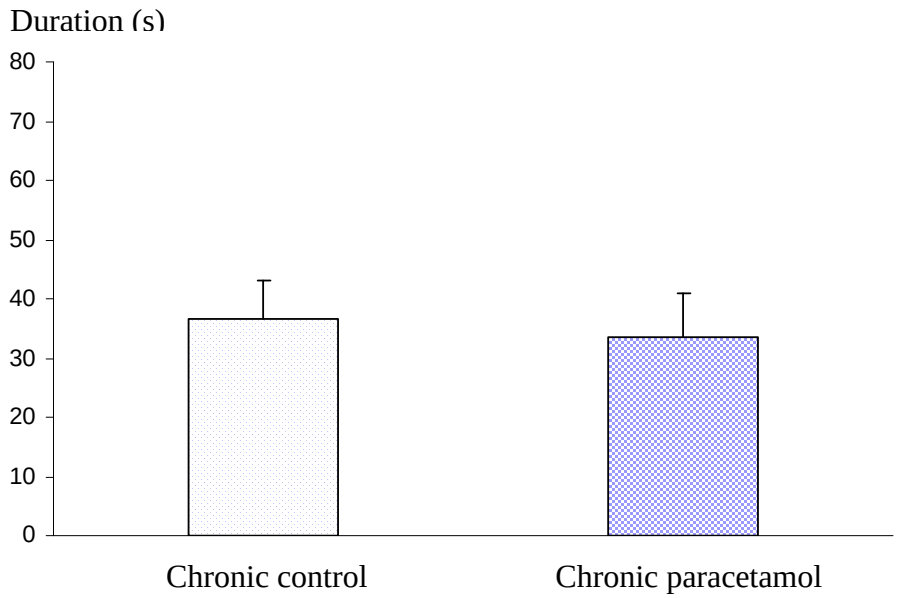

Figure4-21 Bar graphs showing the mean value ± SD of duration of DC shift obtained from chronic control and chronic paracetamol treated group.

- The effect of chronic treatment with paracetamol on Fos expression in cerebral cortex

In order to evaluate the effect of chronic treatment with paracetamol on the activation of neurons in cerebral cortex, the fos expression was measured in cerebral cortex obtained from both control and paracetamol treated groups.

Our immunohistochemical study showed that the induction of CSD evoked Fos expression in cerebral cortex. Fos-ir cells were distributed mainly in cortical hemispheres ipsilateral side to KCl application. Only few Fos-ir cells were observed in the contralateral side to KCl application. Chronic treatment with paracetamol substantially increased the number of CSD-induced Fos-ir cells in layer III and IV of the cerebral cortex as compared with chronic control group (Figure4-22, Table4-11). Fos-ir cells in chronic control and chronic paracetamol rat were 79.00$\pm$17.65 cells per 6.25 x 10^4 μm^2 and 113.86$\pm$15.21 cells per 6.25 x 10^4 μm^2, respectively. Statistical analysis demonstrated significantly difference between two groups (Table4-11). Fos-ir cells were also presented bilateral in cerebral cortex to NaCl application but less than those observed in KCl application (13.41$\pm$6.25 cells per 6.25 x 10^4 μm^2 and 11.42$\pm$3.53 cells per 6.25 x 10^4 μm^2 in chronic control with NaCl and chronic paracetamol with NaCl treated group, respectively) (Figure4-22, Table4-11).

Table4-11 The mean value $\pm$ SD of the number of Fos-ir cells in cerebral cortex sections obtained from chronic control with KCl application, chronic paracetamol with KCl application, chronic control with NaCl application, and chronic paracetamol with NaCl application.

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	Chronic control		Chronic paracetamol	
	KCl	NaCl	KCl	NaCl
Ipsilateral	79.00$\pm$17.65*	13.41$\pm$6.25	113.86$\pm$15.21 *$^{\#}$	11.42$\pm$3.53

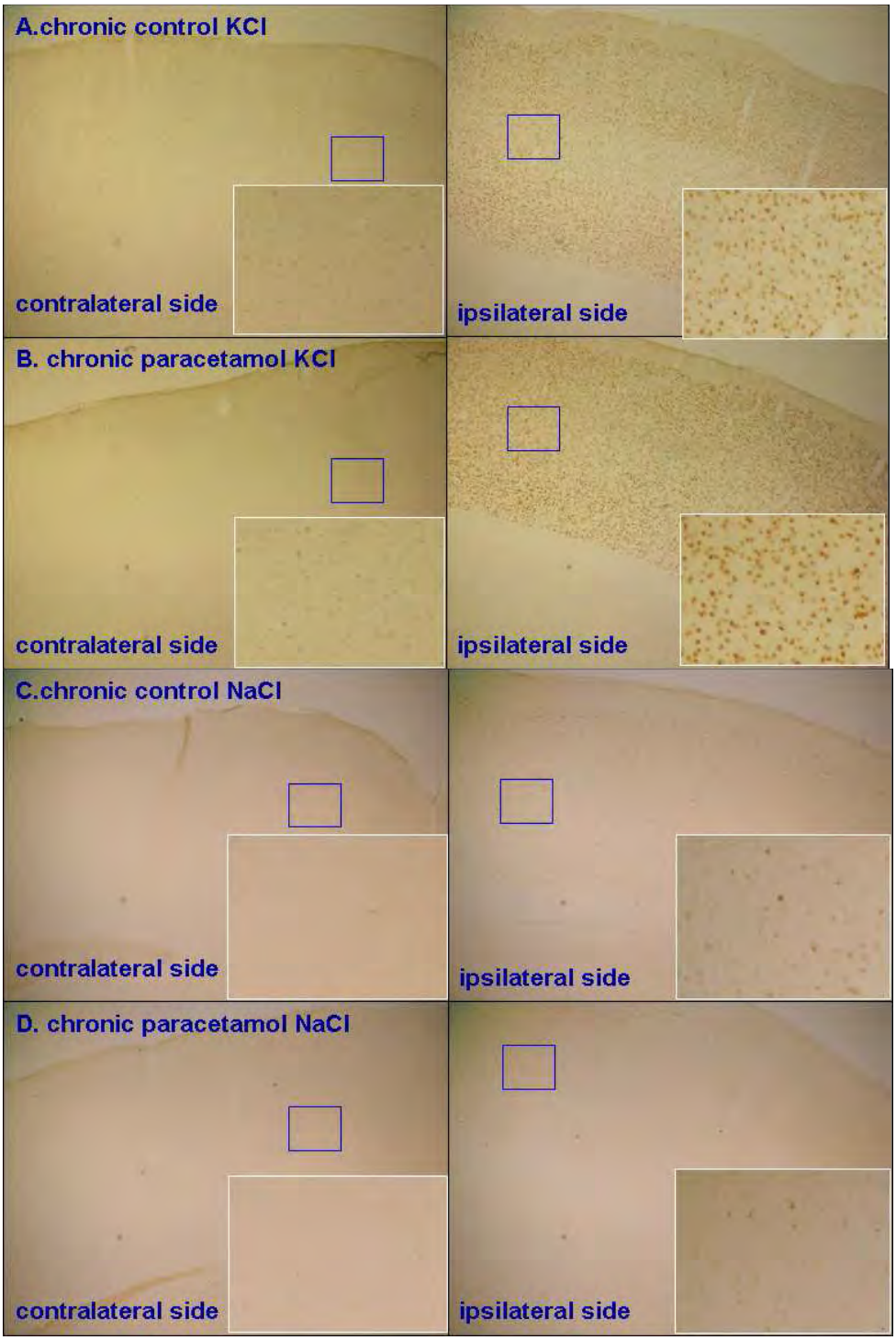

Figure4-22 The photomicrograph showing the Fos-ir cells in the brain sections ipsilateral side and contralateral side to KCl and NaCl application obtained from A. chronic control with KCl application B. chronic paracetamol with KCl application C. chronic control with NaCl application D. chronic paracetamol with NaCl application

- The effect of chronic treatment with paracetamol on 5-HT_{2A} receptor expression in cerebral cortex

In this experiment, 10 sections of brain were collected from each rat. The sections were studied under the light microscope and neurons were classified as immunoreactive or non-reactive based on the immunostaining feature. The 5-HT_{2A}-ir cells were defined as those with dark-brown stained in their cytoplasm. We found that the CSD induced an expression of 5-HT_{2A}-ir cells in the cortical layers III-VI of the cerebral cortex was the same pattern in all experimental group. CSD induced expression of 5-HT_{2A} in the cerebral cortex, which were more prevalent on the side ipsilateral to KCl application (Figure4-23). In chronic control group, the numbers of 5-HT_{2A}-ir cells in the ipsilateral side was 13.00$\pm$2.00 cells per μm^2. The numbers of 5-HT_{2A}-ir cells in chronic paracetamol-treated group were significant higher than those observed from chronic control group (19.67$\pm$4.04 cells per μm^2). The data are shown in Table4-12. The difference in the number of 5-HT_{2A}-ir cells between chronic paracetamol treated group and chronic control groups was statistically significant (Table4-12).

In chronic control NaCl group, the numbers of 5-HT_{2A}-ir cells in the ipsilateral sides was 6.67$\pm$2.52 cells per μm^2. Chronic pretreatment with paracetamol also enhanced the response of trigeminal nociceptive to NaCl application. The numbers of 5-HT_{2A}-ir cells in chronic paracetamol-treated group was 10.33$\pm$1.53 cells per μm^2 for ipsilateral side. The data are shown in Table 4-12. The difference in the number of 5-HT_{2A}-ir cells between chronic paracetamol treated group and chronic control groups was statistically significant (Table4-12).

Table4-12 The mean value $\pm$ SD of the number of 5-HT_{2A}-ir cells in cerebral cortex sections obtained from chronic control with KCl application, chronic paracetamol with KCl application, chronic control with NaCl application, and chronic paracetamol with NaCl application

	Chronic control		Chronic paracetamol	
	KCl	NaCl	KCl	NaCl
Ipsilateral	13.00$\pm$2.00*	6.67$\pm$2.52	19.67$\pm$4.04*$^{\#}$	10.33$\pm$1.53*

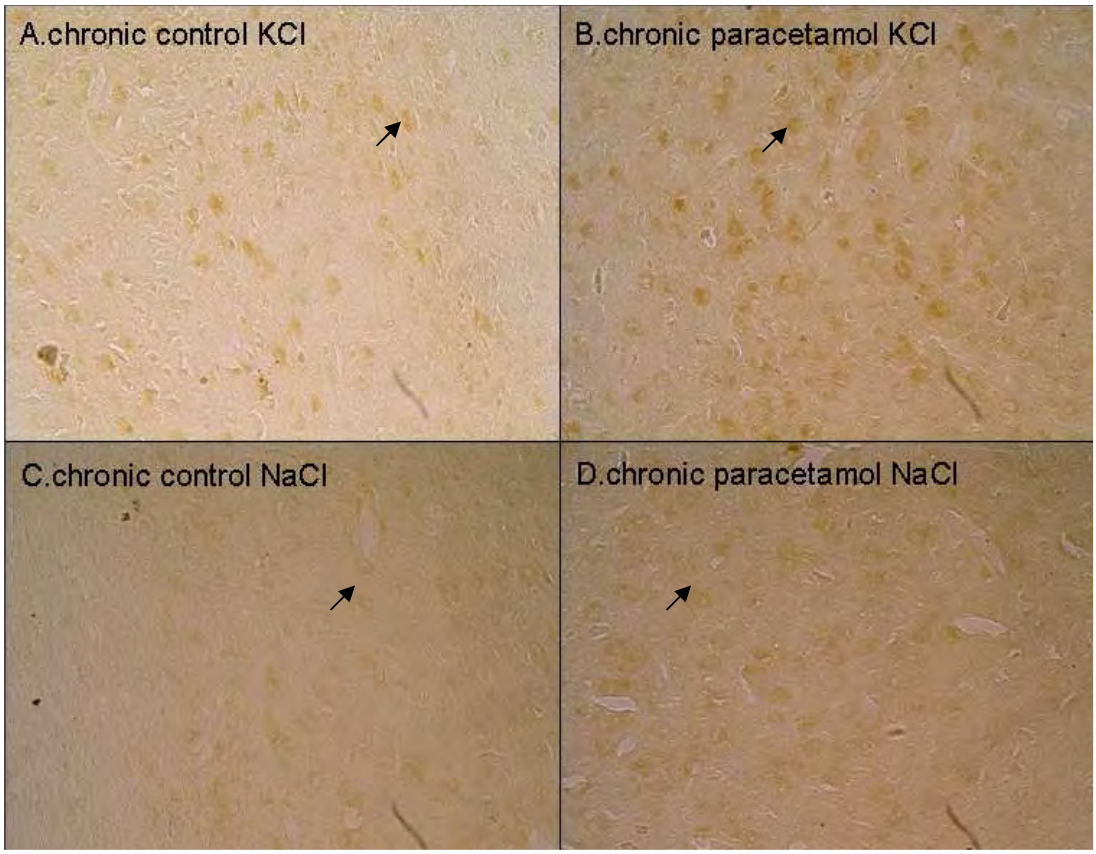

Figure4-23 The photomicrograph showing the 5-HT_{2A}-ir cells (arrow) in the brain sections ipsilateral side to KCl and NaCl application obtained from A. chronic control with KCl application B. chronic paracetamol with KCl application C. chronic control with NaCl application D. chronic paracetamol with NaCl application.

- Effect of chronic treatment with paracetamol on trigeminal neurons activity induced by CSD

- The effect of chronic treatment with paracetamol on Fos expression in TNC

In chronic control group, the numbers of Fos-ir cells in ipsilateral and contralateral sides were 11.51±2.87 cells per slide and 7.40±3.08 cells per slide, respectively. Pretreatment with chronic paracetamol enhanced the response of trigeminal nociception to CSD. The numbers of Fos-ir cells in chronic paracetamol-treated group were 16.43±2.98 cells per slide and 10.21±3.23 cells per slide for ipsilateral and contralateral sides, respectively. The data are shown in Table 4-9. The difference in the number of Fos-ir cells between chronic paracetamol treated group and chronic control groups was statistically significant (Figure4-24, Table4-13).

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In chronic control NaCl group, the numbers of Fos-ir cells in ipsilateral and contralateral sides were 6.31±1.69 cells per slide and 4.09±1.44 cells per slide, respectively. Pretreatment with chronic paracetamol did not effect to the response of trigeminal nociception to NaCl application (Figure 4-24). The numbers of Fos-ir cells in chronic paracetamol-treated group were 8.29±1.71 cells per slide and 4.90±1.27 cells per slide for ipsilateral and contralateral sides, respectively. The data are shown in Table 4-13. The difference in the number of Fos-ir cells between chronic paracetamol treated group and chronic control groups was not statistically significant (Figure4-24, Table4-13).

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Table4-13 The mean value ± SD of the number of Fos-ir cells in the C1 and C2 cervical spinal cord sections obtained from chronic control with KCl application, chronic control with NaCl application, chronic paracetamol with KCl application and chronic paracetamol with NaCl application.

	Chronic control		Chronic paracetamol	
	KCl	NaCl	KCl	NaCl
Ipsilateral	11.51±2.87*	6.31±1.69	16.43±2.98*#	8.29±1.71
Contralateral	7.40±3.08*	4.09±1.44	10.21±3.23*	4.90±1.27

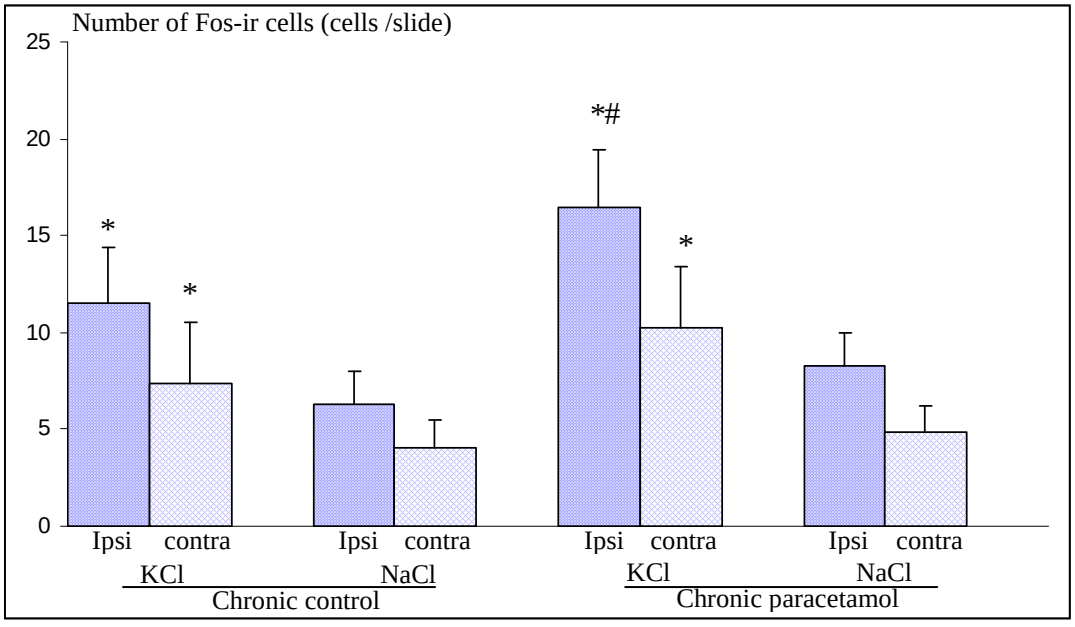

Figure4-24 Bar graph showing the mean value ± SD of the number of Fos-ir cells in the C1 and C2 cervical spinal cord sections obtained form chronic control with KCl application, chronic paracetamol with KCl application, chronic control with NaCl application, and chronic paracetamol with NaCl application. Significant difference was assesses with ANOVA with the Bonferroni test. *p<0.05 compared with chronic control KCl group.

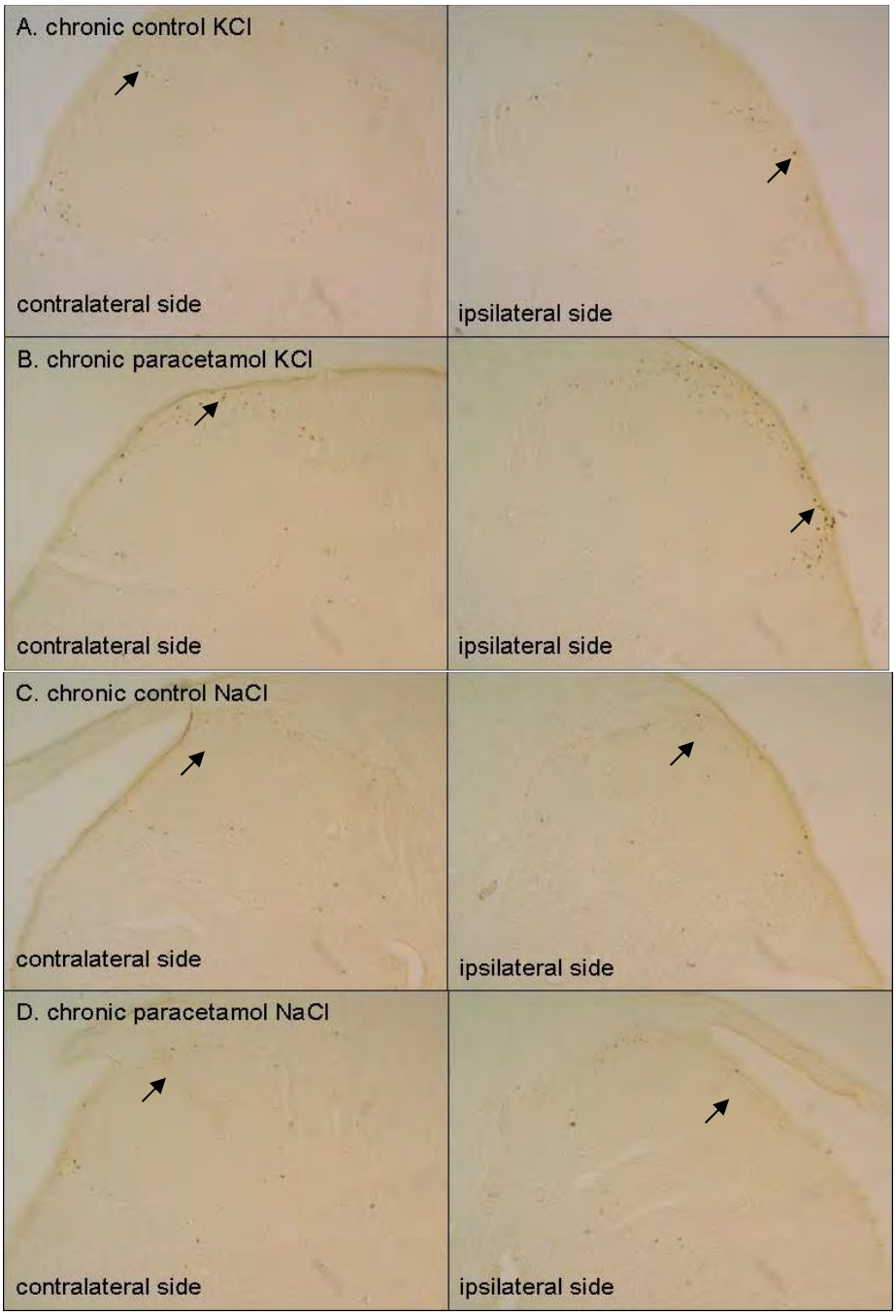

Figure4-25 The photomicrograph showing the Fos-ir cells (arrow) in the C1-C2 cervical spinal cord sections ipsilateral side and contralateral side to KCl and NaCl application obtained from A. chronic control with KCl application B. chronic paracetamol with KCl application C. chronic control with NaCl application D. chronic paracetamol with NaCl application.

- The effect of chronic treatment with paracetamol on 5-HT_{2A} receptor expression in TG

The effect of chronic paracetamol treated rat on the 5-HT_{2A} expression induced by CSD was tested by evaluating the number of 5-HT_{2A}-ir cells in the chronic paracetamol pretreated group and in the chronic control CSD group.

CSD induced expression of 5-HT_{2A} in the TG. 5HT_{2A}-ir cells were confined in TG and were more prevalent on the side ipsilateral to the operation (Figure4-26). In chronic control group, the numbers of 5-HT_{2A}-ir cells in the ipsilateral and contralateral sides were 30.2$\pm$10.2 cells per slide and 22.1$\pm$9.3 cells per slide, respectively. The numbers of 5-HT_{2A}-ir cells in chronic paracetamol-treated group were 40.5$\pm$8.7 cells per slide and 24.1$\pm$11.4 cells per slide for ipsilateral and contralateral sides, respectively. The data are shown in Table 4-14. The difference in the number of 5-HT_{2A}-ir cells between chronic paracetamol treated group and chronic control groups was statistically significant (Table4-14).

In chronic control NaCl group, the numbers of 5-HT_{2A}-ir cells in the ipsilateral and contralateral sides were 23.4$\pm$11.2 cells per slide and 18.5$\pm$10.7 cells per slide, respectively. Pretreatment with chronic paracetamol did not increase the response of trigeminal nociceptive to NaCl application. The numbers of 5-HT_{2A}-ir cells in paracetamol-treated group were 25.5$\pm$13.3 cells per slide and 23.2$\pm$14.2 cells per slide for ipsilateral and contralateral sides, respectively. The data are shown in Table 4-14. The difference in the number of 5-HT_{2A}-ir cells between chronic paracetamol treated group and chronic control groups was not statistically significant.

Table4-14 The mean value ± SD of the number of 5-HT_{2A}-ir cells in the TG sections obtained from chronic control with KCl application, chronic paracetamol with KCl application, chronic control with NaCl application, and chronic paracetamol with NaCl application.

	Chronic control		Chronic paracetamol	
	KCl	NaCl	KCl	NaCl
Ipsilateral	30.2±10.2	23.4±11.2	40.5±8.7*	25.5±13.3
Contralateral	22.1±9.3	18.5±10.7	24.1±11.4	23.2±14.2

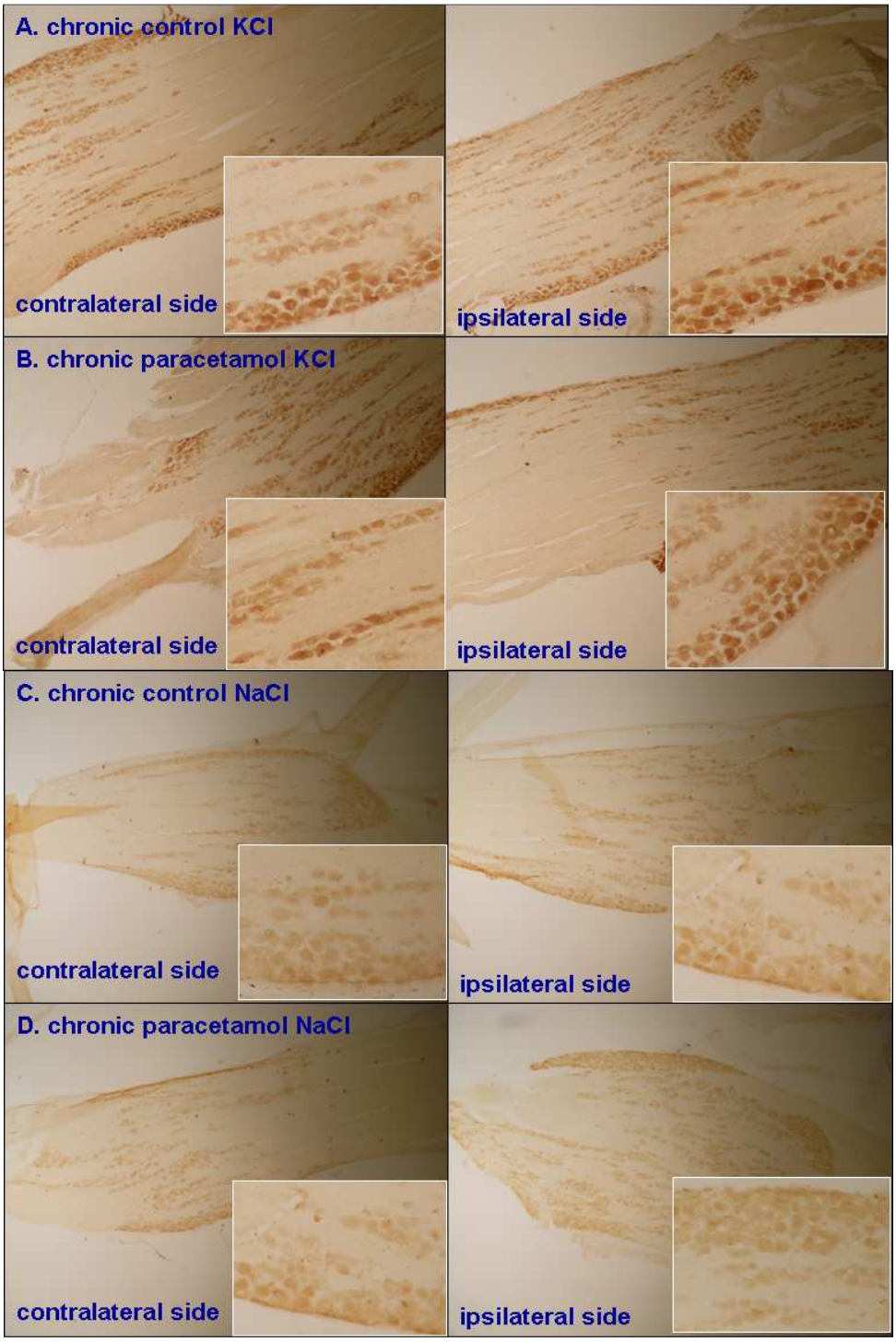

Figure4-26 The photomicrograph showing the 5-HT_{2A}-ir cells in the TG sections ipsilateral side and contralateral side to KCl and NaCl application obtained form A. chronic control with KCl application B. chronic paracetamol with KCl application C. chronic control with NaCl application and D. chronic paracetamol with NaCl application.

- Effect of chronic treatment with paracetamol on cerebral circulation induced by CSD

- The effect of chronic treatment with paracetamol on cortical blood flow

Application of KCl induced the repeated pattern of cerebral hyperemia. The amplitude from each peak was calculated as percent changes from the resting stage. The percent changes from base line were 244.74 ± 43.05 and 199.51 ± 49.20 in chronic control and chronic paracetamol treated group, respectively. Statistical analysis showed significant difference between two groups (Table4-15). The average number of hyperemia cycles within one hour was 11.67 ±1.53 and 13.33 ± 2.88 in chronic control and chronic paracetamol treated groups, respectively. The average duration of these cycles were 141.67 ± 34.97 second and 135.56 ± 21.978 second in chronic control and chronic paracetamol treated groups, respectively. Statistical analysis showed significant difference between these two groups (Table4-15). There was no change of CBF in the NaCl application group (Figure 4-27).

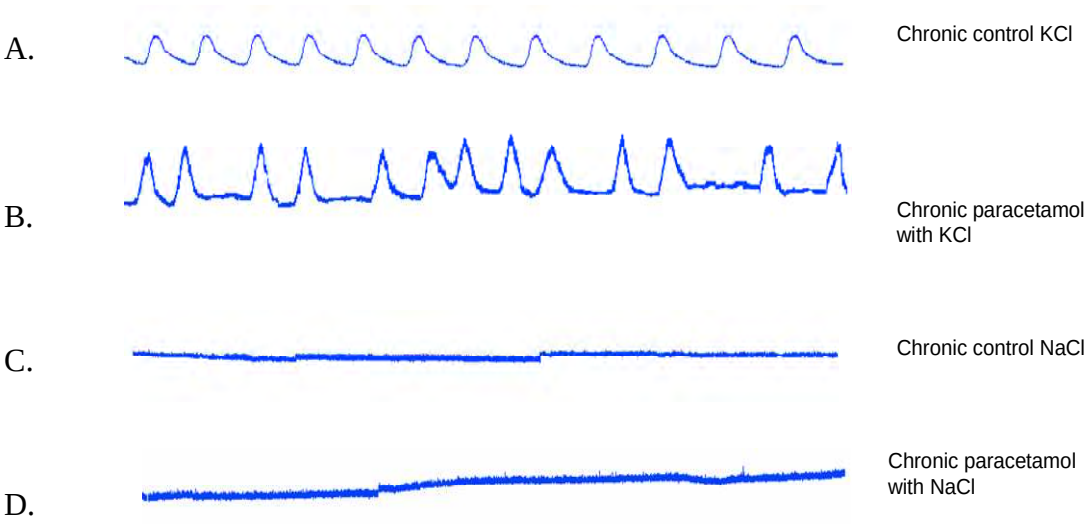

Figure4-27 The tracing showing the CBF changes obtained A. chronic control with KCl application B. chronic paracetamol with KCl application C. chronic control with NaCl application D. chronic paracetamol with NaCl application

Table4-15 The number of peak hyperemia, percent changes from base line and duration of CBF between chronic control and chronic paracetamol treated group.

	Chronic control	Chronic paracetamol	p-value
Number of peak (peak per hour)	11.67 ±1.53	13.33 ± 2.88	0.09
%Change	244.74 ± 43.05	199.51 ± 49.20	0.01
Duration (s)	141.67 ± 34.97	135.56 ± 21.97	0.25

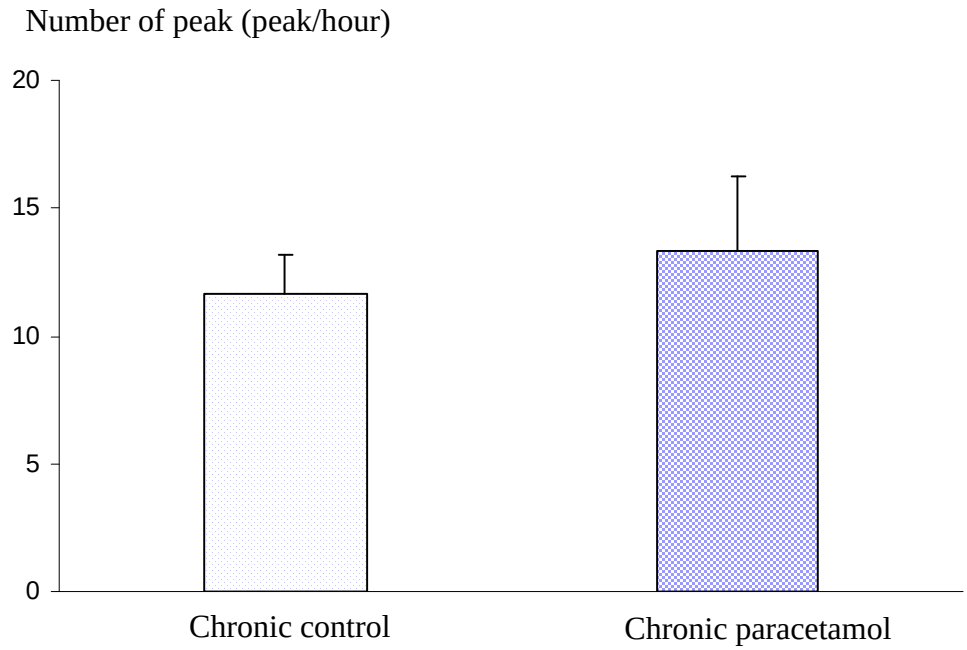

Figure4-28 Bar graphs showing the mean value ± SD of number of peak of CBF obtained from chronic control and chronic paracetamol treated group.

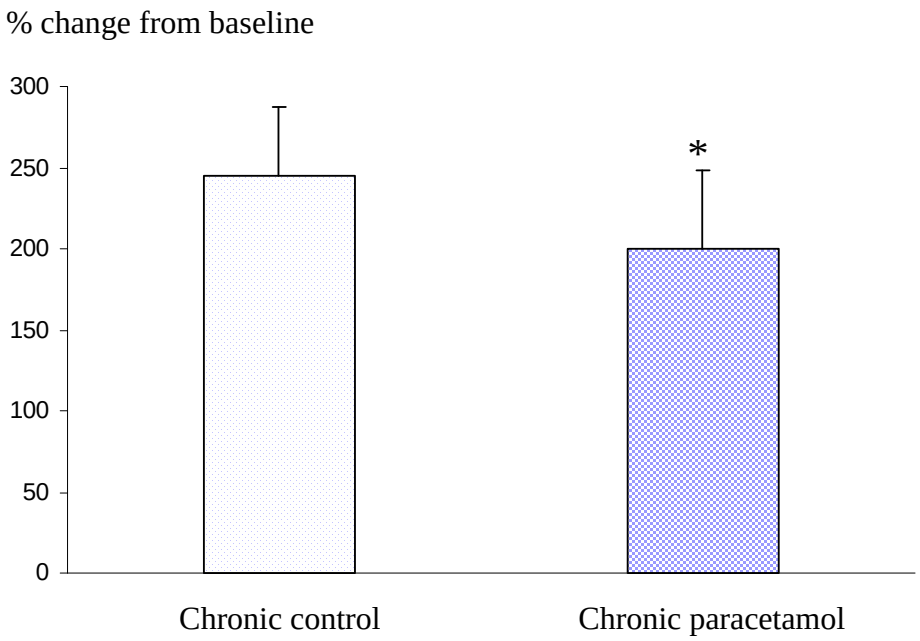

Figure4-29 Bar graphs showing the mean value ± SD of percent change from baseline of CBF obtained from chronic control and chronic paracetamol treated group. Significant difference was assessed with Student t-test. * p< 0.05 compared with chronic control group.

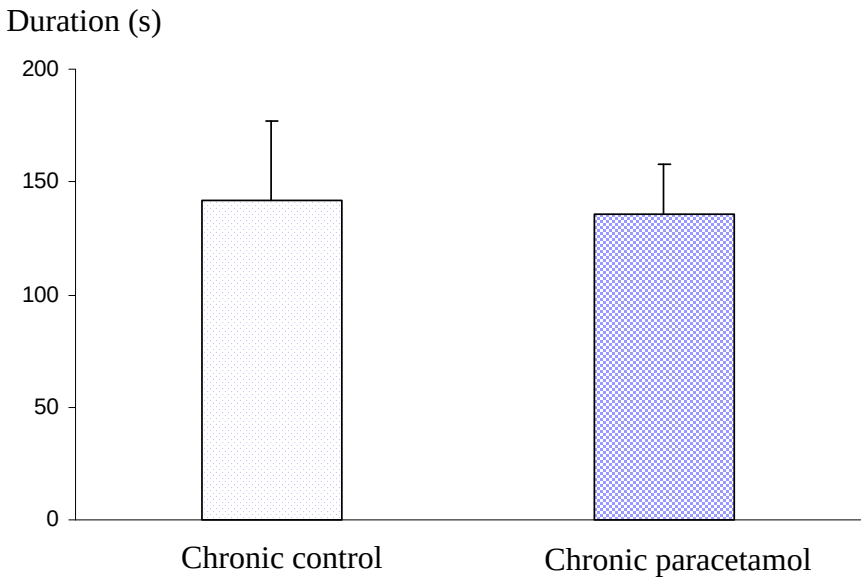

Figure4-30 Bar graphs showing the mean value ± SD of the duration of CBF obtained from chronic control and chronic paracetamol treated group.

-The effect of chronic treatment with paracetamol on ultrastructural of cerebral microvessels

Analysis of 50 randomly selected capillaries and 25 arterioles obtained from the control and chronic paracetamol treated group showed mean values of microvilli per vessel from capillary and arteriole, respectively. The average density of pinocytic vesicle was demonstrated in vesicles per μm^2 for capillary and arteriole, respectively.

The number of microvilli in capillaries and arteriole were 1.27±0.56 and 10.75±5.28 microvilli per vessel in the chronic control group and 3.26±1.33 and 23.75±11.03 microvilli per vessel in the chronic paracetamol treated group, respectively. However the number of microvilli in capillary and arteriole obtained from the chronic control and chronic paracetamol treated group was statistically significant. The data are shown in Table4-16 and Figure4-31.

The average density of pinocytic vesicles in capillaries and arterioles were 34±9 and 32±6 vesicle per μm^2 in the chronic control group and 44±7 and 49±11 vesicle per μm^2 in chronic paracetamol treated group, respectively. The difference in the number of pinocytic vesicles between chronic paracetamol treated group and chronic control groups was statistically significant. The data are shown in Table4-16 and Figure4-32.

- The effect of chronic treatment with paracetamol on ultrastructural of endothelial cell change evoked by NaCl application

The number of microvilli in capillaries and arteriole were 1.52±0.78 and 9.24±4.32 microvilli per vessel in the chronic control group and 2.68±0.17 and 13.50±3.42 microvilli per vessel in the chronic paracetamol treated group, respectively. However the number of microvilli in capillary and arteriole obtained from the chronic control and chronic paracetamol treated group was not significant different. The data are shown in Table4-16 and Figure4-31.

The average density of pinocytic vesicles in capillaries and arterioles were 21±12 and 16±8 vesicle per μm^2 in the chronic control group and 19±5 and 25±4 vesicle per μm^2 in chronic paracetamol treated group, respectively. The difference in the number of pinocytic vesicles between chronic paracetamol treated group and chronic control groups was statistically significant. The data are shown in Table4-16 and Figure 4-32.

Table4-16 The mean value ± SD of the number of microvilli per vessel and the number of pinocytic vesicle/um^2 in the endothelial cell of cerebral capillaries and arterioles obtained from the chronic control with KCl application, chronic paracetamol with KCl application, chronic control with NaCl application and chronic paracetamol with NaCl application.

Group	Control with KCl	Paracetamol treated with KCl	Control with NaCl	Paracetmol treated with NaCl
Microvilli (number of microvilli/ vessel)				
Capillary	1.27±0.56	3.26±1.33*	1.52±0.78	2.68±0.17
Arteriole	10.75±5.28	23.75±11.03*	9.24±4.32	13.50±3.42
Pinocytic vesicles (Number of pinocytic vesicles/µm^2)				
Capillary	34±9*	44±7*$^{\#}$	21±12	22±5
Arteriole	32±6*	49±11*$^{\#}$	16±8	25±4

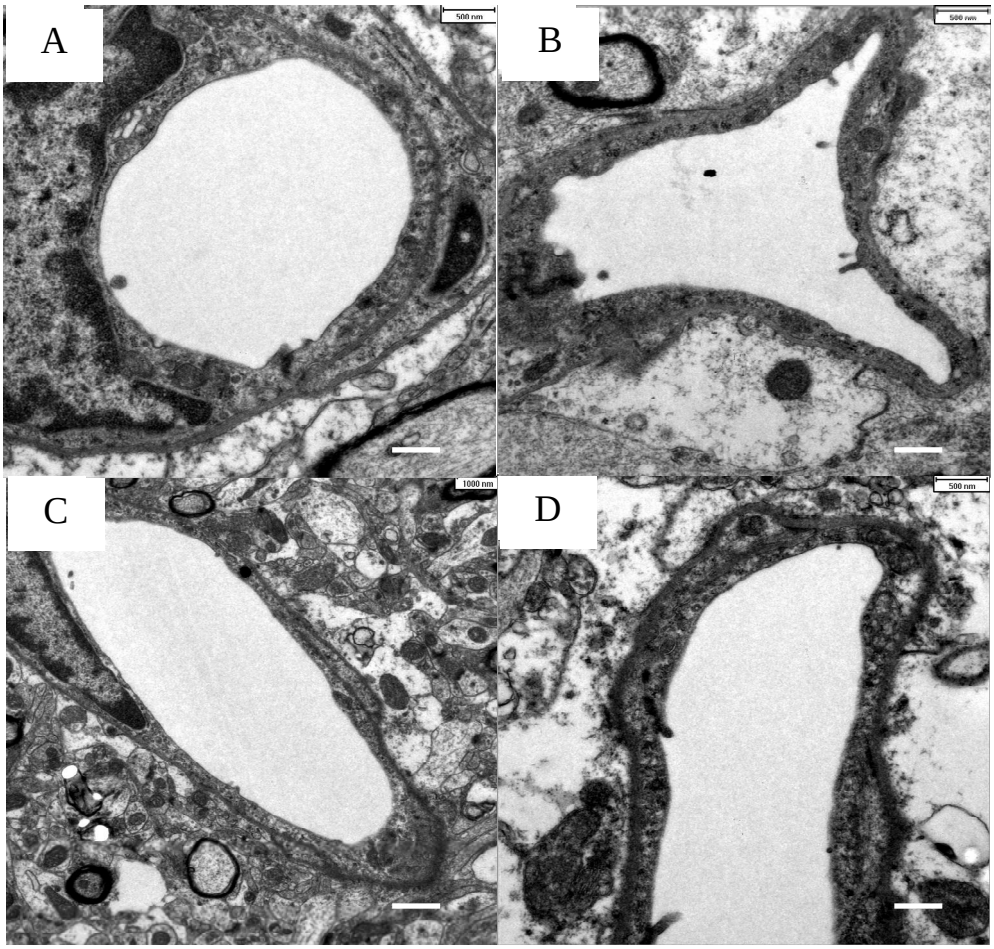

Figure4-31 Electron micrograph of endothelial cell obtained from A. chronic control with KCl application B. chronic paracetamol with KCl application C. chronic control with NaCl application D. chronic paracetamol with NaCl application. (x5000) Bar=500 nm.

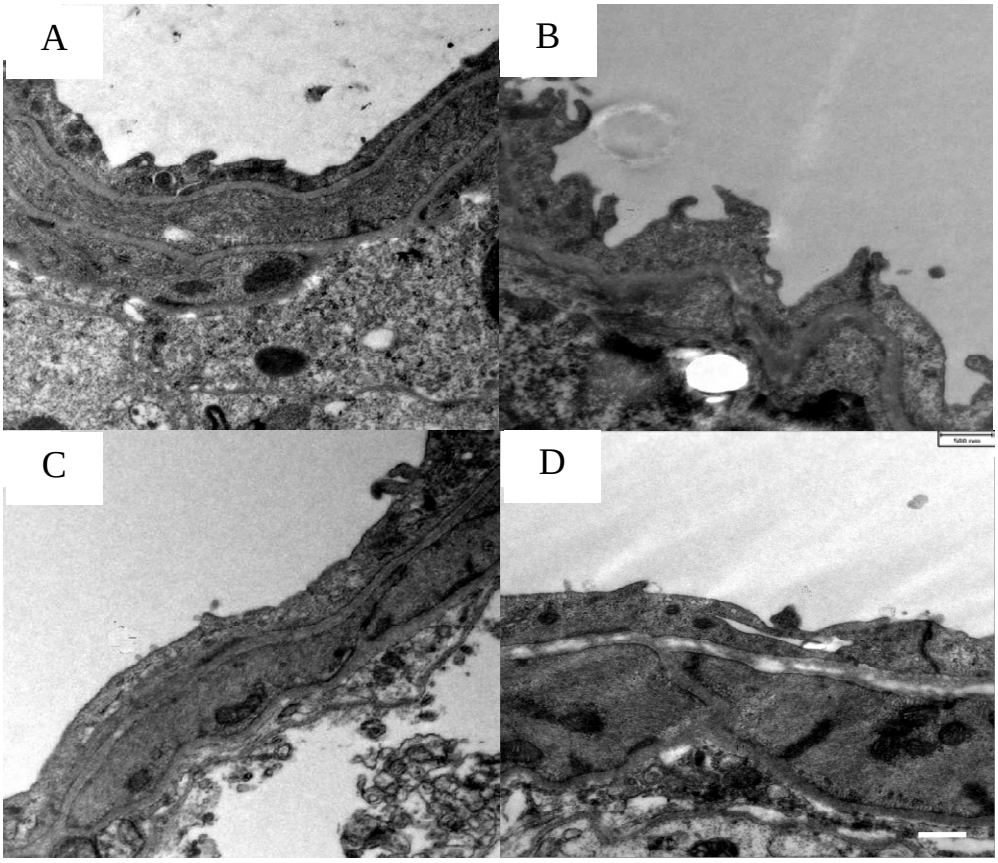

Figure4-32 Electron micrograph of endothelial cell obtained from A. chronic control KCl application B. chronic paracetamol KCl application C. chronic control NaCl application D. chronic paracetamol NaCl application. (x5000) Bar=500 nm.

Experiment III: The study of the effect of 5-HT_{2A} receptor antagonist (ketanserin 1 and 10 mg/kg) on the trigeminovascular nociception induced by CSD in chronic treatment with paracetamol

In the rat with chronic paracetamol with ketanserin treatment, body weight and arterial blood pressure were not difference from chronic paracetamol groups. The body weight in chronic paracetamol, chronic paracetamol with ketanserin 1 mg/kg and chronic paracetamol with 10 mg/kg were 410±20 grams, 398±10.09 grams and 403±12.42 grams, respectively. The arterial blood pressure in chronic paracetamol, chronic paracetamol with ketanserin 1 mg/kg and chronic paracetamol with ketanserin 10 mg/kg were 96.90±19.23, 107.40±6.79 and 113.23±11.79 mmHg, respectively (Table4-17).

Table4-17 The mean value ± SD of body weight and arterial blood pressure of rats in chronic paracetamol, chronic paracetamol with ketanserin 1 mg/kg and chronic paracetamol with ketanserin 10 mg/kg treated group.

Group	Chronic paracetamol	Chronic paracetamol+ ketanserin 1 mg/kg	Chronic paracetamol + ketanserin 10 mg/kg
Body weight (g)	410±20	398±10.09	403±12.42
Blood pressure(mmHg)	96.90±19.23	107.40±6.79	113.23±11.79

- Effect of 5-HT_{2A} receptor antagonist on cortical neurons activity induced by CSD in chronic treatment with paracetamol

- The effect of 5-HT_{2A} receptor antagonist on depolarization shift

In order to evaluate the effect of chronic paracetamol with ketanserin treatment on development of CSD variable including number of peak, peak amplitude, duration, and area under the curve (AUC) were studied. The DC shift was studies in rat given an i.p. injection of ketanserin in varying dose (1 and 10 mg/kg) for 30 minute before KCl application.

In chronic paracetamol administration, the average amplitude, duration and AUC of those CSD waves were 29.76±3.38 mV, 33.54 ± 7.32 and 65.38±16.77 mV-seconds, respectively while those of the chronic paracetamol with ketanserin 1 mg/kg group were 41.2 ± 9.1 mV, 44.58 ± 10.46 and 106.36 ± 26.62 mV-seconds, respectively and in chronic paracetamol with ketanserin 10 mg/kg group were 28.17± 2.63 mV, 49.36 ± 8.51 and 76.53 ± 15.11 mV-seconds, respectively. Statistical analysis showed significant difference between chronic paracetamol and chronic paracetamol with ketanserin 1 mg/kg (Table4-18). The average number of CSD peak in one hour from chronic paracetamol treated group, chronic paracetamol with ketanserin 1 mg/kg and the chronic paracetamol with ketanserin 10 mg/kg were 15.75±1.50, 14.5 ± 3.35 and 7.25 ± 2.36 peak per hour, respectively (Table4-18). Statistical analysis showed significant difference between chronic paracetamol and chronic paracetamol with ketanserin 10 mg/kg (Table4-18).

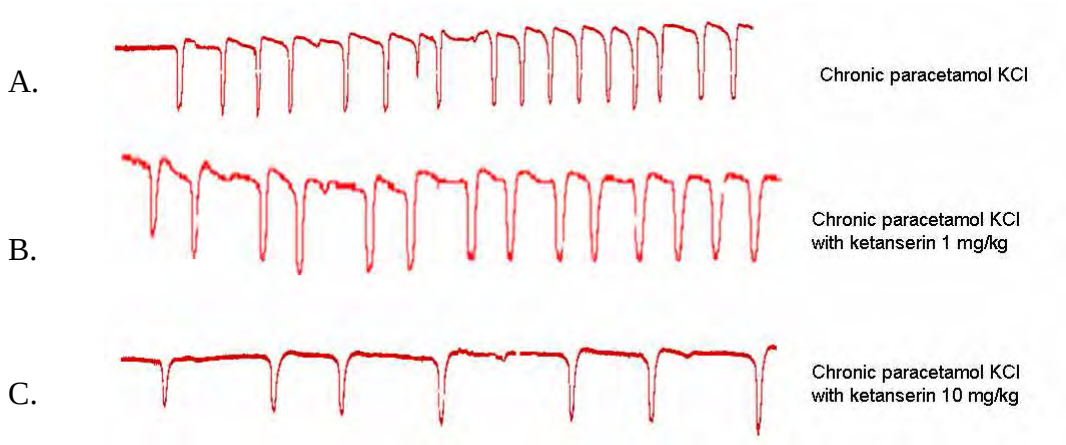

Figure4-33 The tracing showing the DC shift obtained from A. chronic paracetamol treated group B. chronic paracetamol with ketanserin 1 mg/kg treated group C. chronic paracetamol with ketanserin 10 mg/kg treated group

Table4-18 Comparing the electrophysiology variables related to CSD between chronic paracetamol treated group, chronic paracetamol treated with ketanserin 1 mg/kg treated group and chronic paracetamol treated with ketanserin 10 mg/kg treated group.

	Chronic paracetamol	Chronic paracetamol+ ketanserin 1 mg/kg	Chronic paracetamol + ketanserin 10 mg/kg
Number of peak (peak per hour)	15.75 ± 1.50	14.5 ± 3.35	7.25 ± 2.36*#
AUC (mV.s)	65.38 ± 16.77	106.36 ± 26.62*	76.53 ± 15.11#
Amplitude (mV)	29.76 ± 3.38	41.2 ± 9.10*	28.17± 2.63#
Duration (s)	33.54 ± 7.32	44.58 ± 10.46*	49.36 ± 8.51*

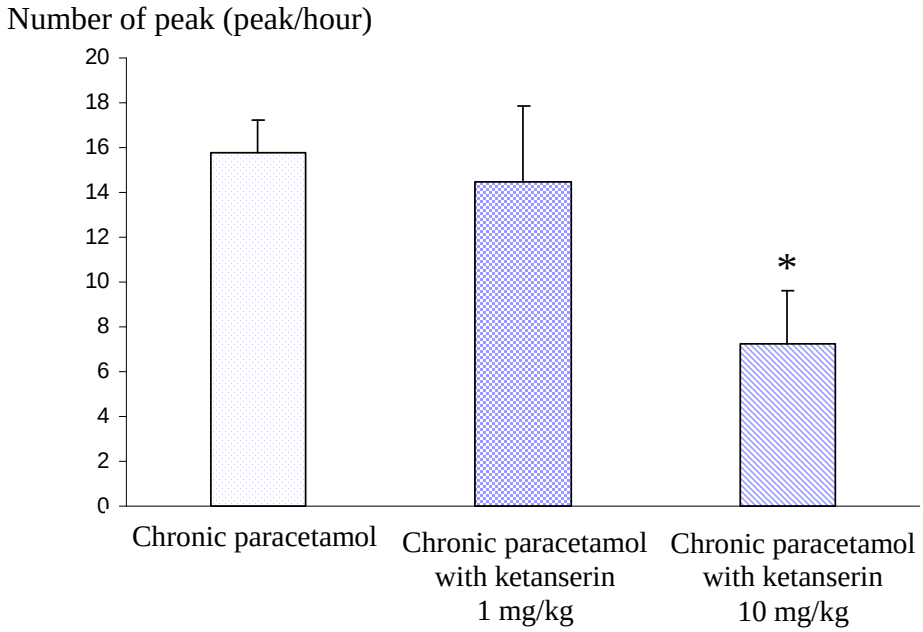

Figure4-34 Bar graphs showing the mean value ± SD of average number of peak of DC shift obtained from chronic paracetamol treated group, chronic paracetamol with ketanserin 1 mg/kg treated group and chronic paracetamol with ketanserin 10 mg/kg treated group. * p< 0.05 compared with chronic paracetamol treated group.

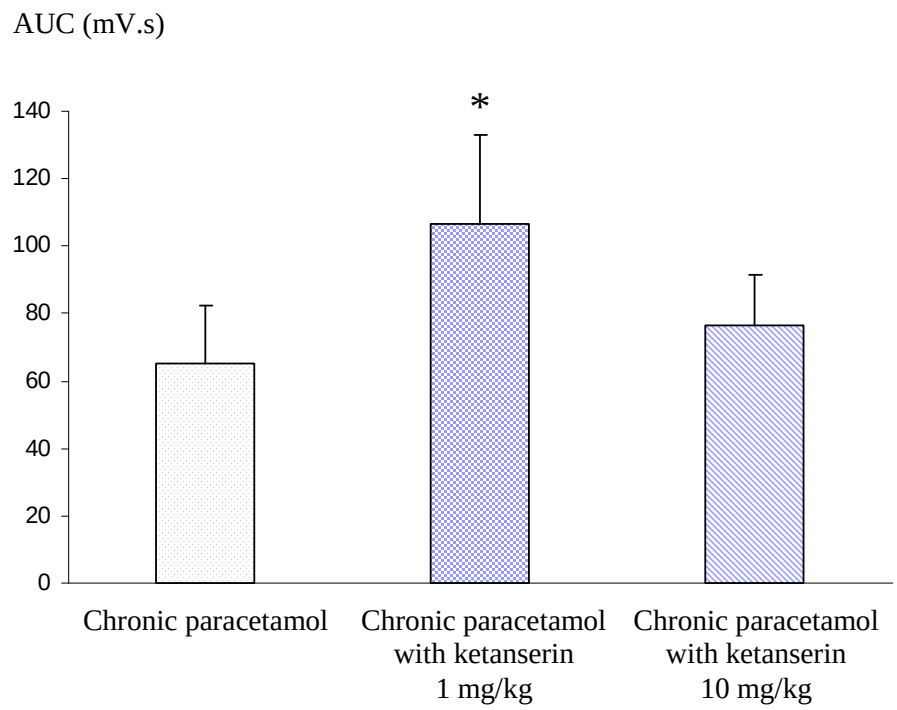

Figure4-35 Bar graphs showing the mean value ± SD of area under the curve of DC shift obtained from chronic paracetamol treated group, chronic paracetamol with ketanserin 1 mg/kg treated group and chronic paracetamol with ketanserin 10 mg/kg treated group. * p< 0.05 compared with chronic paracetamol group.

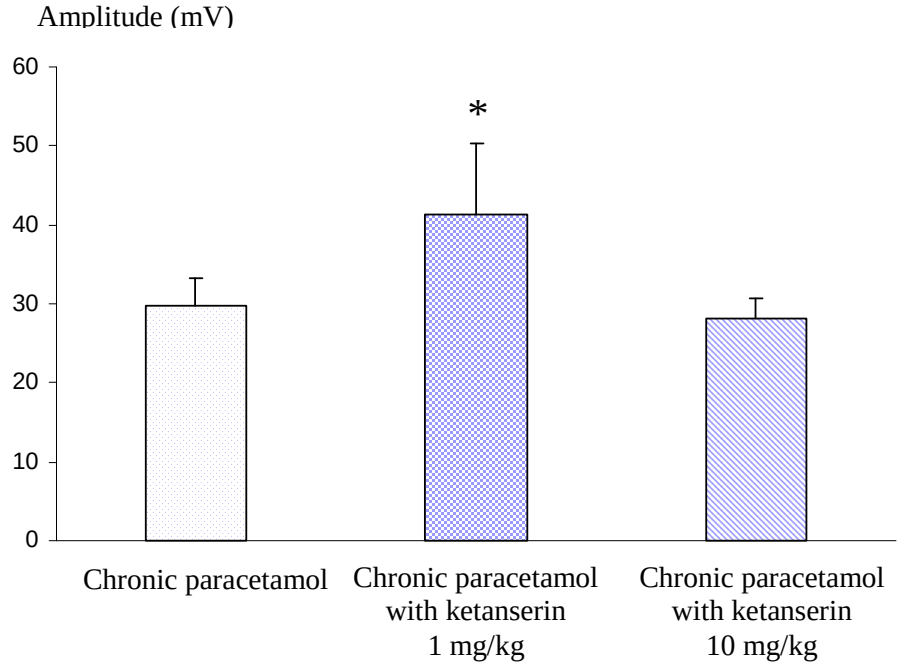

Figure4-36 Bar graphs showing the mean value ± SD of amplitude of DC shift obtained from chronic paracetamol treated group, chronic paracetamol with ketanserin 1 mg/kg treated group and chronic paracetamol with ketanserin 10 mg/kg treated group. * p< 0.05 compared with chronic paracetamol group.

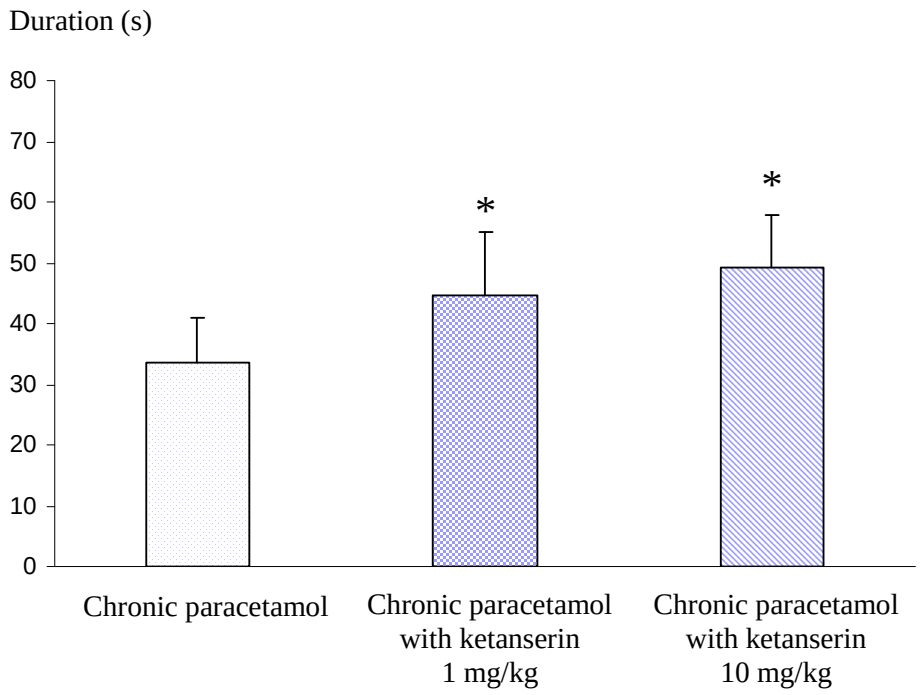

Figure4-37 Bar graphs showing the mean value ± SD of the duration of DC shift obtained from chronic paracetamol treated group, chronic paracetamol with ketanserin 1 mg/kg treated group and chronic paracetamol with ketanserin 10 mg/kg treated group. * p< 0.05 compared with chronic paracetamol treated group.

- The effect of 5-HT_{2A} receptor antagonist on Fos expression in cerebral cortex

In order to evaluate the effect of 5-HT_{2A} receptor antagonist treatment on the activation of neurons in cerebral cortex, the fos expression was measured in cerebral cortex obtained from chronic paracetamol, chronic paracetamol with ketanserin 1 mg/kg and chronic paracetamol with ketanserin 10 mg/kg treated groups.

Our immunohistochemical study showed that the induction of CSD evoked Fos expression in cerebral cortex in all group. Fos-ir cells were distributed mainly in cortical hemispheres ipsilateral side to KCl application in chronic paracetamol group. Treatment with ketanserin 1 and 10 mg/kg in chronic paracetamol substantially decreased the number of CSD-induced Fos-ir cell in ipsilateral side of the cerebral cortex compared with chronic paracetamol group (Figure4-38). Fos-ir cells in chronic paracetamol, chronic paracetamol with ketanserin 1 mg/kg and chronic paracetamol with ketanserin 10 mg/kg rat were 113.86$\pm$15.21 cells per 6.25 x 10^4 μm^2 and 103.60$\pm$22.91 cells per 6.25 x 10^4 μm^2 and 66.36$\pm$19.45 cells per 6.25 x 10^4 μm^2, respectively. Statistical analysis significantly difference only between chronic paracetamol and chronic paracetamol with ketanserin 10 mg/kg (Table4-19)

Table4-19 The mean value $\pm$ SD of the number of Fos-ir cells in cerebral cortex sections obtained from chronic paracetamol treated group and chronic paracetamol with ketanserin 1 mg/kg treated group and chronic paracetamol with ketanserin 10 mg/kg treated group.

	Chronic paracetamol	Chronic paracetamol with ketanserin 1 mg/kg	Chronic paracetamol with ketanserin 10 mg/kg
	KCl	KCl	KCl
Ipsilateral	113.86$\pm$15.21	103.60$\pm$22.91	66.36$\pm$19.45*

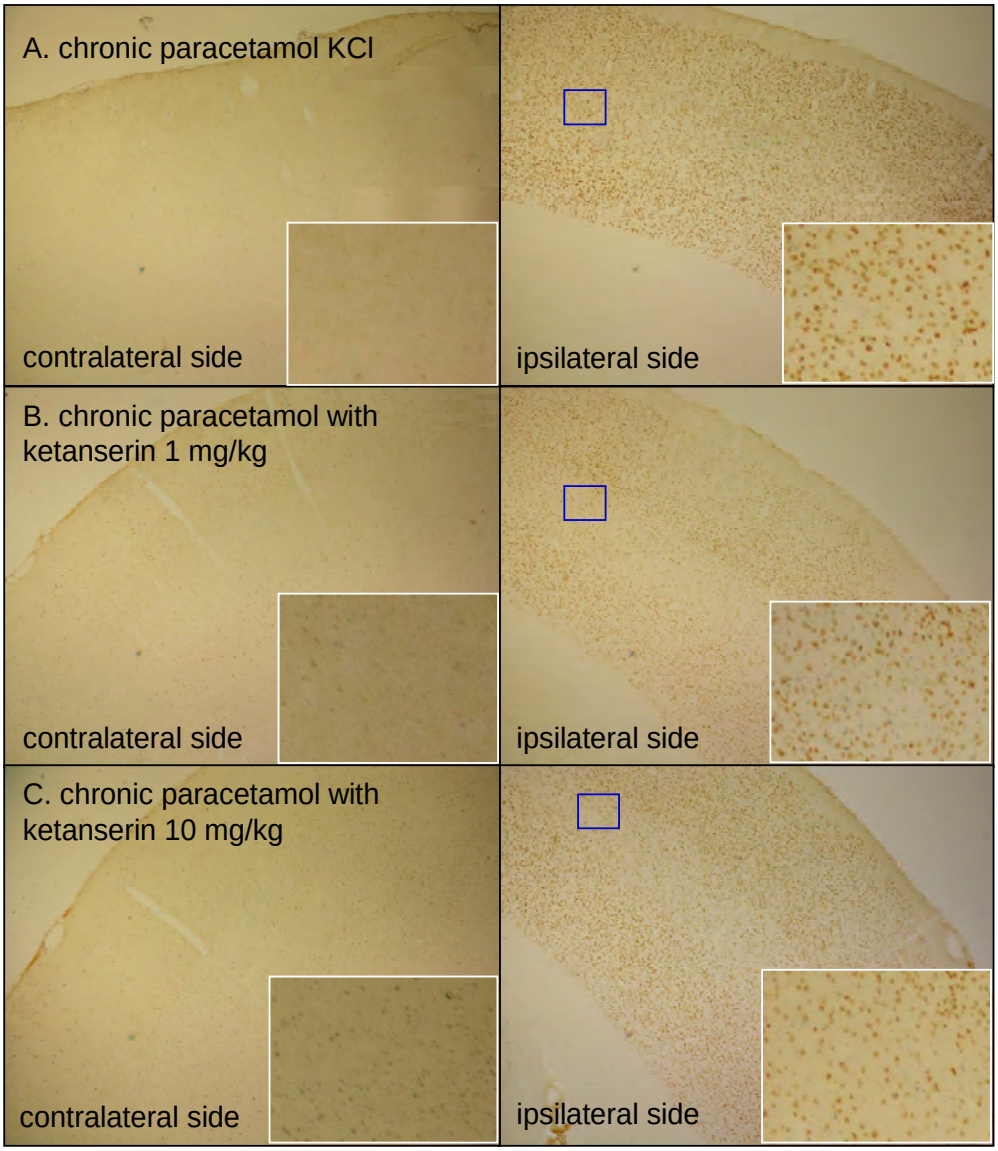

Figure4-38 The photomicrograph showing the Fos-ir cells in the brain sections ipsilateral side and contralateral side to KCl application obtained from A. chronic paracetamol treated group B. chronic paracetamol with ketanserin 1 mg/kg treated group C. chronic paracetamol treated with ketanserin 10 mg/kg treated group.

- The effect of 5-HT_{2A} receptor antagonist on 5-HT_{2A} receptor expression in cerebral cortex

In this experiment, 10 sections of brain were collected from each rat. The sections were studied under the light microscope and neurons were classified as immunoreactive or non-reactive based on the immunostaining feature. The 5-HT_{2A}-ir cells were defined as those with dark-brown stained in their cytoplasm. We found that the CSD induced an expression of 5-HT_{2A}-ir cells in the cortical layers III-VI of the cerebral cortex was the same pattern in all experimental groups. CSD induced expression of 5-HT_{2A} in the cerebral cortex. 5-HT_{2A}-ir cells were confined in cerebral cortex and were more prevalent on the side ipsilateral to the operation (Figure4-39). In chronic paracetamol group, the numbers of 5-HT_{2A}-ir cells in the ipsilateral was 19.67$\pm$4.04 cells per μm^2. Pretreatment with chronic paracetamol with ketanserin 1 mg/kg and 10 mg/kg inhibit the response of trigeminal nociceptive to CSD. The numbers of 5-HT_{2A}-ir cells in chronic paracetamol with ketanserin 1 mg/kg and 10 mg/kg group were 13.33$\pm$1.53 cells per μm^2 and 12.67$\pm$1.53 cells per μm^2 for ipsilateral side, respectively. The data are shown in Table4-20. The difference in the number of 5-HT_{2A}-ir cells between chronic paracetamol treated group and chronic paracetamol with ketanserin 1 mg/kg and 10 mg/kg groups were statistically significant (Table4-20).

Table4-20 The mean value $\pm$ SD of the number of 5-HT_{2A}-ir cells in cerebral cortex sections obtained from chronic paracetamol treated group, chronic paracetamol with ketaserin 1 mg/kg treated group, and chronic paracetamol with ketanserin 10 mg/kg treated group.

	Chronic paracetamol	Chronic paracetamol with ketanserin 1 mg/kg	Chronic paracetamol with ketanserin 10 mg/kg
	KCl	KCl	KCl
Ipsilateral	19.67$\pm$4.04	13.33$\pm$1.53*	12.67$\pm$1.53*

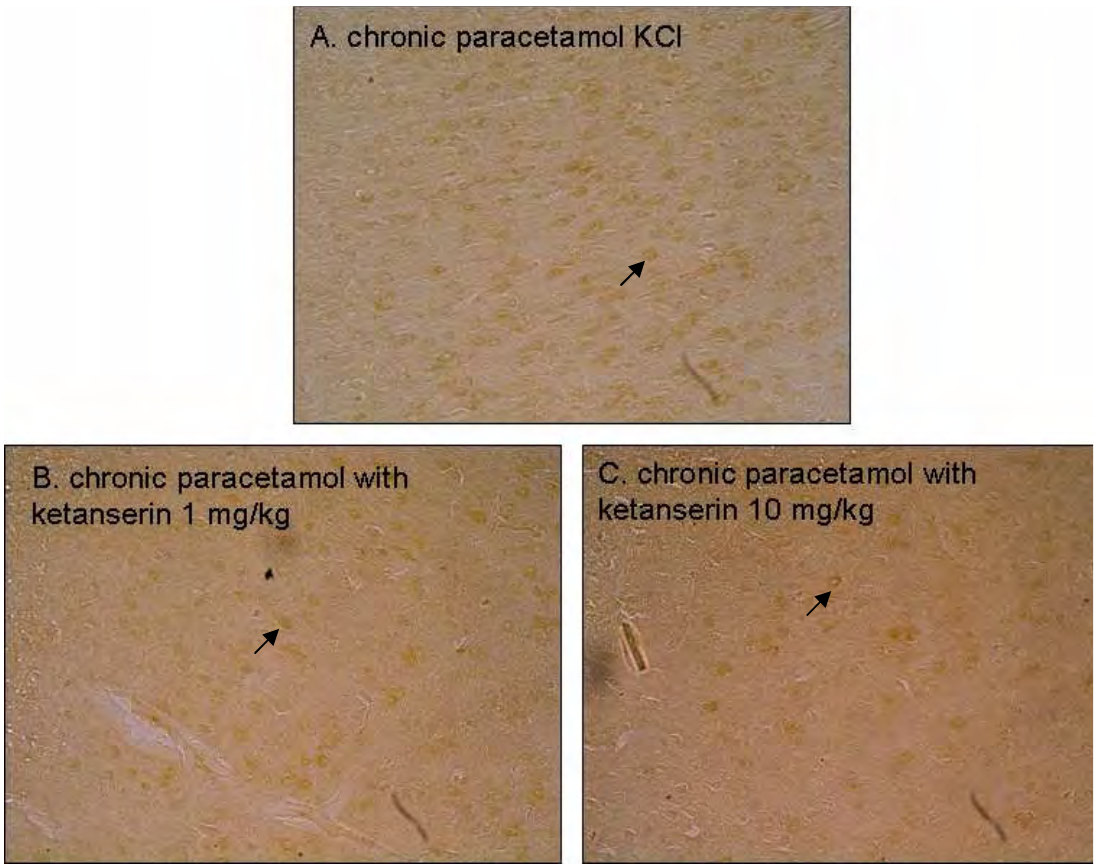

Figure4-39 The photomicrograph showing the 5-HT_{2A}-ir cells in the brain sections ipsilateral side to KCl application obtained from A. chronic paracetamol treated group B. chronic paracetamol with ketanserin 1 mg/kg treated group C. chronic paracetamol with ketanserin 10 mg/kg treated group.

- Effect of 5-HT_{2A} receptor antagonist on trigeminal neurons activity induced by CSD in chronic treatment with paracetamol

- The effect of 5-HT_{2A} receptor antagonist on Fos expression in TNC

In chronic paracetamol treated group, the numbers of Fos-ir cells in ipsilateral and contralateral sides were 16.43±2.98 cells per slide and 10.21±3.23 cells per slide, respectively. In chronic paracetamol with ketanserin 1 mg/kg treated group, the numbers of Fos-ir cells in ipsilateral and contralateral sides were 8.88±5.61 cells per slide and 4.35±2.97 cells per slide, respectively. In chronic paracetamol with ketanserin 10 mg/kg treated group, the numbers of Fos-ir cells were 6.00±3.09 cells per slide and 3.64±2.24 cells per slide for ipsilateral and contralateral sides, respectively. The data are shown in Table4-21. The difference in the number of Fos-ir cells between all groups were statistically significant (Figure4-40) (Figure4-41) (Table4-21).

Table4-21 The mean value ± SD of the number of Fos-ir cells in the C1 and C2 cervical spinal cord sections obtained from chronic paracetamol treated group, chronic paracetamol with ketanserin 1 mg/kg treated group and chronic paracetamol treated with ketanserin 10 mg/kg treated group.

	Chronic paracetamol	Chronic paracetamol with ketanserin 1 mg/kg	Chronic paracetamol with ketanserin 10 mg/kg
	KCl	KCl	KCl
Ipsilateral	16.43±2.98	8.88±5.61*	6.00±3.09*
Contralateral	10.21±3.23	4.35±2.97$^{\#}$	3.64±2.24$^{\#}$

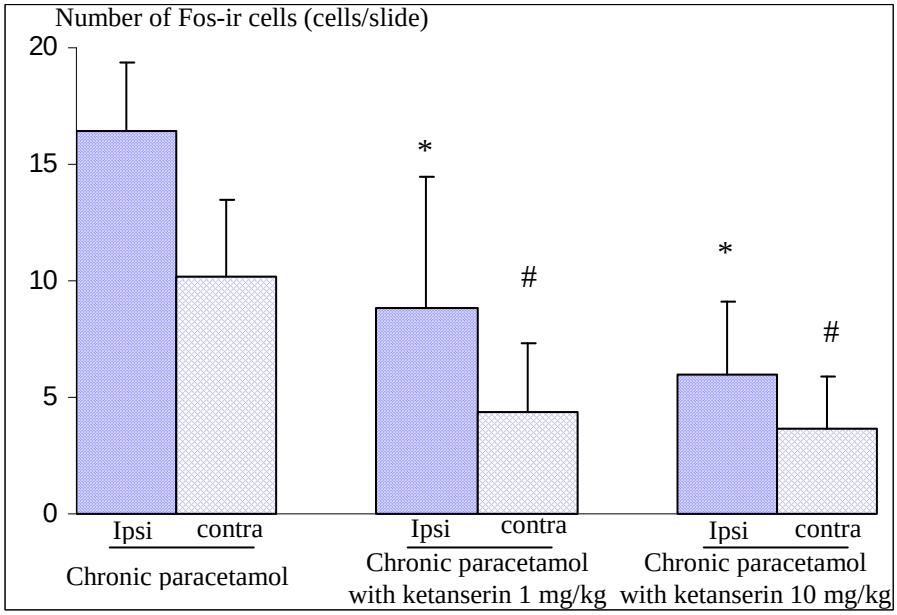

Figure4-40 Bar graph showing the mean value ± SD of the number of Fos-ir cells in the C1 and C2 cervical spinal cord sections obtained form chronic paracetamol treated group, chronic paracetamol with ketanserin 1 mg/kg treated group and chronic paracetamol with ketanserin 10 mg/kg treated group. Significant difference was assesses with ANOVA with the Bonferroni test. *p<0.05 compared with control KCl group. #p<0.05 compared with contralateral side.

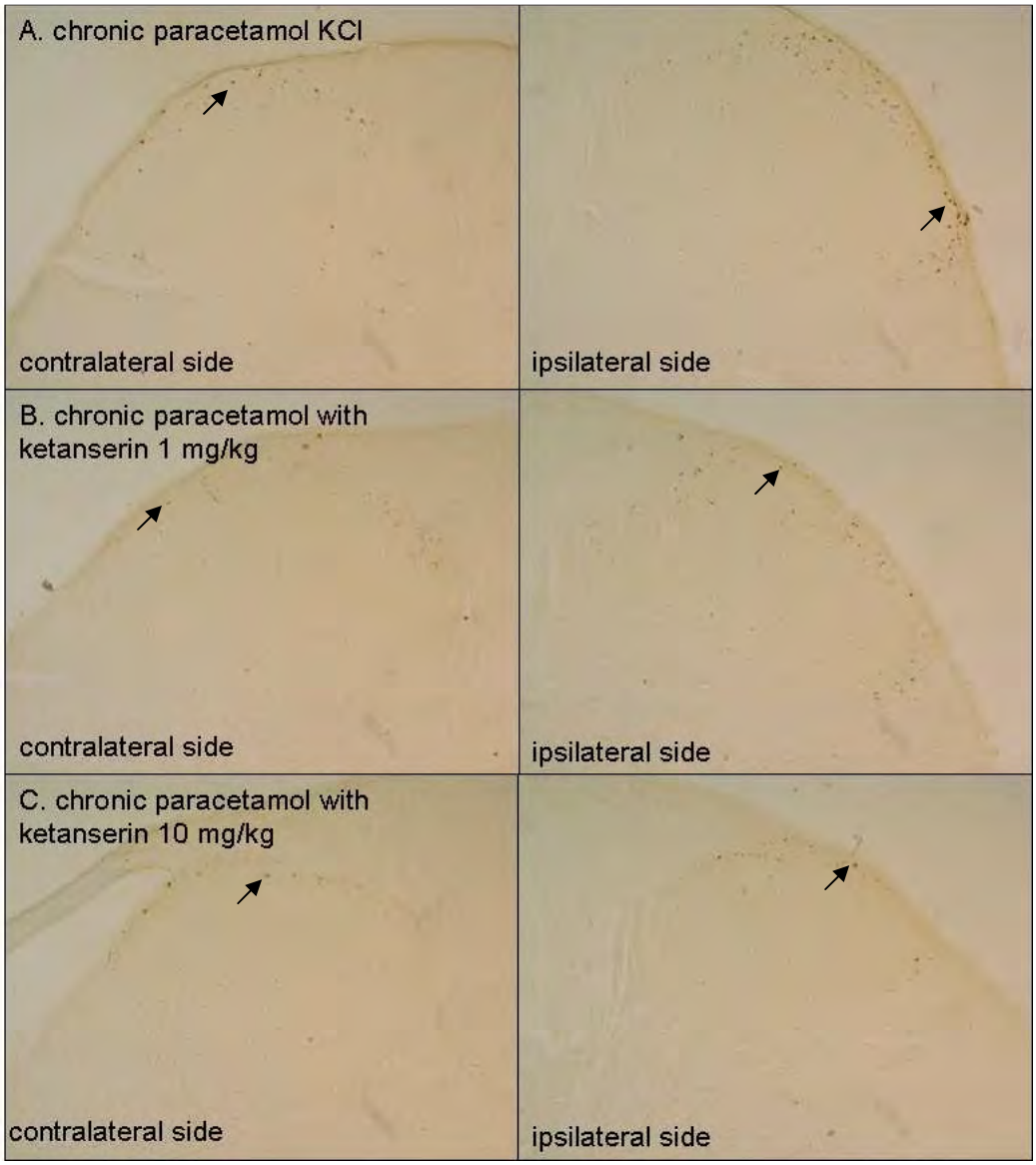

Figure4-41 The photomicrograph showing the Fos-ir cells (arrow) in the C1-C2 cervical spinal cord sections ipsilateral side and contralateral side to KCl application obtained from A. chronic paracetamol treated group B. chronic paracetamol with ketanserin 1 mg/kg treated group C. chronic paracetamol with ketanserin 10 mg/kg treated group.

- The effect of 5-HT_{2A} receptor antagonist on 5-HT_{2A} receptor expression in TG

The 5-HT_{2A}-ir cells were defined as those with dark-brown stained in their cytoplasm. It was shown that the 5-HT_{2A}-ir cells comprised small to medium sized neurons. The large-diameter neurons were usually 5-HT_{2A}-negative. The total of 100 cells was counted from each slide. Data were expressed as mean and standard deviation of percent of 5-HT_{2A}-ir cells. The effect of chronic paracetamol treated on the 5-HT_{2A} expression induced by CSD was tested by evaluating the number of 5-HT_{2A}-ir cells in the chronic paracetamol, chronic paracetamol with ketanserin 1 mg/kg and pretreated group and chronic paracetamol with ketanserin 10 mg/kg.

CSD induced expression of 5-HT_{2A} in the TG. 5-HT_{2A}-ir cells were confined in TG and were more prevalent on the side ipsilateral to the operation (Figure4-42). In chronic paracetamol group, the numbers of 5-HT_{2A}-ir cells in the ipsilateral and contralateral sides were 40.5$\pm$8.7 cells per slide and 24.1$\pm$11.4 cells per slide, respectively. The numbers of 5-HT_{2A}-ir cells in chronic paracetamol with ketanserin 1 mg/kg group were 40.1$\pm$12.5 cells per slide and 21.5$\pm$8.2 cells per slide for ipsilateral and contralateral sides, respectively. The numbers of 5-HT_{2A}-ir cells in chronic paracetamol with ketanserin 10 mg/kg group were 34.2$\pm$10.4 cells per slide and 26.4$\pm$15.3 cells per slide for ipsilateral and contralateral sides, respectively. The data are shown in Table 4-22. The difference in the number of 5-HT_{2A}-ir cells between chronic paracetamol, chronic paracetamol with ketanserin 1 mg/kg and chronic paracetamol with ketanserin 10 mg/kg were not statistically significant (Table4-22).

Table4-22 The mean value $\pm$ SD of the number of 5-HT_{2A}-ir cells in the TG sections obtained from chronic paracetamol treated group, chronic paracetamol with ketanserin 1 mg/kg treated group and chronic paracetamol treated with ketanserin 10 mg/kg treated group.

	Chronic paracetamol	Chronic paracetamol with ketanserin 1 mg/kg	Chronic paracetamol with ketanserin 10 mg/kg
	KCl	KCl	KCl
Ipsilateral	35.2$\pm$8.1	40.1$\pm$12.5	34.2$\pm$10.4
Contralateral	24.1$\pm$11.4	21.5$\pm$8.2	26.4$\pm$15.3

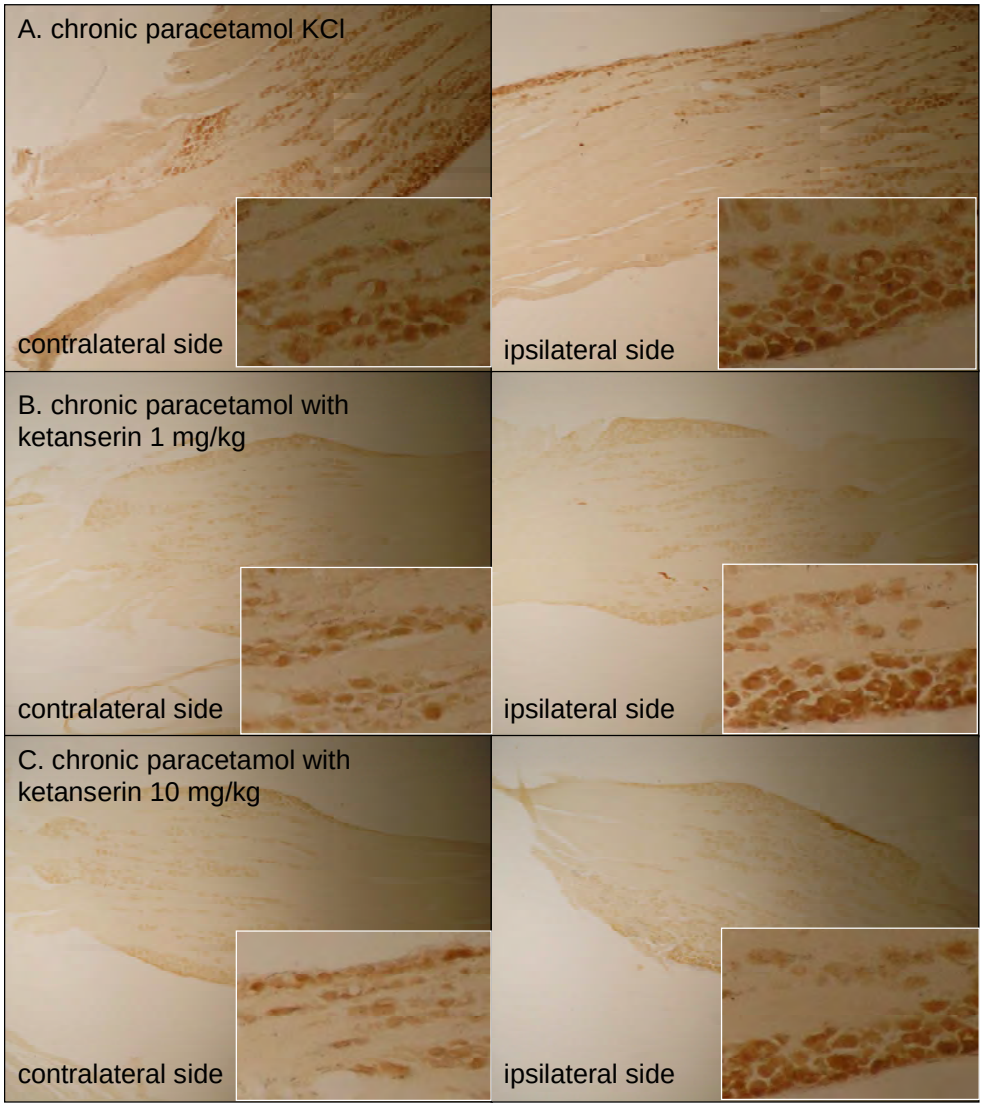

Figure4-42 The photomicrograph showing the 5-HT_{2A}-ir cells in the TG sections ipsilateral side and contralateral side to KCl application obtained form A. chronic paracetamol treated group B. chronic paracetamol with ketanserin 1 mg/kg treated group C. chronic paracetamol with ketanserin 10 mg/kg treated group.

- Effect of 5-HT_{2A} receptor antagonist on cerebral circulation induced by CSD in chronic treatment with paracetamol

- The effect of 5-HT_{2A} receptor antagonist on cortical blood flow

Application of KCl induced the repeated pattern of cerebral hyperemia. The amplitude from each peak was calculated as percent changes from the resting stage. The percent changes from base line were 199.51 $\pm$ 49.20, 212.81 $\pm$ 36.28 and 137.25 $\pm$ 12.01 in chronic paracetamol, chronic paracetamol with ketanserin 1 mg/kg and chronic paracetamol with ketanserin 10 m/kg, respectively (Table4-23). Statistical analysis showed significant difference between chronic paracetamol and chronic paracetamol with ketanserin 10 mg/kg (Table4-23). The numbers of hyperemia cycles within one hour were 13.33 $\pm$ 2.88, 16.33 $\pm$ 2.08 and 7.5 $\pm$ 2.12 peak per hour in chronic paracetamol, chronic paracetamol with ketanserin 1 mg/kg and chronic paracetamol with ketanserin 10 m/kg, respectively. The average duration of these cycles were 135.56 $\pm$ 21.97, 134.16 $\pm$ 21.19 and 112.5 $\pm$ 52.51 second in chronic paracetamol, chronic paracetamol with ketanserin 1 mg/kg and chronic paracetamol with ketanserin 10 m/kg, respectively. Statistical analysis showed significant difference between chronic paracetamol and chronic paracetamol with ketanserin 10 mg/kg (Table4-23).

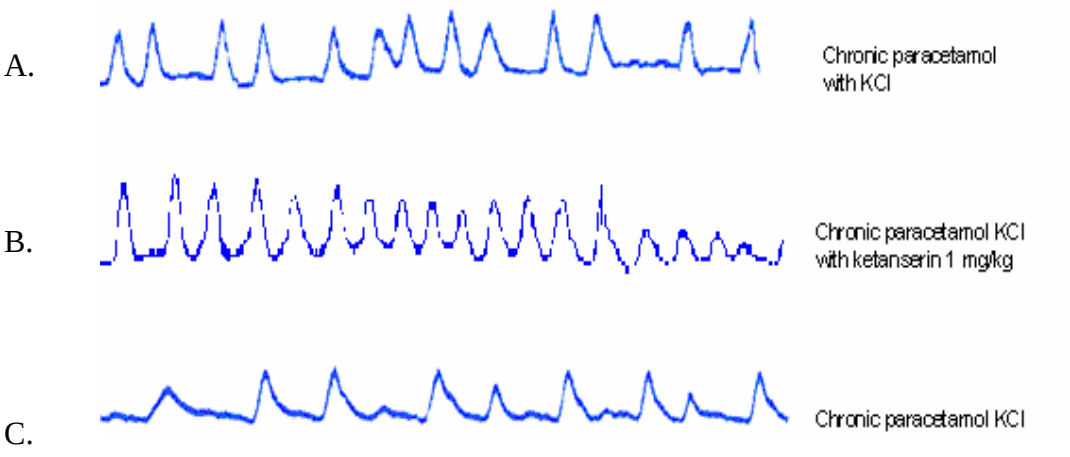

Figure4-43 The tracing showing the CBF changes obtained from A. chronic paracetamol treated group B. chronic paracetamol with ketanserin 1 mg/kg treated group C. chronic paracetamol with ketanserin 10 mg/kg treated group.

Table4-23 The number of peak hyperemia, percent changes from base line and duration of CBF between chronic paracetamol treated group, chronic paracetamol with ketanserin 1 mg/kg treated group and chronic paracetamol with ketanserin 10 mg/kg treated group.

	Chronic paracetamol	Chronic paracetamol+ ketanserin 1 mg/kg	Chronic paracetamol+ ketanserin 10 mg/kg
Number of peak (peak per hour)	13.33 ± 2.88	16.33 ± 2.08*	7.5 ± 2.12*
%Change	199.51 ± 49.20	212.81 ± 36.28	137.25 ± 12.01*
Duration (s)	135.56 ± 21.97	134.16 ± 21.19	112.5 ± 52.51*

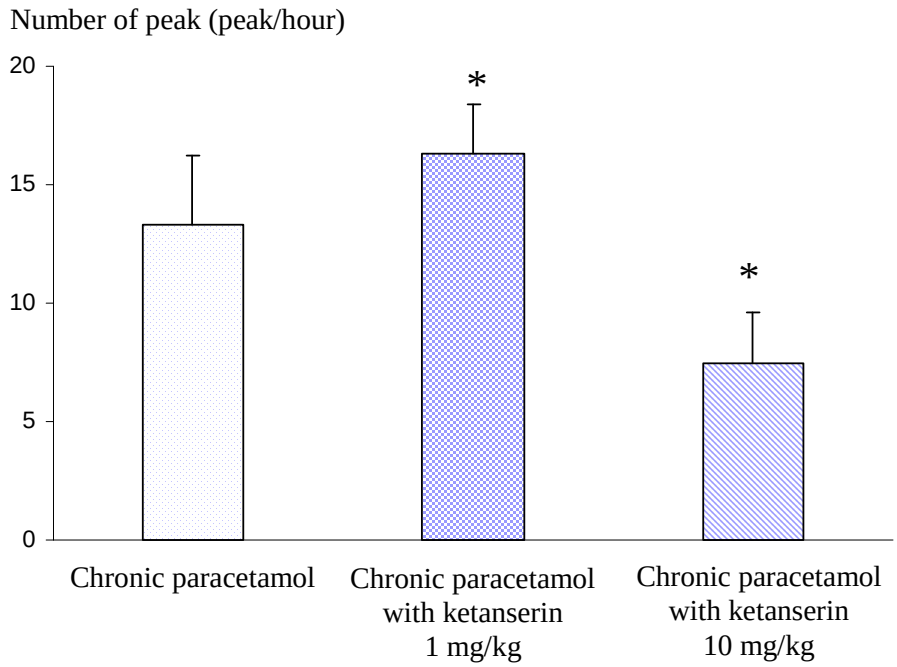

Figure4-44 Bar graphs showing the mean value ± SD of number of peak of CBF obtained from chronic paracetamol treated group, chronic paracetamol with ketanserin 1 mg/kg treated group and chronic paracetamol with ketanserin 10 mg/kg treated group. * p< 0.05 compared with chronic paracetamol group.

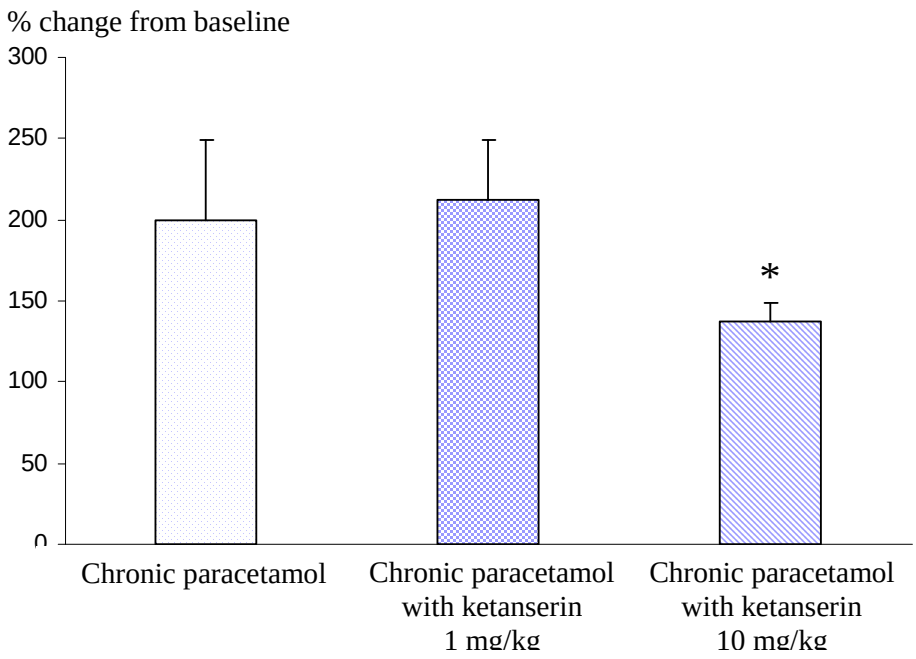

Figure4-45 Bar graphs showing the mean value ± SD of percent change from baseline of CBF obtained from chronic paracetamol treated group, chronic paracetamol with ketanserin 1 mg/kg treated group and chronic paracetamol with ketanserin 10 mg/kg treated group. * p< 0.05 compared with chronic paracetamol group.

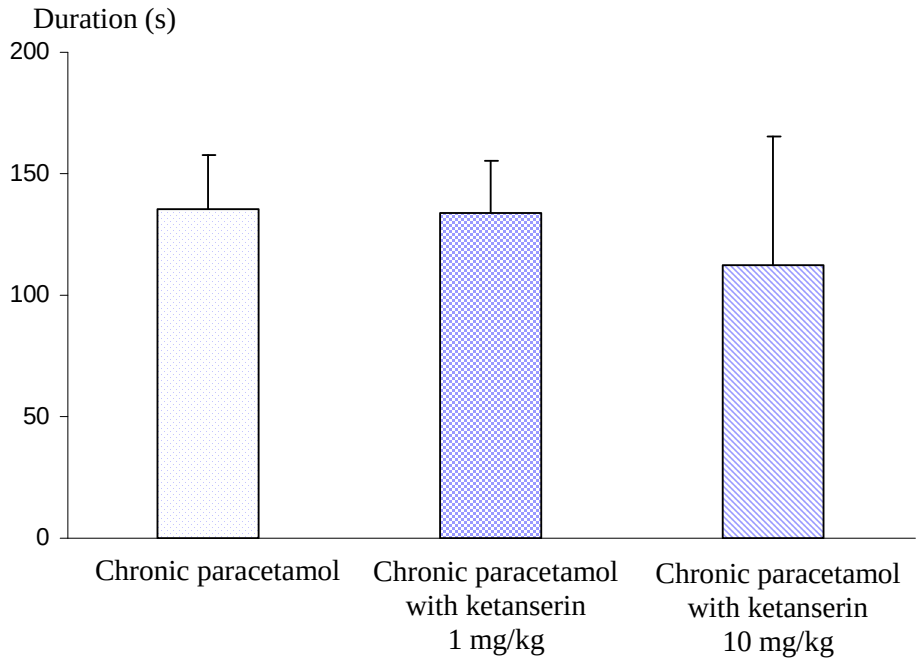

Figure4-46 Bar graphs showing the mean value ± SD of the duration of CBF obtained from chronic paracetamol treated group, chronic paracetamol with ketanserin 1 mg/kg treated group and chronic paracetamol with ketanserin 10 mg/kg treated group.

- The effect of 5-HT_{2A} receptor antagonist on ultrastructural of cerebral microvessels

The number of microvilli in capillaries and arteriole in chronic paracetamol were 3.26±1.33 and 23.75±11.03 microvilli per vessel, respectively. In the chronic paracetamol with ketanserin 1 mg/kg group, the number of microvilli in capillaries and arteriole was 3.02±1.13 and 25.24±7.36 microvilli per vessel, respectively and in the chronic paracetamol with ketanserin 10 mg/kg the number of microvilli in capillaries and arteriole was 2.94±0.82 and 21.67±9.21 microvilli per vessel, respectively. However, the number of microvilli in capillary and arteriole was not significant different in all groups. The data are shown in Table4-24 and Figure4-47.

The average density of pinocytic vesicles in capillaries and arteriole in chronic paracetamol were 44±7 and 49±11 microvilli per vessel, respectively. In the chronic paracetamol with ketanserin 1 mg/kg group, the number of microvilli in capillaries and arteriole were 39±12 and 45±8 microvilli per vessel, respectively and in the chronic paracetamol with ketanserin 10 mg/kg the number of microvilli in capillaries and arteriole were 41±5 and 42±9 microvilli per vessel, respectively. The difference in the number of pinocytic vesicles was not statistically significant. The data are shown in Table 4-24 and Figure 4-48.

Table4-24 The mean value ± SD of the number of microvilli per vessel and the number of pinocytic vesicle/um^2 in the endothelial cell of cerebral capillaries and arterioles obtained from chronic paracetamol treated group, chronic paracetamol with ketanserin 1 mg/kg treated group and chronic paracetamol with ketanserin 10 mg/kg treated group.

Group	Chronic paracetamol	Chronic paracetamol with ketanserin 1 mg/kg	Chronic paracetamol with ketanserin 10 mg/kg
Microvilli (number of microvilli/ vessel)			
Capillary	3.26±1.33	3.02±1.13	2.94±0.82
Arteriole	23.75±11.03	25.24±7.36	21.67±9.21
Pinocytic vesicles (number of pinocytic vesicles/μm^2)			
Capillary	44±7	39±12	41±5
Arteriole	49±11	45±8	42±9

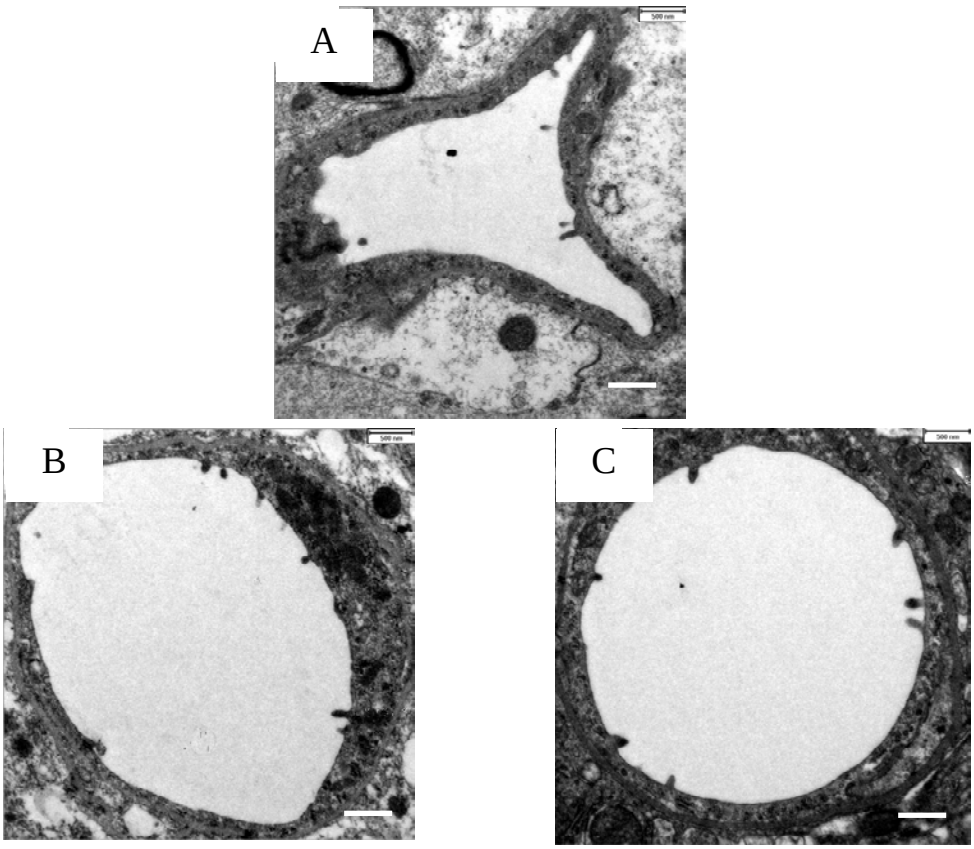

Figure4-47 Electron micrograph of an endothelial cell obtained from A. chronic paracetamol treated group B. chronic paracetamol with ketanserin 1 mg/kg treated group C. chronic paracetamol with ketanserin 10 mg/kg treated group. (x5000) Bar=500 nm.

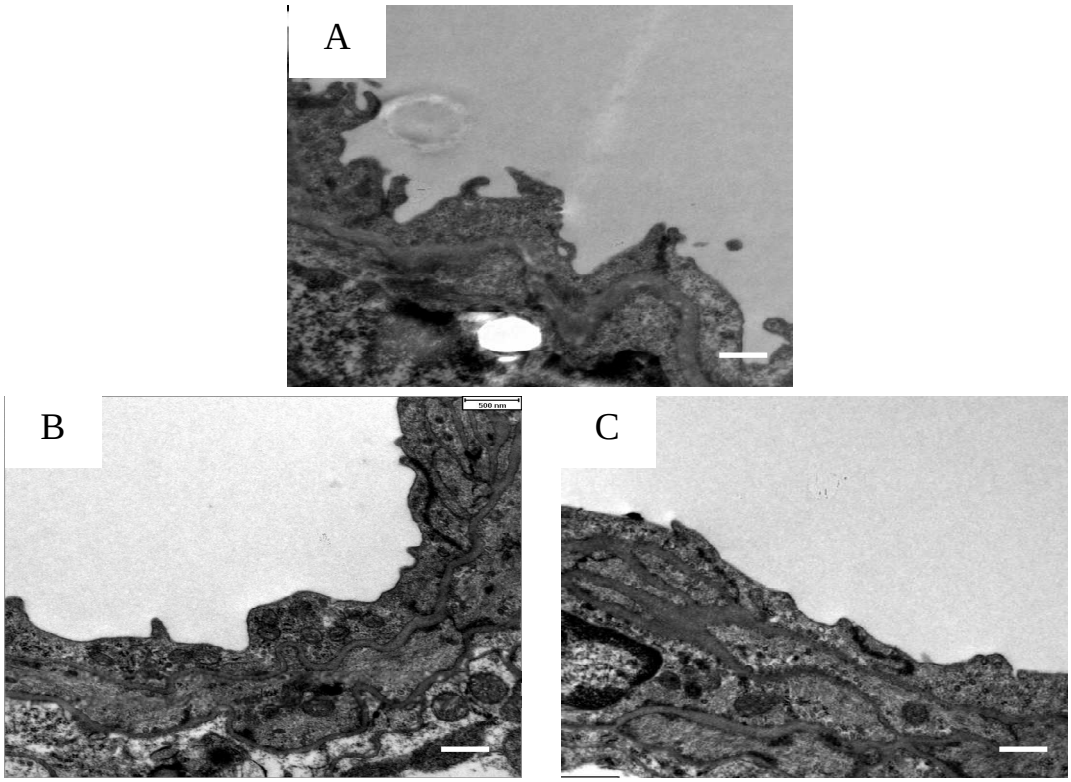

Figure4-48 Electron micrograph of an endothelial cell obtained from A. chronic paracetamol B. chronic paracetamol with ketanserin 1 mg/kg C. chronic paracetamol treated with ketanserin 10 mg/kg. (x5000) Bar=500 nm.

CHAPTER V

DISCUSSION

This study was conducted to investigate the effects of acute and chronic treatement with paracetamol on the trigeminovascular nociceptive system. The first part was conducted to investigate the effect of acute treatment with paracetamol on trigeminovascular nociception evoked by CSD. The second part was conducted to investigate the effect of chronic treatment paracetamol on trigeminovascular nociception evoked by CSD. The third part was conducted to investigate the involvement of 5-HT_{2A} receptor on trigeminovascular nociception evoked by CSD in chronic treatment paracetamol. From the results, it could be discusses as following.

I. The effect of acute treatment with paracetamol on trigeminovascular nociception evoked by CSD.

The present study demonstrated that the acute treatment of a nonnarcotic analgesic, paracetamol, induced changes in importance parameters of the trigeminovascular nociceptive system. Acute treatment with paracetamol reduces the number of CSD-evoked Fos-IR cells in TNC and cerebral cortex without changes in CSD frequency. The cerebral vascular change was also decrease only in the percent change from baseline of CBF but not change in ultrastructral of cerebral endothelial cells. However, 5-HT_{2A} receptor expression showed not significantly difference in acute paracetamol-treated rats compared with the control.

In this study, we observed an increase in the amplitude of CSD without change in it frequency in rats treated with acute treatment with paracetamol. Besides, frequency, changes in the other CSD variables has also been reported. Reduced threshold and increased propagation velocity have been reported in R192Q knock-in mice, an animal model for familial hemiplegic migraine (van den Maagdenberg et al., 2004). Estrogens and progesterone have been reported to enhance the repetition rate as well as the amplitude of spreading depression in neocortical slices treated with hypotonic artificial cerebrospinal fluid or KCl microinjection. Base on these findings, modification of CSD frequency is unlikely to be the mechanism underlying analgesic effect of paracetamol in CSD-evoked trigeminal nociception.

The present study clearly showed that acute treatment with paracetamol is effective in attenuating the effect of CSD in activating the trigeminal nociceptive pathway. Due to the lack of anti-inflammatory activity of paracetamol, its anti-nociceptive effect should be explained by central mechanism. Since paracetamol has low binding affinity to amine or opioid transmitters, it is unlikely that this agent modifies the endogenous pain control system by direct alteration of these important nociceptive control transmitters. Other transmitter systems i.e. nitric oxide may be involved in this anti-nociceptive process. In 1994, Björkman et al showed that the antinociceptive effect of paracetamol was readily reversed by administration of the natural substrate for nitric oxide synthase (NOS), L-arginine (Björkman et al 1994). Pretreatment with NO antagonist also potentiates the anti-nociceptive effect of

suboptimal dose of paracetamol (Bujalska and Gumulka 2001, Bujalska 2004). These observations are supported by the finding that paracetamol is able to inhibit NO synthesis in the rat spinal cord slice (Godfrey et al 2007). These evidences suggested that the analgesic effect of paracetamol may be related to inhibition of NO generation. In the vascular compartment, acute treatment with paracetamol reduces the percent change from baseline of CBF induced by CSD but not change in ultrastructral of cerebral endothelial cells.

Recent evidences have suggested the roles of the serotonin system in antinociceptive mechanism of paracetamol (Mallet et al., 2008). Previously experiments have shown that treatment with paracetamol can alter the expression of 5-HT_{2A} receptor in frontal cortex (Srikiatkhachorn et al 1999 and 2000). Since this class of receptor has strong excitatory effect on post-synaptic neurones, the change in its density is likely to alter the level of cortical excitability. However, the present study failed to demonstrate any significant changes in the 5-HT_{2A} receptor expression in TG and in cerebral cortex in the acute paracetamol treated rat.

II. The effect of chronic treatment with paracetamol on trigeminovascular nociception evoked by CSD.

The present study showed that the chronic treatment with paracetamol induced changes of the trigeminovascular nociceptive system. Long term exposure to paracetamol increases the CSD frequency, and CSD-evoked Fos expression in TNC and cerebral cortex. Moreover, chronic treatment with paracetamol leads to increase the expression of 5-HT_{2A} receptor in TG and cerebral cortex and vascular endothelial cell compared with the chronic controls rats. Concerning CBF, paracetamol reduces the percent change from baseline of CSD-evoked cortical hyperaemia regardless of the duration of exposure.

CSD is believed to be the physiological mechanism underlying aura phase of migraine. Changing in CSD frequency may reflect the excitability of cerebral cortex and susceptibility to develop the attacks of migraine. Several factors have been reported to affect the frequency of CSD waves. In general, factors or conditions that reduce neuronal excitability will reduce the frequency of CSD while those which increase the excitability will also increase the CSD frequency. For instance, CSD frequency is reduced in rats treated with anaesthetics such as halothane, isofurane and sevoflurane (Kitahara et al., 2001). Therefore, the increase in the CSD frequency observed in this present study may indicate that the long term treatment with paracetamol may lead to the state of cortical hyperexcitability in these rats. This postulation is supported by our findings which demonstrate an increase in the number of Fos-ir cells in both cerebral cortex and TNC in animals with prolonged paracetamol treatment.

In the vascular compartment, chronic treatment with paracetamol reduces the percent change from baseline of CBF induced by CSD. On the other hand, chronic paracetamol exposure leads to an increase CSD-evoked the changes of ultrastructral of cerebral endothelial cells. After CSD activation, perivascular nerve terminals release several substances, i.e. substance P, that promote vasodilatation and increase vascular permeability. Previous evidence confirmed the role of perivascular nerve, namely trigeminal and parasympathetic nerves, in contribution to CSD-associated hyperaemia (Reuter et al, 1998). These released peptides are also algogenic and can

further sensitize the surrounding nociceptors. As shown here, CSD can induce substantial changes in ultrastructure of cerebral microvessels. Although the endothelial tight junction was still intact and cerebral endothelial cells increased pinocytosis and perivascular astrocytic footplates were mildly swollen. These findings reflected increased transendothelial transport. These ultrastructural changes were non-specific and can be found in various conditions, e.g. concussive brain injury, hypertensive encephalopathy (Wei, 1980, Hazema et al, 1978). It is known that paracetamol can induce nitric oxide production. Therefore, long term treated with paracetamol may induce vasorelaxation and increase transendothelial transports.

It is widely recognized that 5-HT has diverse modulatory effects on cortical neurons depending on the receptor subtypes. Pattern of 5-HT receptors distribution in the CNS are complex and depend on subtypes of receptor. 5-HT_2 receptors reveal a specific distribution in many brain areas including the frontal cortex (Leysen and Powels, 1990), and the cortex is one of the most important axonal projection targets for neurons derived from brainstem. Actually cerebral cortex presents highest density of 5-HT_2 receptors. The present of very high densities of these receptors in the neocortex, especially over the pyramidal cell layers, which receive afferents from several central structures, suggest their involvement in the regulation of many brain functions, including nociceptive modulation. The previous studies show that the reduction in the number of 5-HT_{2A} receptors in the cortical, but not in the pontine area could depend on the different density of receptors in these areas and would emphasize the role of the cortex as the end terminal for the serotonergic antinociceptive system. Mechanisms of paracetamol induces 5-HT_{2A} receptors plasticity are not well understood. It has been recently shown that paracetamol has no binding affinity to either 5-HT receptors or transporters (Raffa and Codd, 1996). Since there is no evidence that paracetamol acts directly on any subtypes of 5-HT receptors, the plasticity of such receptors should be resulted from other indirect interactions. Many findings have high lighten the complexity of the adaptive mechanisms of the 5-HT system, but the monoamine adaptation theory implies that a persistent exposure to agonist or endogenous neurotransmitters results in receptor down regulation. Chronic paracetamol administration of a more selective 5-HT_2 receptor agonist, such as DOI, has been shown to decrease 5-HT_2 receptor density (Bukholts et al., 1988; Mc Kenna et al., 1989). Generally, activation of 5-HT_1 receptor exerts inhibitory effect while

activation of 5-HT_2 receptor leads to cortical activation (Araneda et al., 1991). Therefore, the increased cortical excitability observed in the chronic paracetamol treated group may have resulted either from 5-HT_2 receptor activation. The pattern of paracetamol induced 5-HT_{2A} receptors adaptation observed in this study is interest. As the results, we have showed that 5-HT_{2A}-ir cells were increased in TG in 30 days of paracetamol administration. If analgesic efficacy is seemed to depend on 5-HT_{2A} receptors regulation, the reverse of such down regulation observed in 30 day treated group would result in the reduction of analgesic efficacy demonstrated by the tail flick test (Srikiatkhachorn et al., 2000).

It has been accepted that prolonged reductions of pain sensation by any processes including chronic analgesic consumption often lead to functions changes to restore sensitivity. The phenomenon of analgesic rebound headache (Kudrow, 1982) many represent a situation where such a compensatory mechanism plays a major role in perpetuation of pain. An up regulation of 5-HT_{2A} receptors has been reported in migraine patient with analgesic overuse headache (Srikiatkhachorn et al., 1994). As well as in 2000, Srikiatkhachorn et al reported that 5-HT_{2A} receptor was up-regulated in the condition with 5-HT depletion state such as migraine patients with analgesic overuse and rats chronically treated with paracetamol. In 2002, Sandrini et al investigated that analgesic effect of rofecoxib, a cyclo-oxygenase II inhibitor, was associated with increased 5-HT levels and down-regulation of 5-HT_2 receptor in frontal cortex. These observations indicate the relationship between chronic analgesic overuse and 5-HT_{2A} receptor expression. Since this subtype of 5-HT receptor involves in pain facilitation, an increase in receptor numbers observed in these patients may results in a hyperalgesic state and the development of chronic daily headache. These data provide further evidence for trigeminovascular nociception and a central 5-HT dependent antinociceptive effect of paracetamol. Therefore, in addition to an increase in cortical hyperexcitability, chronic paracetamol treatment also interferes with trigeminovascular nociceptive control and 5-HT dependent antinociceptive system due to excessive analgesic may result in an increase in headache frequency. The changes in 5-HT_{2A} receptor involve in trigeminovascular nociception may be an important mechanism related to the loss of analgesic efficacy and, in its more extreme form, may produce analgesic related painful conditions included analgesic overuse headache.

III. The effect of 5-HT_{2A} receptor antagonist on trigeminovascular nociception evoked by CSD in chronic treatment with paracetamol.

In order to test whether, the 5-HT_{2A} receptor involve in the enhancement of the trigeminovascular nociception in chronic treatment with paracetamol. The effect of pretreatment with ketanserin at either low dose 1 mg/kg or high dose 10 mg/kg was evaluated. Our results showed that pre-treatment with ketanserin 1 mg/kg reduce the number of CSD-evoked Fos-IR in the TNC without changes in CSD frequency. However, pre-treatment with ketanserin 10 mg/kg inhibit the CSD frequency and CSD-evoked Fos expression in TNC and cerebral cortex. Moreover, both of ketanserin concentrations lead to decrease the expression of 5-HT_{2A} receptor in cerebral cortex but not in TG. The cerebral vascular change was also decrease only in the percent change from baseline of CBF in ketaserin 10 mg/kg treated rats but was not change in ultrastructral of cerebral endothelial cells.

Although there are several lines of evidences indicate the relation between the 5-HT_{2A} receptor and nociception, the role of this receptor in the nociception is still unclear. Several studies demonstrated that 5-HT_{2A} receptor may have some role in anti-nociception (Bardin et al., 2000). Recent studies have demonstrated that the intrathecally administered 5-HT receptor agonists suppressed inflammatory pain or neuropathic pain, which were reversed by ketanserin (Obata et al., 2001, Sasaki et al., 2001). In 2001, Courade and coworker show that ketanserin significantly reduced the paracetamol induced anitinociception. However the study by Srikiatkhachorn and coworker in 2002 demonstrated that chronic paracetamol administration can up-regulate the expression of 5-HT_{2A} receptor in frontal cortex. In clinical study, the maximum number of binding of this receptor on the platelets is increased in migraine patients with analgesic overuse. This up-regulation becomes normalized after drug withdrawal. In the animal experiment, this up-regulated in frontal cortex of rat chronically treated with paracetamol.

In this experiment, the extensive Fos expression in somatosensory cortex and TNC induced by chronic treatment with paracetamol was attenuated by pre-treatment with 5-HT_{2A} receptor antagonist, ketanserin 1 and 10 mg/kg. This result can indicate that the upregulation of the 5-HT_{2A} receptor is involved in the enhancement of the trigeminovascular nociception observed in the rats with the chronic treatment of paracetamol.

CHAPTER VI

CONCLUSION

The results of this study demonstrated that the acute and long term treatment with paracetamol can alter trigeminovascular nociception induced by CSD. The acute treatment with paracetamol can attenuate the CSD-evoked trigeminal nociception as well as minimise the expression of fos in the trigeminal system without interfering with the process of CSD development. According to these results, it can be concluded that the modulation of the cortical neuron activities is not the mechanism involved in the anti-nociceptive effect of paracetamol when using it as acute treatment.

Interestingly, we found that the chronic treatment with paracetamol significant enhance the trigeminovascular nociception induced by CSD as indicated by increase in the cortical neuron activity, trigeminal nociception and ultrastructural changes of the cerebral endothelial cells. The chronic treatment with paracetamol also induced the up-regulation of 5-HT_{2A} receptors in both cerebral cortex and trigeminal ganglion cells. The enhancement of trigeminal nociception in chronic paracetamol-treated group was attenuated by pretreatment with ketanserin, the 5-HT_{2A} receptor antagonist. These observations reflect that up-regulation of 5-HT_{2A} receptor is at least take part in the enhancement of the trigeminovascular nociception.

The above findings indicate that the chronic treatment of paracetamol induces the hypersensitivity of the trigeminovascular nociceptive system in the response to CSD in both vascular and neural compartments. These alterations are related with the up-regulation of 5-HT_{2A} receptor. Therefore, the 5-HT_{2A} receptor plasticity may be one mechanism involve in the pathophysiology of analgesic overuse headache.

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