ผลของเมลาโทนินในการควบคุมการเกิดความเจ็บปวดจากหลอดเลือดในสมอง

นางสาว ศุภางค์ มณีศรี

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EFFECT OF MELATONIN ON THE CONTROL OF CEREBROVASCULAR NOCICEPTIVE SYSTEM.

Supang Maneesri

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By	Supang Maneesri
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Thesis Advisor	Professor Anan Srikiatkhachorn, M.D.
Thesis Co-advisor	Associate Professor Suthiluk Patumraj, Ph.D. Associate Professor Pansiri Phansuwan, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor's Degree

.....Dean of the Graduate School
(Professor Suchada Kiranandana, Ph.D.)

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.....Thesis Co-advisor
(Associate Professor Suthiluk Patumraj, Ph.D.)

.....Thesis Co-advisor
(Associate Professor Pansiri Phansuwan, Ph.D.)

.....Member
(Associate Professor Boonyong Tantisira, Ph.D.)

.....Member
(Associate Professor Chuthamanee Suthisisang, Ph.D.)

ศุภางค์ มณีศรี: ผลของเมลาโทนิน ในการควบคุมการเกิดความเจ็บปวดจากหลอดเลือดสมอง (EFFECT OF MELATONIN ON THE CONTROL OF CEREBROVASCULAR NOCICEPTIVE SYSTEM) อ. ที่ปรึกษา: ศ. นพ. อนันต์ ศรีเกียรติขจร, อ. ที่ปรึกษาร่วม: รศ. ดร. สุทธิลักษณ์ ปทุมราช, รศ. ดร. ปานสิริ พันธุ์สุวรรณ; 202 หน้า ISBN 974-17-5461-2

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาบทบาทของเมลาโตนินต่อระบบควบคุมการเกิดความเจ็บปวดจากการกระตุ้นหลอดเลือดในสมองในหนูทดลองสองภาวะ คือภาวะปกติและภาวะที่ได้รับการกระตุ้น โดยกลุ่มที่ได้รับการกระตุ้นแบ่งสัตว์ทดลองออกเป็นสองกลุ่มใหญ่ๆ คือกลุ่มสัตว์ทดลองที่ได้รับการกระตุ้นโดยการวางผลึกโปแตสเซียมคลอไรด์ที่ผิวสมอง เพื่อให้เกิดปรากฏการณ์คอร์ติคัล สเปรดดิ้ง ดีเพรสชั่น (Cortical spreading depression; CSD) และกลุ่มสัตว์ทดลองที่ได้รับสารกลีเซอรีลไตรไนเตรททางหลอดเลือดดำ เพื่อกระตุ้นหลอดเลือดสมองด้วยไนตริคออกไซด์ โดยการศึกษาทั้งหมดทำการตรวจวัดการเปลี่ยนแปลงของหลอดเลือดสมองและระบบการไหลเวียนเลือดเฉพาะที่ของเปลือกสมองด้วยกล้องฟลูออเรสเซนต์และ Laser Doppler Flowmetry (LDF) ตรวจวัดการเปลี่ยนแปลงของเซลล์เยื่อบุหลอดเลือดด้วยกล้องจุลทรรศน์อิเลคตรอนแบบส่องผ่าน และตรวจวัดการกระตุ้นระบบรับความเจ็บปวดจากหลอดเลือดสมอง โดยการตรวจหาเอนไซม์นอสและโปรตีนฟอสในกลุ่มเซลล์ไตรเจมินัลนิวเคลียสคอดาลลิส

ผลการศึกษาพบว่าเมลาโตนินมีผลทำให้หลอดเลือดสมองหดตัวในภาวะปกติและในกลุ่มสัตว์ทดลองที่ได้รับการกระตุ้นโดยโปแตสเซียมคลอไรด์มีการเพิ่มขึ้นของอัตราการไหลเวียนเลือดเฉพาะที่ในลักษณะที่เป็นวงจร ส่วนการกระตุ้นด้วยกลีเซอรีลไตรไนเตรททำให้หลอดเลือสมองเกิดการขยายตัว ผลจากการกระตุ้นสัตว์ทดลองทั้งสองวิธีทำให้ระดับเอนไซม์นอสและโปรตีนฟอสในกลุ่มเซลล์ไตรเจมินัลนิวเคลียสคอดาลลิสเพิ่มขึ้น ส่วนผลการศึกษาด้วยกล้องจุลทรรศน์อิเลคตรอนพบว่าเซลล์เยื่อบุหลอดเลือดในกลุ่มสัตว์ทดลองทั้งสองกลุ่มมีจำนวน pinocytic vescicle และ microvilli เพิ่มขึ้นอย่างชัดเจน

นอกจากนี้ยังพบว่าสัตว์ทดลองที่ได้รับเมลาโตนินก่อนการกระตุ้นมีการเปลี่ยนแปลงของอัตราการไหลเวียนเลือดเฉพาะที่และการขยายตัวของหลอดเลือดจากการกระตุ้นลดลงเมื่อเปรียบเทียบกับกลุ่มที่ไม่ได้รับเมลาโตนิน และการศึกษาด้วยกล้องจุลทรรศน์อิเลคตรอนให้ผลในทำนองเดียวกัน โดยพบว่าเซลล์เยื่อบุหลอดเลือดในกลุ่มที่ได้รับเมลาโตนินมีการเปลี่ยนแปลงลดลง

นอกจากนี้ยังพบว่าการให้เมลาโตนินในสัตว์ทดลองสามารถลดการกระตุ้นระบบรับความเจ็บปวดจากหลอดเลือดสมองได้โดยพบว่าเอนไซม์นอสและโปรตีนฟอสในไตรเจมินัลนิวเคลียสคอดาลลิสจากการกระตุ้นทั้งสองวิธีลดลง ในกลุ่มที่ได้รับเมลาโตนิน

จากการที่เมลาโตนินสามารถยับยั้งการกระตุ้นต่อระบบหลอดเลือดสมองและระบบรับความเจ็บปวดจากหลอดเลือดสมองในสัตว์ทดลองที่ถูกกระตุ้นทั้งสองวิธี บ่งชี้ว่าเมลาโตนินนั้นน่าจะมีผลต่อต้านการปวดศีรษะไมเกรนได้

หลักสูตร.....สหสาขาสรีรวิทยา.....ลายมือชื่อนิสิต.....
สาขาวิชา..... สรีรวิทยา .....ลายมือชื่ออาจารย์ที่ปรึกษา.....
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEYWORD MIGRAINE/ MELATONIN/ NITRIC OXIDE/ CORTICAL SPREADING DEPRESSION/ FOS/ nNOS
SUPANG MANEESRI: EFFECT OF MELATONIN ON THE CONTROL OF CEREBROVASCULAR NOCICEPTIVE SYSTEM. THESIS ADVISOR: PROF. ANAN SRIKIATKHACORN, M.D. THESIS CO-ADVISOR: ASSOC. PROF. SUTHILUK PATUMRAJ, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. PANSIRI PHANSUWAN, Ph.D. 202 pp ISBN 974-17-5461-2.

Clinical evidences show the inverse relationship between migraine attack and sleep. Melatonin, a pineal hormone which mainly being produced at night, has been hypothesized to involve in migraine pathophysiology. Several biological effect of melatonin, i.e. vasoconstriction, pain modulation, antioxidant, etc. imply its potential benefit in migraine prevention. Its nocturnal surge may play a role in inhibiting the development of migraine during sleep period. This study was conducted to investigate the effects of melatonin in controlling the trigeminovascular nociceptive system.

Adult male Wister rats weighing 250-300 g was divided into multiple groups of five rats. Two experimental models for migraine, namely cortical spreading depression (CSD) and nitric oxide-donor (nitroglycerine, GTN) infusion models were chosen in this study. CSD was induced by topical application of three milligram of solid potassium chloride on frontal cortex. Melatonin was given (30 milligram intravenously) prior to CSD induction or NTG infusion. Cerebral microcirculation was assessed using laser Doppler flowmetry (LDF) and intravital fluorescent videomicroscopy. Ultrastructure of cerebral microvessels was studied using electron microscopy. Trigeminal nociception was determined by the expression of c-fos in trigeminal nucleus caudalis (TNC). Expression of nitric oxide synthase (NOS) enzyme in TNC was studied using immunohistochemical method.

The results demonstrated that topical and systemic administration of melatonin resulted in the immediate cortical vasoconstriction. Pretreatment with melatonin showed strong attenuation effect on CSD-evoked cortical hyperemia. Melatonin also inhibited NTG-induced pial microvascular dilation. Our electron microscopic study revealed that pretreatment with melatonin could minimize the effect of GTN in induction of microvilli formation and pinocytosis in cortical endothelial cells. These changes in the vascular compartment were parallel to the changes in neural compartment. Reduction of CSD-evoked Fos immunoreactive in TNC neurons was evident in melatonin pretreated group reflecting the attenuation of trigeminal nociceptive activation. The same result was observed in nitric oxide-donor infusion model. Melatonin also minimized the effect of CSD and GTN on inducing NOS expression.

These findings revealed the potential anti-migraine effect of melatonin in two models. It also indicated that such effect of melatonin is mediated via the modulation of both vascular and neuronal compartments of trigeminovascular system.

Department.... Inter-department (Physiology) .....Student's signature.....
Field of study .....Physiology .....Advisor's signature .....
Academic year .....2003.....Co-advisor's signature .....
Co-advisor's signature .....

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Table of contents

List of tables

List of figures

List of abbreviations

$^{\circ}C$	= celsius
5-HIAA	= 5-hydroxyindoleacetic acid
5-HT	= serotonin
AA-NAT	= N-acetyltransferase
aCSF	= artificial cerebrospinal fluid
AP	= area postrema
ATP	= adenine triphosphate
BK_{Ca}	= Ca^{2+} activated potassium
BOLD	= blood oxygen level dependent
BSA	= bovine serum albumin
BW	= bodyweight
Ca^{2+}	= calcium
CaCl	= calcium choride
cAMP	= cyclic adenosine monophosphate
CBF	= cortical blood flow
CSD	= cortical spreading depression
Fos-IR cells	= Fos immunoreactive cells
cGMP	= cyclic guanosine monophosphate
CGRP	= calcitonin gene related peptide
CO_2	= carbondioxide
CSD	= cortical spreading depression
CSF	= cerebrospinal fluid
DAB	= diaminobenzidine
eNOS	= endothelium nitric oxide synthase
FITC	= fluorescineisothiocyanate-dextran
fMRI	= functional magnetic resonance imaging
GMP	= Guanosine monophosphate
GTN	= Glyceryl trinitrate
H_2O_2	= hydrogen peroxide
HCl	= hydrochoric acic
HIOMT	= hydroxyindole-O-methyltrasferase
IHS	= International Headache Society
iNOS	= inducible nitric oxide synthase
K_2PO_4	= potasssium phosphate
KCl	= potassium choride
kg	= kilogram
L/D	= light/dark
LC	= locus coeruleus
LFPX	= light induced functional pinealectomy

L-NAME	= Nω-Nitro-L-Arginine Methyl Ester
MA	= migraine with aura
MCA	= middle cerebral artery
mg	= milligram
Na_2HPO_4	= di-sodium hydrogen phosphate
NaCl	= sodium choride
$NaCO_3$	= sodium carbonate
NaOH	= sodium hydroxide
NF κβ	= nuclear factor-κβ
NHS	= normal horse serum
NI	= neurogenic inflammation
nm	= nanometer
NMDA	= N-methyl-D-aspartate
nNOS	= neurons nitric oxide synthase
NO	= nitric oxide
NOS-IR cells	= NOS immunoreactive cells
NR	= nucleus ruber
NTC	= nucleus trigeminalis caudalis
NTS	= nucleus tractus solitarius
$ONOO^-$	= peroxynitrite
SP	= substance P
PAG	= periaqueductal gray
PBN	= parabrachial nucleus
PBS	= phosphate buffer
PKA	= protein kinase A
PKC	= protein kinase C
PPE	= plasma protein extravasation
PT	= pars tuberalis
PUFA	= polyunsaturated fatty acid
PVH	= paraventricular nucleus of the hypothalamus
rCBF	= regional cerebral blood flow
SCN	= suprachiasmatic nucleus of the hypothalamus
sGC	= soluble guanylate cyclase
SN	= substantia nigra
SNAT	= serotonin-N-acetyltransferase
SPX	= surgical pinealectomy
TNC	= trigeminal nucleus caudalis
TNF	= tumor necrosis factor
VLM	= ventrolateral medulla
β-END	= β-endorphin
μm	= micrometer

CHAPTER 1
Introduction

Migraine is a syndrome that affects a substantial fraction of world's population with a higher prevalence in female than male. Migraine is characterized by an attack of intense, pulsatile and throbbing headache, which is typically unilateral and is accompanied by anorexia, nausea, vomiting and photo- and /or phonophobia. In about one third of the patients, the headache is preceded by aura symptoms, consisting of certain sensory, motor and/or focal neurological symptom (migraine with aura). The majority of patients, however, do not present with such symptoms (migraine without aura) (Olesen et al., 1990; Raskin, 1998).

Several Theories have attempted to explain migraine etiology. Wolff and Graham postulated that the constriction of intracerebral vessels cause aura in migraine with aura and the headache due to reflex vasodilation (Wolff, 1963). This vascular hypothesis was deemed valid for many years, although the results from the cerebral blood flow studies did not support this theory (Olesen et al., 1981; Marshall, 1982; Raskin, 1998).

Secuteri postulated that the decrease of serotonin was responsible for the migraine headache and the dilatation was not essential. This theory is support by the improvement of headache after administration of serotonin precursors, the production of headache with serotonin synthesis inhibitors and the alleviation of headache after administration of serotonin receptor agonists such as sumatriptan (Polini et al., 1974; Silberstein, 1992; Lance et al., 1990).

In 1940, Lashley and Leao and other researchers described a cortical spreading depression across the occipital and cerebral cortex preceded by an excitatory wave front (Leao, 1944; Lance, 1985; Raskin, 1998). Related research describes the spreading wave of neuronal depression originating in the posterior cerebral regions (Lauritzen et al., 1984). The results from cerebral blood flow, electroencephalogram and magnetoencephalographic studies support this theory (Lauritzen et al., 1984). They suggest that migraine etiology is due to a brain parenchymal disturbance leading to the spreading depolarization with vasodilation being secondary (Lauritzen et al., 1984; Okada et al., 1988; Gardner-Medwin et al., 1991).

The most significant diagnostic finding is the fact that migraine is frequently triggered by a variety of external and internal factor such as certain foods, perfumes, flickering lights, loss of sleep, traveling through time zones, hypoglycemia, menses etc. (Raskin, 1998; Peres et al., 2001; Toglia, 2001). Even though the current theory is generally accepted, it does not explain why or how external stimuli trigger the migraine attack.

Melatonin, the main indole compound produced by pinealocyte, which plays a central role in circadian organization of several biological rhythms (sleep-wake, seasonal variations) in several species including man, has been hypothesized to involve in the pathophysiology of migraine (Toglia, 1986; Gagnier, 2001).

Several clinical observations show that the attack of migraine has a diurnal pattern. The attack occurs most frequently during daytime and hardly occurs during sleep. The reduction in migraine attacks during sleep may relate to the nocturnal increase in melatonin. Furthermore several changes in melatonin metabolism are evident in migraine patients (Claustrat et al., 1989; Murialdo et al., 1994).

In 1989, Claustrat and co-workers had examined melatonin levels in migraine sufferers and a control group. They found that the entire migraine group had lower nocturnal plasma melatonin levels than those in the control group (Claustrat et al., 1989). In another study, Brun and colleagues collected urine samples and measured the melatonin level in 10 female patients suffering menstrual associated migraines without aura. The migraine patients had lower mean nocturnal melatonin throughout the entire menstrual cycle with no difference between luteal and follicular phases, whereas the controls displayed only the lower follicular phase of melatonin (Brun et al., 1995). Furthermore, Murialdo and colleagues examined a urinary excretion of melatonin in patients with menstrual related migraine. They found that the melatonin levels in migraine patients were significant lower than those of the controls in all phases of the menstrual cycle. Also the mean urine melatonin levels measured during the pain episode were significantly lower than those during the pain free period (Murialdo et al., 1994). The study of the nocturnal plasma melatonin profile and melatonin kinetics during melatonin infusion in migraine patients demonstrate that four of the six patients reported headache relief in the morning after starting melatonin infusion (20 μg at 4 μg per hour) (Claustrat et al., 1989).

Furthermore several studies of the effect of melatonin on blood vessels have demonstrated that melatonin can contract isolated blood vessels. Melatonin is known to constrict large-diameter cerebral arteries in vitro (Geary et al., 1997) and in vivo experiments which also demonstrate the vasoconstrictive effect of melatonin on small-diameter cerebralcerebral arterioles (Regringny et al., 1999). This vasoconstrictor effect is mediated by inhibition of Ca2+ activated large conductance K+ channels following activation of the melatonin receptor (Geary et al., 1997; Regringny et al., 1999).

Moreover melatonin demonstrates the anti analgesic effect in several pain models (Lakin et al., 1981; Sugden, 1983; Golombek et al., 1991; Yu et al., 1999, 2000b). A number of behavioral studies have shown that melatonin administered peripherally exerts an antinociceptive action against thermal and chemical stimuli in mice (Lakin et al., 1981; Sugden, 1983; Golombek et al., 1991). Yu et al. have also observed that the i.p. injection of melatonin resulted in a dose-dependent antinociception in rats and mice in a nociceptive test, electrical stimulation (Yu et al., 1999). However, the site and mechanism of action of melatonin to induce analgesia remain to be clarified. Several studies have shown that peripheral administration of naloxone may blunt the analgesic effect induced by melatonin (Lakin et al., 1981; Golombek et al., 1991; Yu et al., 1999). All observations point to a significant interaction between melatonin and opioid peptides in the brain (Kumar et al., 1982; Xu et al., 1995; Yu et al., 2000a).

These data prompted us to test the hypothesis of the anti-migraine effect of melatonin. We designed the experiment to investigate the effect of melatonin on the control of the cerebrovascular nociceptive system in two experimental animal models. The Cerebrovascular responses were studied using the fluorescence video microscope and the laser Doppler flowmetry. The c-fos and nNOS immunoreactivity study were used to evaluate the neural activity in pain pathway.

CHAPTER 2
Review Literatures

Migraine, a complex and multi factorial syndrome has been established as a disabling but treatable neurological disorder. Migraine is defined clinically as an episodic unilateral headache with a characteristic pulsating quality, associated with: nausea, photophobia and autonomic dysfunction.

Since 1988 migraine is defined by the criteria set by the Headache Classification Committee of the International Headache Society (IHS). According to the criteria migraine is mainly divided into 2 subtypes (migraine with aura and migraine without aura). Migraine with aura is an "idiopathic, recurring disorder manifesting with attacks of neurological symptoms unequivocally localizable to the cerebral cortex or brain stem, usually gradually developed over 5-20 minutes and usually lasting less than 60 minutes. Headache, nausea and/or photophobia, usually follow neurological aura symptoms directly or after a free interval of less than an hour. The headache usually lasts 4-72 hours, but may be completely absent". Correspondingly migraine without aura is an "idiopathic, a recurring headache disorder manifesting in attacks lasting 4-72 hours. Typical characteristics of headaches are its unilateral location, pulsating quality, moderate or severe intensity, aggravation by routine physical activity, and association with nausea, photo- and phonophobia". The diagnostic criteria for migraine according to HIS are listed in Table 2-1. These criteria have been a major improvement for migraine research and have been shown to perform adequately both in science and in the clinic.

Table 2-1. International Headache Society Criteria for Migraine with and without Aura (Headache classification committee, 1988).

1.1 Migraine without aura

A. At least 5 attacks fulfilling B-D

B. Headache attacks lasting 4-72 hours
(untreated or unsuccessfully treated)

C. Headache has at least two of the following characteristics:

1. Unilateral location
2. Pulsating quality
3. Moderate or severe intensity (inhibits or prohibits daily activities)
4. Aggravation by walking stairs or similar routine physical activity

D. During the headache at least one of the following:

1. Nausea and/or vomiting
2. Photophobia and phonophobia

E. At least one of the following:

1. History, physical- and neurological examinations do not suggest a secondary cause of headache
2. History and/or physical- and/or neurological examinations do suggest such disorder, but it is ruled out by appropriate investigations
3. Such disorder is present, but migraine attacks do not occur for the first time in close temporal relation to the disorder

1.2 Migraine with aura

A. At least 2 attacks fulfilling B

B. At least 3 of the following 4 characteristics:

1. One or more fully reversible aura symptoms indicating focal cerebral cortical and/ or brain stem dysfunction
2. At least one aura symptom develops gradually over more than 4 minutes or, 2 or more symptoms occur in succession
3. No aura symptom lasts more than 60 minutes. If more than one aura symptom is present, accepted duration is proportionally increased
4. Headache follows aura with a free interval of less than 60 minutes. (It may also begin before or simultaneously with the aura)

C. Same as 1.1.E, see above

2.1 Migraine pathophysiology

Despite this increasing attention, little is still 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:

The Cortical Spreading Depression Theory

Cortical spreading depression (CSD) was first characterized and detected by Leao (Leao, 1944). From these initial studies, research efforts by various workers have resulted in better understanding of this phenomenon. Spreading depression is a transient disturbance of mechanisms maintaining homeostasis which spread across the brain at a defined rate of between 3-5 mm/minute (Figure 2-1) (Leao, 1944). These perturbations in ionic homeostasis are characterized by a negative shift of extracellular DC potential, suggesting a cell depolarization, accompanied by marked alterations in ionic concentrations in extracellular space, notably an increase in K+ and H+ and a decrease in Ca2+ ions (Lauritzen, 1987). The precise mechanisms involving the spread of activity are though to involve activation of the NMDA receptors, as propagation and induction of spreading depression are both blocked by various competitive and non-competitive NMDA antagonists (Lauritzen et al., 1982).

During CSD, depolarization of neurons and glial cells occurred, giving rise to spike activity followed by neuronal silence. The sequence of brief excitation followed by a short-lasting depression is believed to be the basis of sensory symptoms during the migraine aura. Cerebral blood flow during and after spreading depression in rats has been studied by using autoradiography methods. These studies demonstrated that cortical blood flow is reduced by 20 to 25 percent following induced CSD. However, CSD has been shown to induce transient vasodilatation of the pial circulation in a number of anesthetized animals (Lauritzen, 1987, Shibata et al., 1990, Lauritzen, 1994). Apart from the blood flow changes, CSD is associated with marked disturbances of extracellular and intracellular ion concentrations, for example, the 10-fold increases of K+ and 10-fold decreases of Ca2+ (Gardner-Medwin, 1991; Ferreira et al., 2000). These perturbations in ionic homeostasis may be caused by a negative shift of extra cellular direct circuit potential, leading to a cell depolarization. The precise mechanisms involving the spread of activity are thought to involve activation

of N-methyl-D-aspartate (NMDA) receptors, as propagation and induction of spreading depression are both blocked by NMDA receptor blockers such as MK801 and DL-2-aminophonovaleric acid (Lauritzen, 1994; Ferreira et al., 2000).

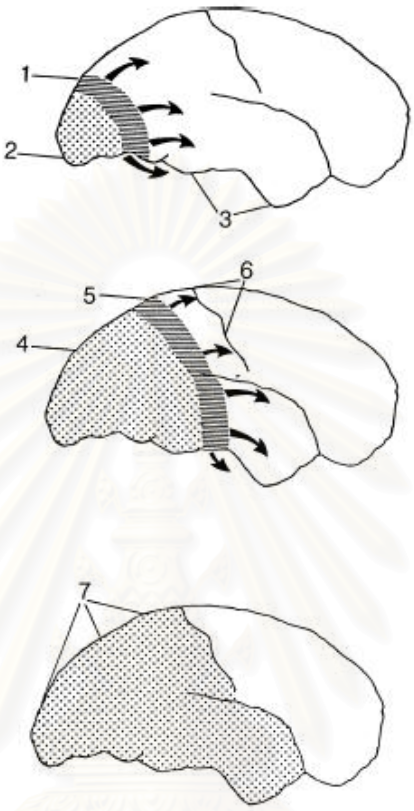


Figure 2-1. Hypothesis of development of a MA attack based on aspects of CSD and migraine.

The figure represent lateral views of the human brain at different time intervals after the start of the attack, spaced by approximately 30 min.. The dotted area represents the region of reduced rCBF, the striped area represents the region of neuronal depolarization during the first minutes of CSD, and arrows represent the direction of progression of CSD. 1. Initially during an MA attack a CSD is elicited at the occipital pole, spreading anteriorly at the lateral, mesial, and ventral sides of the brain. At the CSD wave front, transient ionic and metabolic disequilibria trigger the perturbed neuronal function, rCBF changes and neurological symptoms. 2. Following CSD, cortical rCBF decreases by 20 ± 30% for 2 ± 6 h. 3. rCBF in regions not invaded by CSD remains normal until encountered by CSD. 4. The region of reduced rCBF expands as the CSD moves anteriorly. 5. Somatosensory symptoms from the extremities appear when the CSD invades the primary sensory cortex at the post

central gyrus. 6. CSD usually stops on reaching the central sulcus, but in many patients it does not even propagate this far. The ventral spread of CSD causes activation of pain-sensitive fibers and headache. 7. Full-scale attack. The CSD has stopped and is now detectable as a persistent reduction of cortical rCBF. At this time the patient suffers from headache but has no focal deficits (Lauritzen, 1994, 2001).

Migraine aura and CSD

This CSD hypothesis is supported by the clinical observation of slowly spreading symptoms in migraine with aura. A gradual spread of reduced blood flow was observed in patients starting in the occipital region and advancing anteriorly. The rCBF measurements showed a gradually enlargement of the hypoperfused region in migraine patients resembling that of a CSD seen in the animal brain. They speculated that the aura in migraine patients occurs secondary to the spreading of oligaemia. This theory states that migraine results from an evolving process in the cerebral cortex that occurs secondarily to the decreased cortical function, decreased cortical metabolism, and/or vasoconstriction of cortical arterioles (Lauritzen et al., 1994; Olesen et al., 1981; Peuter et al., 1998).

Clinical observations, neuro-imaging studies, and blood flow measurements clearly indicate that migraine aura originates from the cerebral cortex. The visual aura, which is the most common form of migraine aura, started at the visual field center and propagated to the peripheral (temporal) parts within 10-15 minutes. The aura symptoms indicates a wave of intense excitation in the primary visual cortex that moved at the speed of 3 mm/minute, followed by a longer period of inhibition (Lashley, 1941) (Figure 2-2). Similar calculations can be made with respect to somatosensory symptoms developing along the sensory homuculus (Lord, 1986). The very orderly development of the aura makes a vascular origin a remote possibility, while a primary disturbance of cortical nerve cell function, probably cortical spreading depression (CSD) is a more attractive explanation (Leao and Morison, 1945).

Figure 2-2. Successive maps of a scintillation-scotoma to show the characteristic distribution of the fortification figures. In each case the asterisk indicates the fixation point. Knowledge of the retinotopic organization of the visual cortex allowed Lashley (1941) to calculate the speed of propagation of the excitation-depression wave as ~3 mm/min (Lashley, 1941).

Electrophysiological evidences support CSD theory in migraine

Bures et al. 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. The results obtained from experiments in human cortical tissue supports the development of a CSD, but the report from Mayevsky et al. remains the only description of electrical evidence of CSD from the human neocortex in vivo.

Cerebral blood flow changes in CSD

Olesen et al. provided the first evidence in human patients during migraine attacks of slowly spreading cortical hypo perfusion 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. 1984; 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., 1984). 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.

How CSD /aura leads to headache

The relationship between CSD and how it leads to headache has been difficult to explain. The current theory is that spreading depression depolarizes the sensory nerve fibers of the trigeminovascular system and sets up a painful sterile inflammatory state around the artery (Moskowitz, 1993). Recently it has been hypothesized that Ca^{2+} signals from the cortical parenchyma may be transmitted to the pia-arachnoid by gap-junction communication or extracellular movement of adenine triphosphate (ATP), and that this could induce some of the neurovascular changes in migraine. In addition, recent studies with functional magnetic resonance imaging (fMRI) have shown aura-related activation of brainstem centers nucleus ruber (NR) and substantia nigra (SN). Aura-induced dysfunction of these centers could then play a role during migraine headache and headache-associated symptoms (nausea, vomiting, dysautonomia). Thus the connection between migraine aura and headache

has been hypothesized to be both local (aura-induced trigeminovascular neurogenic inflammation) and central (dysfunction of brainstem centers).

Nitric Oxide Theory

Physiology of Nitric Oxide

Nitric oxide (NO) has recently been recognized as an important messenger molecule having a broad spectrum of functions in many biological systems ranging from physiological control to the pathological cytotoxic effect (Tassorelli et al., 1999; Moncada et al., 1991).

The free radical NO is a unique bioactive mediator because it is a gas with no known storage mechanism. NO is the lowest molecular weight of any human cell secretory product. NO can easily cross membranes by diffusion. NO is highly reactive and extremely labile, so its biological half-life is within a range of only a few seconds, and it is oxidized to stable nitrite (NO^{-2}) and nitrate (NO^{-3}) (Griffiths 2000). NO disappears within moments of its production, e.g., in synapses. Therefore, NO action is very transient and directly controlled by the generation of NO.

NO is generated from the terminal guanidine nitrogen of L-arginine. The family of enzymes that catalyzes NO synthesis is known as nitric oxide synthase (NOSs) (Knowles et al., 1994). NOS activity is present in many tissues of several species including the endothelium, brain, peripheral nerves, vascular smooth muscle, myocardium, macrophage, neutrophils and microglias (Knowles and Moncada., 1994).

Purification and cloning of NOS has revealed the existence of at least three types of NOS. (Knowles and Moncada, 1994) (Table 2-2) Two are constitutive, calcium/calmodulin dependent, and release NO from endothelium (eNOS) and neurons (nNOS). This release is accelerated in response with several membrane bound receptors by, for example, glutamate, bradykinin, 5-HT, acetylcholine, histamine, endothelin-1, substance P and probably the calcitonin gene related peptide (CGRP) (Ayajiki et al., 1992; Glusa et al., 1993; Toda, 1990). An increased flow velocity and subsequent increase in shear stress in endothelial cells may also stimulate eNOS (Lucher and Vanhauttle1990). Another NOS is inducible and calcium independent (iNOS). It is not normally present in significant amounts, but is expressed after stimulation by, for example, endotoxin and cytokines. After induction

it generates NO for prolonged periods and in large amounts (Busse and Mulsch., 1990).

Table 2-2. Summarizes the properties of the different NOS isoforms.

	Neural (nNOS, type 1)	Inducible (iNOS, type 2)	Endothelial (eNOS, type 3)
First identified in	Neurons	Macrophages	Endothelium
Other cells expressing	Myocytes Astrocytes	Astrocytes Microglia	Neurons
Intracellular localization	Soluble or membrane bound	Soluble or membrane bound	Largely membrane bound
Ca^{2+} dependency	Activity depends on elevated Ca^{2+}	Activity is independent of elevated Ca^{2+}	Activity depends on elevated Ca^{2+}
Expression	Constitutive Inducible under certain circumstances e.g. trauma	Inducible	Constitutive
Amounts of NO released	Small, pulses	Large, continuous	Small, pulses
Proposed function	Regulation	Host defense	Regulation
Activators	Glutamate Noradrenaline	Lipopolysaccharide	Acethylcholine

Most physiological actions of NO are mediated via activation of soluble guanylate cyclase and a consequence increase in cyclic GMP, eventually leading to a decrease in intracellular calcium in the target cells (Moncada et al., 1991; Mayer, 1994). Neuronal NOS is thought to be most widely distributed among isoforms, and in addition to its neural functions, it regulates the secretion in the kidney (macula densa), epithelial cells of the lung, stomach, pancreatic islets, uterus as well as the non-vascular smooth muscle functions (Tassorelli et al., 1999).

In the brain, especially in the cerebellum, NO mediates the ability of the excitatory neurotransmitter glutamate to stimulate cGMP levels via the NMDA (N-methyl-D-aspartate) receptors (Griffiths 2000). The NO/cGMP signal transduction system may be involved in a hypnotic action of volatile, intravenous anesthetics and alpha-2-agonists, recognized by their increased activity after inhibition of NOS in

rats. The inhibition is reversible with L-arginine. NO may also participate in two forms of long-term synaptic modulation, long-term potentiation in the hippocampus (Griffiths 2000), and long-term depression in the cerebellum. During development, NO may influence synaptic formation, apoptosis, and columnar organization of cortex (Tassorelli et al., 1999; Griffiths 2000).

NO has an amazing number of physiological effects throughout the body, of which several are theoretically implicated in the pathophysiology of several types of headaches (Moncada et al., 1991). Thus NO causes endothelium-dependent vasodilatation, which is important in cerebrovascular regulation, and in addition neurogenic vasodilatation may be mediated via perivascular which operates through NO (Toda et al., 1991, 1997). Furthermore, NO is reported to be an important mediator in the pain perception (hyperalgesia) (Wolff and Thompson, 1991).

Nitric oxide in migraine

Histamine and nitrate have long been accepted to induce headaches in migraineurs and in cluster headache sufferers (Secuteri et al., 1987; Dahl et al., 1990). Glyceryl trinitrate (GTN), a nitric oxide donor, has proven to be a suitable and reliable substance for experimental studies of NO-donor induced headaches (Iversen et al., 1989, 1996). Because of its lipid solubility it may deliver NO to several tissues, including those that are protected by the blood brain barrier. Several observations support the assertion that GTN induced headaches by liberating NO. The results demonstrate that a headache induced by GTN in healthy control is very short lived while the metabolites other than NO have a longer half-life (Iversen et al., 1989, 1996).

Histamine may also cause headaches via liberation of NO. They suggest that activation of the endothelial H1 receptor induces the formation of endogenous NO (Ayajiki et al., 1992; Toda et al., 1990).

Several studies have demonstrated that the migraine patients experience a migraine-like headache in association with nitroglycerine and histamine administration more often than those in normal individuals (Sicuteri et al., 1987). The study of intravenous of four increasing doses of GTN reveals that migraine patients have a headache of stronger intensity and with more migraine characteristics than those in control individuals. More over they found that in most migraineurs, in addition to the headaches experience in close association of GTN infusion, they

complained of a delayed headache, which they labeled as typical migraine (Olesen et al., 1993; Thomsen et al., 1994).

It can be concluded that migraine patients are hypersensitive to NTG induced headaches and most likely therefore to NO in two ways (Olesen et al., 1993). First, they experience more severe immediate headaches than those in normal control. Second, most migraineurs, in contrast to control individuals, subsequently experience a delayed migraine headache.

In patients of migraine with aura, GTN induces the headache response as in patient of migraine without aura, but without aura symptom (Christiansen et al., 2000). This result suggests that NO is involved in the pain mechanism in both types of migraine.

Experimental evidences suggest a coupling between CSD, NO release and headaches (Christiansen et al., 1999), as predicted from the finding that brain extracellular L-arginine decreases during CSD and that brain cortical cGMP increases by up to 400% (Gault et al., 1994). The results from recent study confirmed that CSD elicits a widespread release of NO and cGMP levels (Read et al., 2000).

An increase headache response in migraine patients after GTN infusion could reflect a greater general sensitivity to NO. The latter is supported by an increase dilatation of the middle cerebral artery during infusion of GTN in migraineurs (Thomsen et al., 1993). Interestingly, it has been reported that this vascular effect is more pronounced on the headache side during GTN infusion (Bellantonio et al., 1997; Thomsen and Olesen., 2002).

The studies in platelets also confirmed the physiological hypersensitivity to NO in migraine patients. The decreased aggregability to collagen and increased arginine level in platelets obtained from the migraineurs are demonstrated (D'Andrea et al., 1994). The increased in concentration of the metabolites of NO (nitrate and nitrite) in platelets from migraineurs supports the assertion that NO is produced during the migraine attack (Shimomura et al., 1999). Other indirect measurements of NO synthesis in platelets and in serum also provide the evidence of activation of the NO pathway in a migraine attack (Gallai et al., 1996; Nattero et al., 1996).

In addition to experimental and clinical human studies, the results from animal and in-vitro experiments also confirm the role of NO in migraine. A recent study showed that inhibition of NO synthesis could reduce the c-fos expression in the trigeminocervical complex of the cat (Hoskin et al., 1999). Further sumatriptan, which

effectively reduces migraine headaches, can reduce the concentration of cortical NO when given as pretreatment before spreading depression is induced in the rat and cat (Read et al., 2000).

These cumulative data strongly confirm that NO is an important mediator involved in the pain mechanism in migraine.

The vascular theory

The pulsating quality of headaches indicates a vascular connection. However, it is still debated whether vasoconstriction or vasodilatation can account for a migraine headache.

Vasoconstriction: Based on the theory of Wolff, developed in the 1940s and 1950s, migraine was considered to be a vasospastic disorder; cerebral vasoconstriction was postulated to occur during the migraine prodrome and vasodilatation to occur during the headache phase (Wolff 1987). In apparent support of this theory, Olesen and colleagues found that there is indeed a decrease of regional cerebral blood flow (rCBF) during the aura phase, supporting the local vasoconstriction theory of aura. They also showed that the decrease of rCBF in the posterior hemisphere continued throughout the headache phase, which was confirmed by others (Lance 1981).

Vasodilatation: One of the mechanisms of pain production in migraine is the dilatation of arteries lying outside the brain, especially the scalp arteries. There is an alleged correlation between the severity of a headache and the pulse amplitude of scalp arteries. Artificial distention of the superficial temporal artery can reproduce a migraine headache; physical compression or chemical constriction of scalp arteries often alleviates a headache at least temporarily (Drummond et al., 1983).

Moreover, distention of major cerebral vessels by balloon dilatation leads to pain referred to the ophthalmic division of the trigeminal nerve (Nicholson et al., 1993). The direct measurement confirms that extracranial blood flow is often elevated during migraine attacks (Moskowitz et al., 1989).

Studies using combined transcranial sonography and the rCBF technique showed that the unilateral headache is associated with dilatation of large intracranial arteries on the headache side (Friberg et al., 1991) (Figure 2-3). It is well known that the large intra cranial arteries contain nociceptive fibers derived from the trigeminal

ganglion. So, it is likely that the migraine headache is associated with the dilatation of the large intracranial arteries.

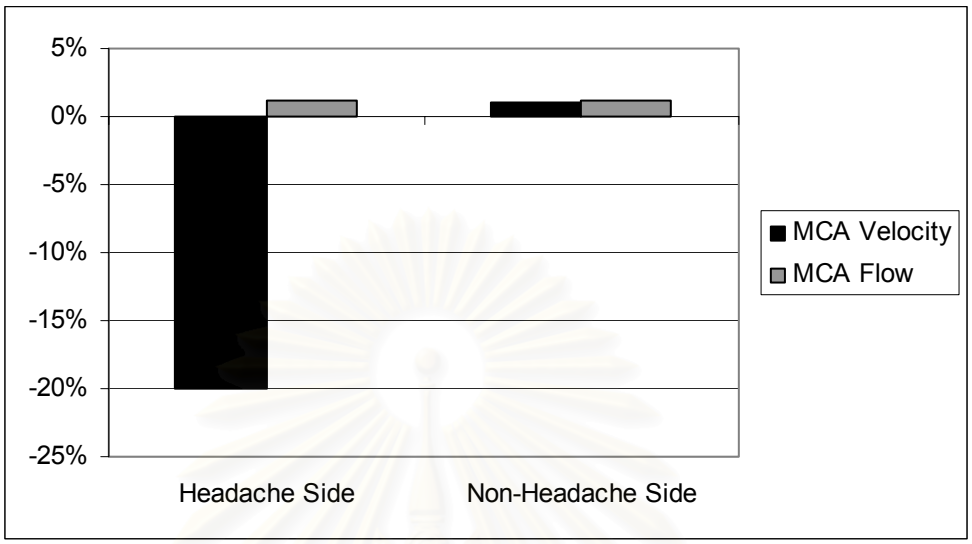


Figure 2-3. Middle cerebral artery, MCA, velocity recorded with trans cranial ultrasonography was significantly reduced on the headache side but normal on the non-headache side. rCBF was normal on both sides. The data indicated a 20% dilatation of the MCA on the headache side, which was normalized after treatment with sumatriptan causing headache recovery (Friberg et al., 1991).

A headache following the administration of vasodilators, such as nitrates, has been used to support the importance of a vasodilatation cause. Several studies have determined that the mechanism of dilatation by NTG and sodium nitropruside in the cat is via the activation of perivascular sensory nerves, with subsequent release of CGRP. This in turn relaxes vascular smooth muscle via activation of the soluble form of guanylate cyclase. This would cause the vessels to change in diameter and trigger the nociceptive perivascular fiber. It is highly likely that it is not the vasodilatation per se that causes a headache in response to NTG, but that the activation of trigeminal perivascular sensory afferent nerve fibers by NO may participate (Thomsen et al., 1993; Wei et al., 1992).

In conclusion, the ability of these changes in cerebral blood flow (vasoconstriction / vasodilatation) to induce the symptoms of migraine has been questioned. The observed decrease in blood flow does not appear to be significant enough to cause focal neurological symptoms. Secondly, the increase in blood flow

per se is not painful, and vasodilatation alone cannot account for the local edema and focal tenderness often observed in migraine patients. Thus, it is unlikely that simple vasoconstriction and vasodilatation are the basic pathophysiological abnormalities in migraine. However, it is clear that cerebral blood flow is altered during certain migraine attacks.

Neurogenic inflammation theory

The neurogenic inflammation theory accounts for several phases of migraine and is supported by many lines of evidences. Several studies in animals have shown that upon stimulation of the trigeminovascular system, the complex of trigeminal sensory afferents that innervate the dura mater and the larger blood vessels of the brain, neuropeptides such as substance P (SP) and calcitonin gene related peptide (CGRP) are released at the afferent terminal site, causing neurogenic inflammation (NI) in the perivascular space of blood vessels of the meninges. Plasma protein extravasation (PPE) and vasodilatations are proven to result from neurogenic inflammation (Siberstein, 1992). Using animal models it has been shown that the classic ergot alkaloids, sumatriptan and also the new generation, centrally active triptans inhibit dural PPE which is induced by trigeminal afferent stimulation. Also, non-steroidal-anti-inflammatory-drugs inhibit dural PPE and have been reported to be effective in the treatment of migraine.

Moskowitz has provided an elegant series of experiments whose results suggest that the pain of migraine may be a form of sterile neurogenic inflammation (Moskowitz, 1993). Neurogenic plasma extravasations can be seen during electrical stimulation of the trigeminal ganglion in the rat. Plasma extravasations can be blocked by several anti-migraine drugs (Buzzi et al., 1990; Cutrer et al., 1995; Kallela et al., 1998). These experiments provided evidence that neurogenic inflammation can be prevented by blocking transmission in the nerve fibers, and they explain a peripheral mechanism of action for acute-headache medications that work by blocking nerve-fiber transmission in the trigeminal system rather that through a vascular route. Recently, the role of neurogenic inflammation in the pathogenesis of migraine has been questioned since most anti-migraine drugs not only ameliorate PPE but also induce vasoconstriction. Bosentan, which blocks PPE without having vasoconstrictive

effects, was ineffective in alleviating migraine attacks when given during the headache phase which implies that NI is not involved in migraine. It seems that blocking neurogenic inflammation without vasoconstriction is not enough to abort a migraine attack (May et al., 1996). However, it cannot be excluded that NI precedes the headache phase of migraine.

The Neurovascular Hypothesis

Trigeminal nerve fibers innervate blood vessels in the meninges, the extracranial arteries, and those in the circle of Willis. These nerve fibers contain nociceptors that are capable of generating pain impulses. Further, the endings of these nerve fibers contain several peptide neurotransmitters (Feindel et al., 1960; Mayberg et al., 1984) (Figure 2-4).

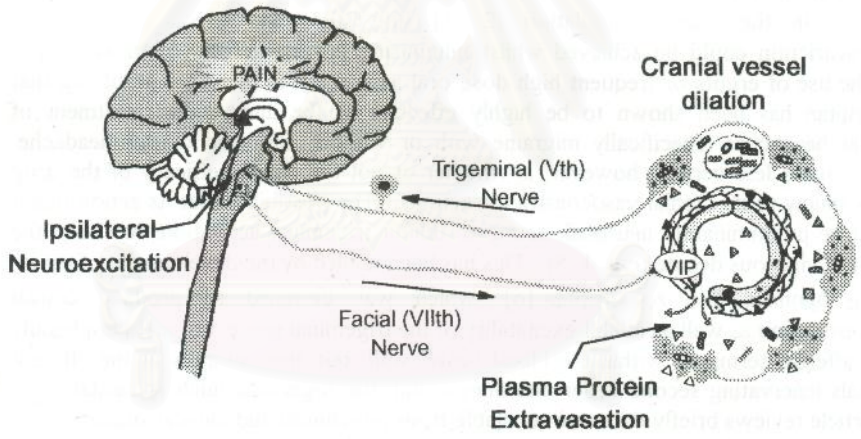


Figure 2-4. The trigemino vascular system (modified from Moskowitz et al., 1989).

The neurovascular hypothesis proposes that either migraine triggers or CSD can activate trigeminal nerve axons, which then release neuropathies (such as substance P, neurokinin A, and CGRP) from axon terminals near the meningeal and other blood vessels.

These vasoactive polypeptides further dilate blood vessels and cause a rapid-onset inflammatory reaction consisting of plasma extravasations and mast cell changes in the perivascular area (Moskowitz et al., 1989). These changes result in

dilated, swollen, and inflamed blood vessels and transduce pain at the trigeminal nerve endings. The pain is then carried through the trigeminal nerve (first-order neuron) into the second-order neurons in the brain stem. Vasodilatation and neurogenic inflammation sensitize the first-order neurons rapidly, often within 30 minutes, resulting in a throbbing head pain that is aggravated by non-nociceptive stimuli, including pulsations of the arteries, and activities that increase intracranial pressure, including physical exercise, bending down, coughing, and a sneezing (Moskowitz and Cutrer, 1993). This sensitization of the first-order neurons explains why migraine pain is aggravated by physical activity and why migraine patients prefer to stay quiet and not move during a headache.

Activated first-order neurons transmit pain to the second-order neurons in the trigeminal nuclei. C-fos studies have shown activation of the trigeminal nucleus caudalis. The activated second-order trigeminal neurons have functional connections to other important brain stem centers, particularly to nausea and vomiting centers such as the nucleus tractus solitarius, the activation of which results in nausea and vomiting. Specific anti-migraine agents, namely, the triptans, have been shown to bind to the trigeminal nucleus caudalis as well as to its functional connections such as the nucleus tractus solitarius. Therefore, these agents reduce nausea and vomiting in addition to reducing pain (Saxena and Tfelt-Hansen, 2000).

According to this theory, vasodilatation alone cannot trigger migraine headaches but it has to accompany with trigeminal nerve activation. Although the cause of this activation is not known, it may be due to ionic and metabolic disturbances in the brain function, such as those associated with CSD. It has also been proposed that abnormal activity in the brain stem sensory nuclei may cause antidromic activation of trigeminal sensory pathways.

The Serotonergic Abnormalities Hypothesis

Serotonin, 5-HT, has long been involved in the pathophysiology of migraine. Shortly after its discovery, 5-HT was implicated in the pathophysiology of migraine based on the original finding by Sicuteri and colleagues (Olesen et al., 2000) of an increased urinary excretion of 5-HT's primary metabolite, 5-hydroxyindoleacetic acid (5-HIAA) during migraine attacks.

A most convincing piece of evidence, in favor of migraine being a low serotonergic syndrome has been provided from physiologic studies on the evaluation of auditory evoked potentials. The amplitude of these evoked potentials has shown to be inversely related to central serotonergic neurotransmission. In migraine patients, a marked increase in amplitude was observed between attacks, in support of a low 5-HT transmission and abnormal cortical processing of sensory information. The implication of serotonergic pathways in this response has further been confirmed by the observation that 5-$HT_{1B/1D}$ receptor agonists, which can penetrate the brain and activate cortical inhibitory prejunctional 5-$HT_{1B/1D}$ autoreceptors, can decrease the amplitude of auditory evoked potentials in migraine sufferers. These observations all converge to support the notion that migraine is associated with a cortical hypersensitivity to stimulate the headache-free interval, which is, at least in part, due to low 5-HT transmission (Wang et al., 1996; Srikiatkhachorn et al., 1998).

Such a scenario of a chronically low availability of the HT could result in a sensitization of all or specific populations of 5-HT receptors in migraine sufferers. Interestingly pharmacologic and molecular evidence seems to support the original hypothesis that migraine is the consequence of an inappropriate increased sensitivity of 5-HT_{2B} receptors (Kalkman 1994). More specifically, these researchers suggested that activation of cerebrovascular endothelial 5-HT_{2B} receptors, following and increase bioavailability of 5-HT at the onset of a migraine attack would induce the endothelial production and release of nitric oxide (NO) (Srikiatkhachorn et al., 2000). Nitric oxide can dilate cranial blood vessels and activate trigeminovascular afferents, thus invoking two aspects of the neurogenic inflammation response thought to be involved in pain generation and transmission. This mechanism reinforces the view that central dysfunctions in migraine will lead to perturbations of the cranial circulation. Indeed, activation of 5-$HT_{1B/1D}$ receptors causing cranial vasoconstriction and inhibition of trigeminovascular activity leads to alleviation of migraine symptoms (Kalkman, 1994; Lance 1993).

Arguments in favor of the biochemical triggering event being an increase in 5-HT availability include the observations that several drugs that release 5-HT from neurons and blood platelets (fenfluramine and reserpine) and some 5-HT reuptake inhibitors (zimeldine and femoxatine) are all able to provoke migraine attacks and more frequently so in migraine subjects than in controls (Humphrey, 1991). The same may be the case with red wines, which also release 5-HT from blood platelets, and

possibly from neuronal stores. However, fenfluramine and reserpine used for extended periods can confer resistance to migraine headaches, and 5-HT given during an attack will induce a headache relief. It seems therefore that the mobilization of 5-HT from intracellular stores at an early stage will trigger migraine attacks. However the receptors inducing the migraine attack (possibly the 5-HT2B) are different from those (5-HT1family) that relieve migraine headaches (Humphrey, 1991; Olesen et al., 2000, Srikiatkhachorn et al., 2002).

It has been claimed that changes in circulating 5-HT alone cannot explain all the facets of a migraine attack (Humphrey, 1991). Despite the fact that a low brain serotonergic activity has been evidenced between attacks on the basis of an increase amplitude of auditory action potentials, an increase in central 5-HT neurotransmission at the onset and during an attack has not yet been demonstrated. Thus, whether the pharmacologic manipulations with 5-HT agonists and 5-HT releasing agents bear any relevance to a spontaneous migraine attack is still largely speculative. However, a positron emission tomography study performed in migraine patients during their spontaneous migraine attacks showed an increased cerebral blood flow in the brain stem, including in an area corresponding closely in location to the serotonergic dorsal raphe nucleus (Olesen et al., 2000). This fascinating observation may suggest that serotonergic dorsal raphe neurons become active and release 5-HT during a migraine attack, and as such they have been considered as part of the migraine generating center. These neurons project to many areas of the central nervous system, including the cerebral cortex and the area postrema, where abnormal information processing has been documented during migraine. They are also intricately related to the cranial vasculature, which constitutes the primary focus of pain generation in migraine. These characteristics, together with the known interactions between serotonergic, dopaminergic, and noradrenergic systems in the brain, suggest that dysfunctions of the 5-HT system are likely to have functional repercussions much larger than those expected on the basis of 5-HT neurotransmission alone. This may explain some of the complexity of the neurochemical changes associated with migraine, most specifically those related to other amines (Olesen et al., 2000).

Genetic

Several lines of evidences have indicated that hereditary factors play a role in the individual susceptibility to develop a migraine attack. Significant data exists for many years to indicate that migraine is a genetically transmitter syndrome. Indeed, Living (1873) noted the frequent occurrence of “migraine” within families and that the disorder was often transmitted from parent to child. The study of twin pairs by Lucas in 1977 also provided very important information about the contribution of hereditary factors to a migraine headache. This interesting finding was that monozygotic twins, who were separated at birth and raised apart not only were concordant for the occurrence of migraine, but also for the age at onset of the attacks. This observation in twins strongly supports a genetic component of migraine. Moreover, the hypothesis that migraine is inherited has been supported by numerous studies (Lucas, 1977; Russell and Olensen, 1995). Some authors have even considered a positive family history as a prerequisite for the diagnosis of migraine (Sjaastad and Stovner, 1993). Traditionally, family studies have been used to determine whether a migraine headache has a genetic basis.

Prior to recent technical advances in the field of molecular genetics, it was essentially impossible to even consider migraine as a candidate disease for a formal genetic analysis. The wide spectrum of clinical symptomatology, the likely role of environmental factors such as stress and diet and perhaps most important, the high prevalence of migraine within the general population are all factors which make the genetic analysis of migraine a difficult task. However, the development of the polymerase chain reaction technique has led to an exponential growth in the ability to generate data on the human genome since; the molecular genetic analysis of migraine can be performed. The report by Joutel and colleagues in 1993 showed the genetically linking of a region of DNA on human chromosome 19 to the clinical diagnosis of familial hemiplegic migraine (Joutel et al., 1993). May and co-workers (1995) provided evidence for the involvement of a gene on chromosome 19 in the etiology of common forms of migraine. They suggested that the putative 19p13 gene plays a role in a number of migraine families (May et al., 1995). They identified some mutations of brain-specific P/Q-type calcium channel $\alpha 1$ subunit gene on chromosome 19p13.1 and suggested that these mutations may play a role in the migraine pathogenesis

(Ophoff et al., 1997). However, it remains to be elucidated whether this genetic contribution constitutes a mild risk factor for migraine in the general population.

Anti-migraine drugs

On the basis of the current understanding of the pathophysiology of migraine, drugs or substances used as anti-migraine agents need to have mechanism of actions on at least these criteria:

- Constriction of cranial vessels.
- Inhibition of NO activated cascade.
- Inhibition of activation of the trigeminovascular system induced by CSD.
- Inhibition of activated peripheral trigeminal nerve terminals.
- Reduction in neuronal activity in central trigeminal neurons.
- Inhibition of neurogenic inflammation.

Melatonin, the indole amine produced by pineal gland, has been focus on the possibility to be anti-migraine agent since several actions of this amine have several effects suitable for being the anti migraine agent.

2.2 Melatonin

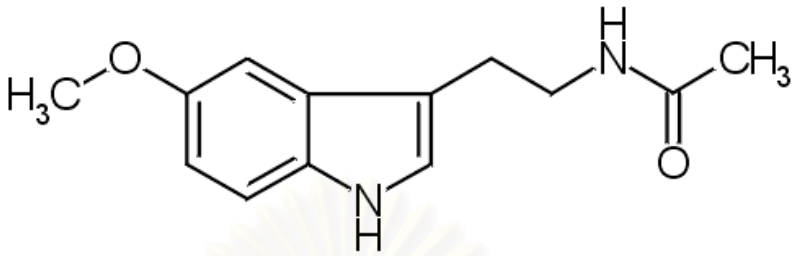


Figure 2-5. Structure of melatonin or (N-Acetyl-5-methoxytryptamine).

Melatonin or 5-methoxy-N-acetyltryptamine (Figure 2-5) is an important hormone that plays a role in regulating the neuroendocrine system. It plays an important role in the regulation of the circadian sleep-wake cycle. It also plays an important role in the control of several essential functions such as metabolism, sex drive, reproduction, appetite, balance, muscular coordination and the immune system in fighting to diseases triggered by bacteria, viruses, chemical pollutants and excessive free radical activity. It is normally released during the night in response to environmental changes in light levels by the pineal gland which is a tiny gland buried deep in the brain behind the eyes of mammals.

The Pineal Gland

The pineal gland is a small secretory organ regulated by either direct exposure to light, or influenced by visual pathways. In fish and amphibians, regulation of the pineal takes place by direct exposure to light, because of its location on the surface of the brain (Axelrod et al., 1965; Cahill 1996; Iigo et al., 1997). In birds and reptiles, the signal is usually a combination of direct photoreception and light-induced hormonal signals (Veylon 1980; Tosini et al., 2001). In humans, the pineal gland lies in the center of the brain, behind the third ventricle (Figure 2-6). The gland consists of two types of cells: pinealocytes, which predominate and produce both indolamine (mostly melatonin) and peptides (such as arginine vasotocin), and neuroglia cells (Brzezinski, 1997). Because of the location deep in the midbrain, the pineal gland's functions are regulated exclusively by signals arising from the retina (Reiter, 1981).

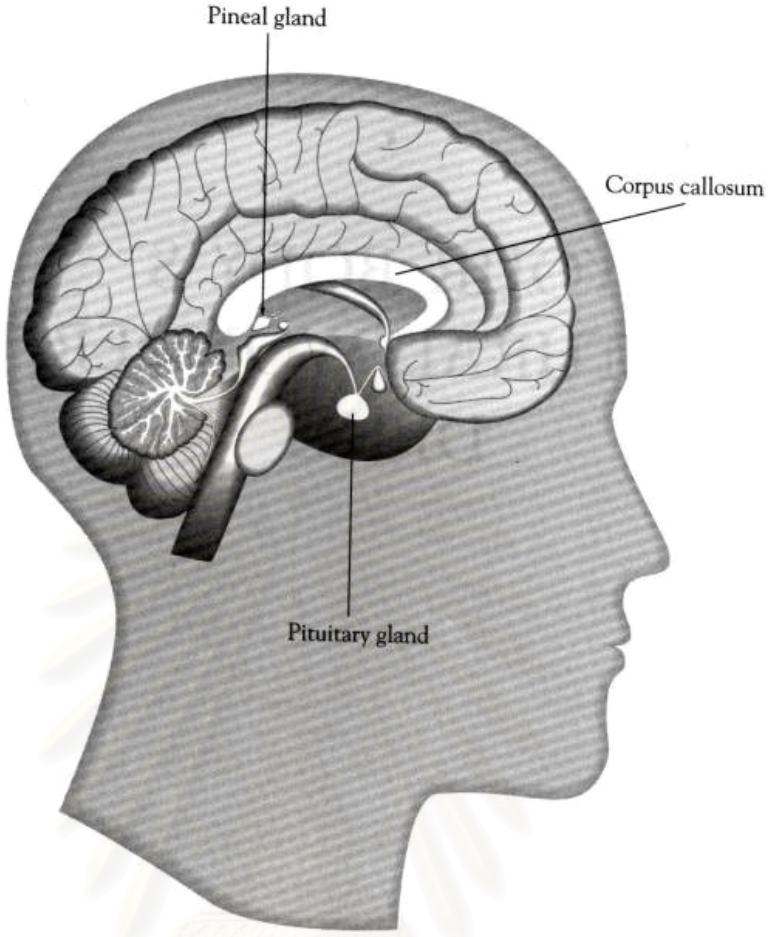


Figure 2-6. The location of pineal gland.

The mammalian pineal gland is a neuro endocrine transducer. Photic information from the retina is transmitted to the pineal gland though the suprachiasmatic nucleus of the hypothalamus and the sympathetic nerve system (Figure 2-7). The neural input of the gland is norepinephrine, and the output is melatonin. The synthesis and release of melatonin are stimulated by darkness and inhibited by the light. During daylight hours, the retina photoreceptor cells are hyperpolarized, which inhibit the release of norepinephrin. The retinohypothalamic-pineal system is quiescent, and low level of melatonin is secreted (Brzezinski, 1997). With the onset of the darkness, the photo receptors release norepinephrin, thereby the activation of the system and the number of $\alpha 1$ and $\beta 1$-adrenergic receptors increase. The activity of arylalkylamine-N-acetyltransferase, the enzyme that regulates the rate of melatonin synthesis, is increased. These phenomena initiate the synthesis and release of melatonin (Pangerl et al., 1990).

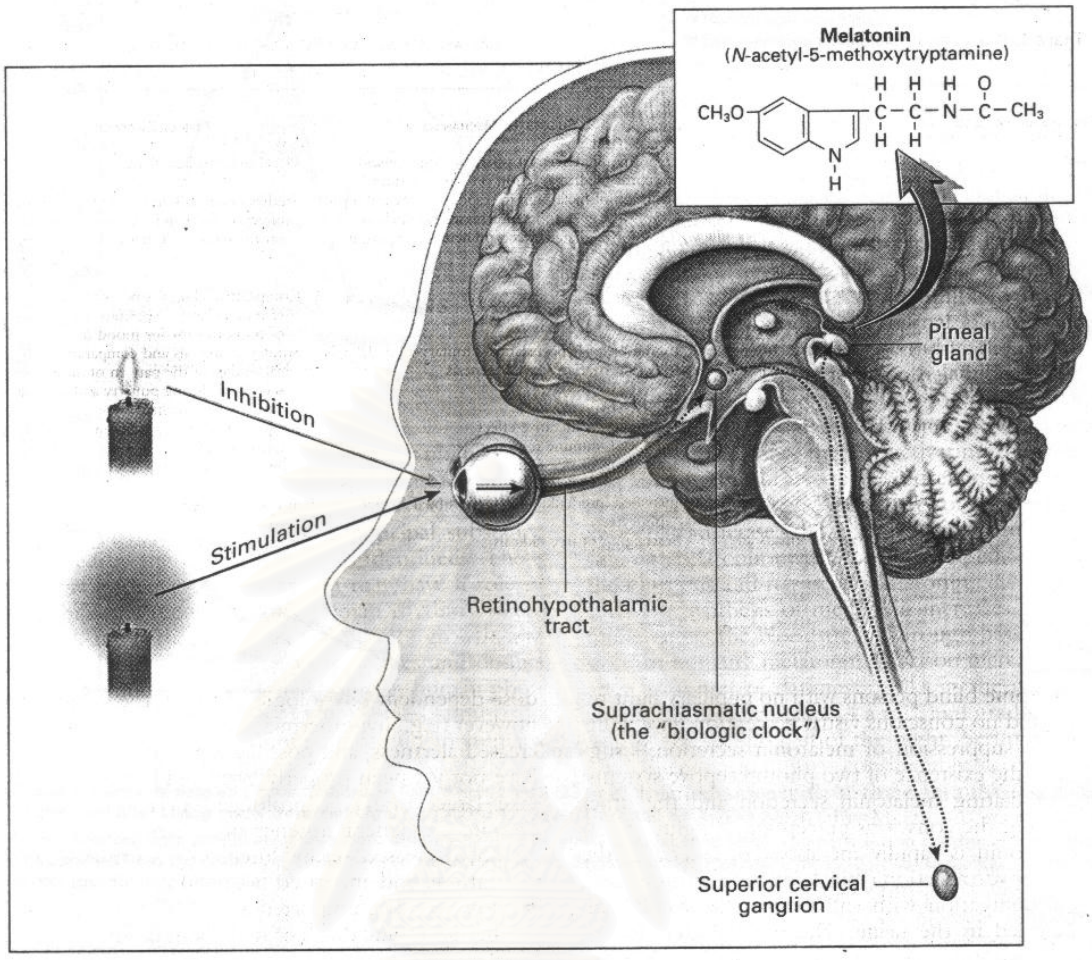


Figure 2-7. Physiology of melatonin secretion. Melatonin (insert) is produced in the pineal gland. The production and secretion of melatonin are mediated largely by postganglionic retinal nerve fibers that pass through the retinohypothalamic tract to the suprachiasmatic nucleus, then to the superior cervical ganglion, and finally to the pineal gland. This neuronal system is activated by darkness and suppressed by light. The activation of α-1 and β-1 adrenergic receptors in the pineal gland raises cyclic AMP and calcium concentrations and activates arylalkylamine N-acetyitransferase, initiating the synthesis and release of melatonin. The daily rhythm of melatonin secretion is also controlled by an endogenous, free-running pacemaker located in the suprachiasmatic nucleus.

Biological Synthesis of Melatonin

The biosynthesis of melatonin (Figure 2-8) is initiated by the uptake of the essential amino acid tryptophan into pineal parenchymal cells (Sugden, 1983). Tryptophan is the least abundant of essential amino acids in normal diets. It is converted to another amino acid, 5-hydroxytryptophan, through the action of the

1983). Tryptophan is the least abundant of essential amino acids in normal diets. It is converted to another amino acid, 5-hydroxytryptophan, through the action of the enzyme tryptophan hydroxylase and then to 5-hydroxytryptamine (serotonin) by the enzyme aromatic amino acid decarboxylase.

Pineal tryptophan hydroxylase, the rate limiting enzyme in serotonin production and the enzyme that catalyses the conversion of tryptophan to 5-hydroxytryptophan, was initially reported to exhibit no change in activity during the light-dark cycle (Deguchi, 1977). However, later some studies suggested that tryptophan hydroxylase activity increases during darkness in the rat pial gland (Shibuya et al., 1978; Sitaram et al., 1978).

Serotonin concentrations are higher in the pineal than in any other organ or in any brain region. They exhibit a striking diurnal rhythm remaining at a maximum level during the daylight hours and falling by more than 80% soon after the onset of darkness as the serotonin is converted to melatonin, 5-hydroxytryptophol and other methoxyindoles. Serotonin's conversion to melatonin involves two enzymes that are characteristic of the pineal: SNAT (serotonin-N-acetyltransferase) which converts the serotonin to N-acetylserotonin, and HIOMT (hydroxyindole-O-methyltrasferase) which transfers a methyl group from S-adenosylmethionine to the 5-hydroxyl of the N-acetylserotonin.

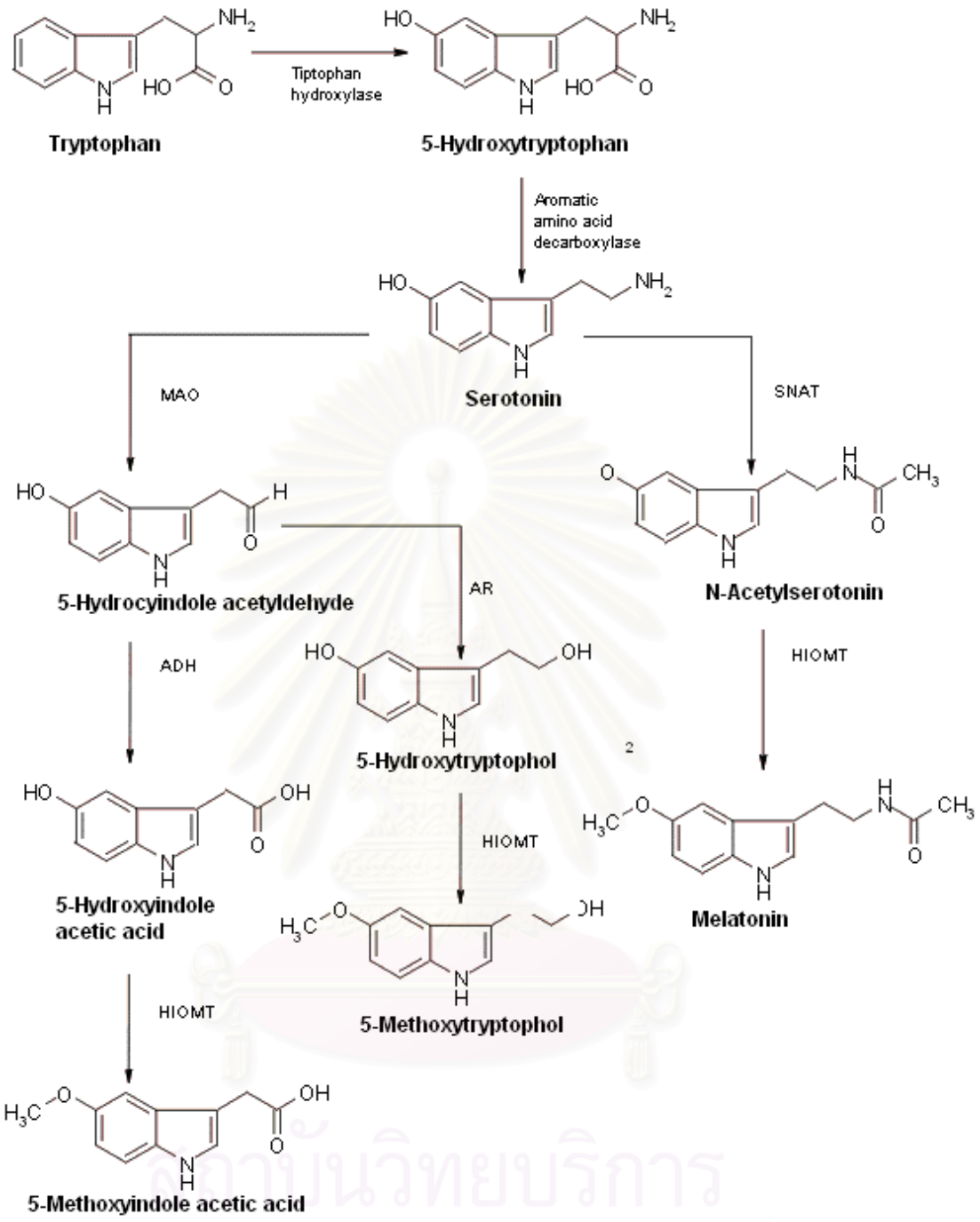


Figure 2-8. Biosynthesis of melatonin.

Melatonin, after the synthesis, enters the bloodstream though passive diffusion. In humans, melatonin increases soon after the onset of darkness, peak in the middle of the night (between 2 to 4 a.m.), and gradually falls during the second half of the night. Serum melatonin concentrations vary considerably according to age. Infant younger than tree months of age secrete very little melatonin. Melatonin secretions

increase and become circadian in older infants. The peak nocturnal concentrations are highest (325 pg/ml) at the age of one to three years, after that they decline gradually (Waldhauser et al., 1985). In normal young adults, the average daytime and peak night time values are 10 and 65 pg/ml, respectively. The 24-hour cycle of melatonin production in normal young adult is shown in Figure 2-9 below;

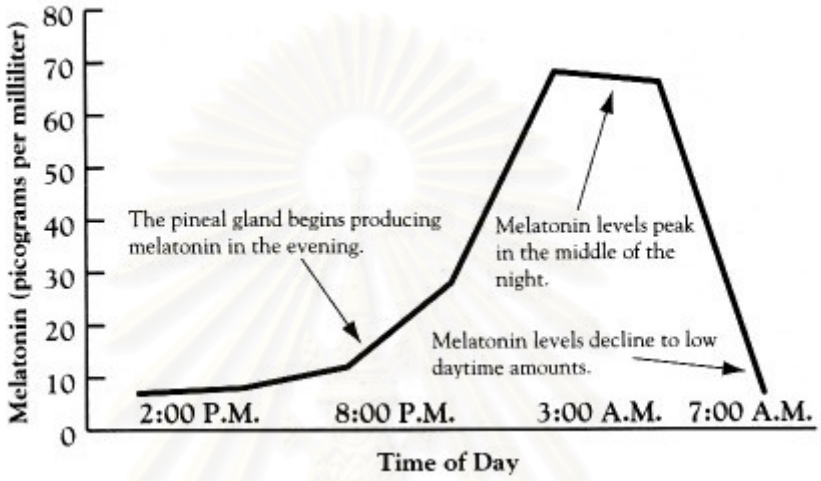


Figure 2-9. The amount of melatonin secretion from the human pineal gland during various time of the Day.

Melatonin levels rise at night because of an increase in the transcription and translation of serotonin N-acetyltransferase (AA-NAT), the rate-limiting enzyme in melatonin synthesis. Light given during the dark phase of a light/dark (L/D) cycle can suppress AA-NAT, thus reducing melatonin synthesis and release from the pineal gland (Minneman et al., 1974).

Melatonin secretion and regulation of its levels in blood

Whether there is the specific mechanism governing the release of melatonin from the pineal gland remains unknown. Even the specific fluid, e.g. blood and cerebrospinal fluid (CSF), into which melatonin normally discharged has been debated (Reiter et al., 1975) although there is almost universal agreement that it is released directly into the blood vascular system and only secondarily into other body fluids. Also the higher concentration of melatonin in CSF than in the plasma in some

cases could be taken as evidence as the CSF may be an important secretory rout for melatonin and for its transport to its effectors sites (Hedlund et al., 1976; Shaw et al., 1989).

As pineal melatonin production increases at night, there is a parallel rise in blood levels of the hormone (Wilkinson et al., 1977; Reiter et al., 1986). The nocturnal surge of blood melatonin is a consequence of pineal secretion which is documented by the fact that pinealectomy or the sympathetic innervation of the gland prevents the night time rise in plasma melatonin.

The circulating melatonin in the blood is bound to albumin (Pardridge et al., 1980). Melatonin appearing half-life in the blood is rather short (10-40 minutes) (Kopin et al., 1961) and during the single passage through the liver, 90% of the melatonin is cleared (Pardridge et al., 1980). About 75% of the melatonin taken up to the hepatic cells is converted to 6-hydroxymelatoninby microsomal enzymes; this compound is subsequently conjugated to either sulfate (70%) or to glucoronide (6%) (Kopin et al., 1961; Reiter et al., 1991). In humans the percentage of 6-hydromelatonin bound to glucoronide may be slightly higher (Jones et al., 1969). In the brain, melatonin is converted to N-acetyl-5-methoxykenurenamine (Kopin et al., 1961; Hirata et al., 1974). Other minor metabolites of melatonin are produced in various organs. All the metabolites are excreted in the urine and, as with circulating melatonin concentrations are higher than those during the day (Fellenberge et al., 1981; Reiter et al., 1991).

Circulating melatonin levels are influenced by a variety of perturbations, the most obvious of which is the light-dark environment. It has already been noted that pineal melatonin production, and therefore its concentration in the blood, is greatest during the night. Furthermore, prolonging the daily dark period generally leads to a proportional prolongation of elevated nocturnal melatonin levels (Figure 2-10).

The exposure of animals to light at night when melatonin levels are high abruptly curtails pineal melatonin production and causes a rapid decline in tissue and blood level of the hormone. The responses are similar in animal and man in terms of the rapidity of the melatonin changes (Lewy et al., 1980; Reiter et al., 1983).

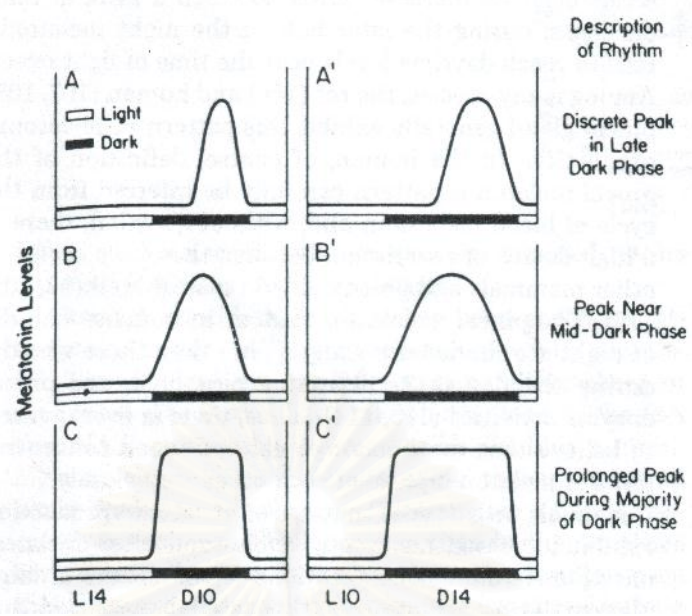


Figure 2-10. Provisional classification of different patterns of pineal melatonin production in various species as they occur in animals kept under either long day (A, B, and C) or short day conditions (A', B', and C'). Increasing the duration in darkness is invariably associated with prolongation of the melatonin peak. (Reiter; 1989).

Melatonin in CSF

The characteristic of melatonin that apparently allows for its rapid escape form the pinealocyte into the blood vascular system, i.e. its lipophilicity, also probably permits its ready entrance into other body fluids, even though the bulk (70%) of the melatonin in the blood is bound to albumin (Cadinali et al., 1972). On occasion it has been speculated that melatonin may, under some circumstances, be secreted directly into the CSF of mammals, however, the consensus is that the indole arrives in the CSF indirectly after its secretion into the blood. The site of transfer of melatonin into the brain ventricles is likely via choroids plexus (Reiter et al., 1975; Reiter et al., 1991; Mess et al., 1975).

Melatonin levels in the CSF exhibit a rhythm similar to that in the blood (Hedlun et al., 1976). The CSF melatonin rhythm is eliminated in animals maintained under continue lighting, whereas the rhythm persists when the animals are kept under persistent darkness (Figure 2-11).

There would certainly be some physiological advantage to having melatonin present in the CSF. First it would have ready access to the neural site as which it acts; second, it would escape the rapid metabolic fates to which it is subjected in the blood; finally, because of the relatively small amount of CSF compared to blood, the transfer of only minute quantities from the blood to the ventricle would bring the levels up to physiologically active concentrations (Reiter, 1991).

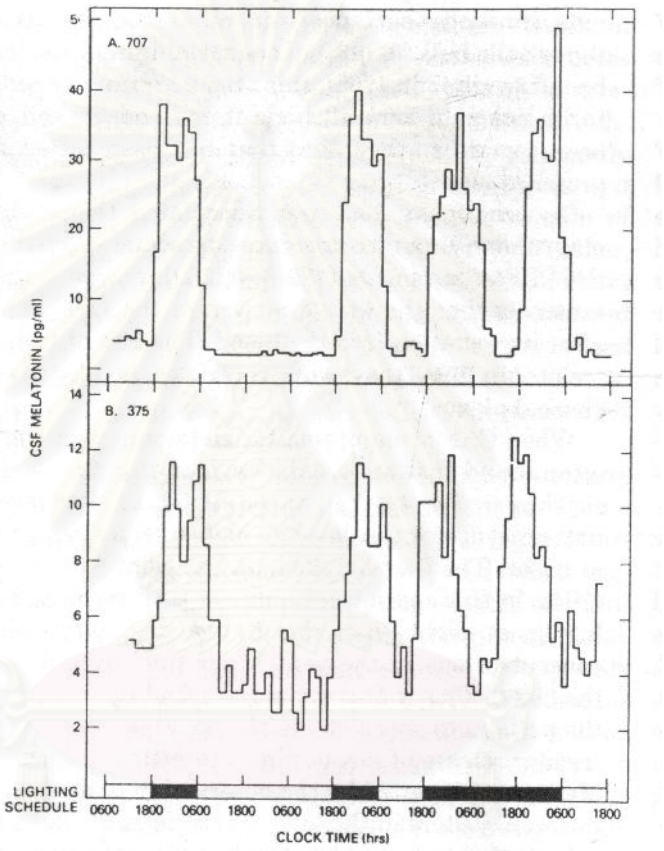


Figure 2-11. CSF melatonin levels during the day (open bars on horizontal axis) and at night (solid bars) in two (707 and 375) rhesus monkeys. Continual light exposure (long open bar) prevented the rhythms while they persisted in continuous darkness (long solid bar) (Hedlun et al., 1976).

Melatonin receptor

The NU-IUPHAR subcommittee on Melatonin Receptors has recently revised the nomenclature of the three known melatonin receptors formerly known as MEL1A (Mel1a or ML1A) are now MT1 receptors, MEL1B (Mel1b or ML1B) are now MT2, and unclone ML2 receptors are now called MT3 receptors (Alexander and Peters,

1998). The agonist, antagonist and distribution of these receptors are demonstrated in Table 2-3.

Table 2-3. Melatonin receptors.

Melatonin receptors			
Currently accepted name	MT_1	MT_2	MT_3
Previous names	MeL_{1a} ML_{1A} MEL_{1A}	MeL_{1b} ML_{1B} MEL_{1B}	ML_2
Structural information	350 aa (human)	363 aa (human)	
Full Agonists	Melatonin (M 5250) 2-Iodomelatonin (I 1899) N-Propionyl melatonin N-Butanoyl melatonin 6-Chloromelatonin (C 0331) 2-Methyl-6,7-dichloromelatonin S20098 GR 196429 8M-PDOT (-)-AMMTC	Melatonin (M 5250) 2-Iodomelatonin (I 1899) N-Propionyl melatonin N-Butanoyl melatonin 6-Chloromelatonin (C 0331) 2-Methyl-6,7-dichloromelatonin S20098 GR 196429 8M-PDOT (-)-AMMTC IIK7 (I 5531)	2-Iodomelatonin (I 1899) 6-Chloromelatonin (C 0331) Melatonin (M 5250) N-Acetylserotonin (A 1824) 5-MCA-NAT
Partial agonists	5-Methoxyluzindole N-Acetyltryptamine (A 7342)	5-Methoxyluzindole N-Acetyltryptamine (A 7342)	Not known
Antagonists	Luxindole (L 2407) S20928	Luzindole (L 2407) S20928 4P-PDOT 4P-ADOT K185 (K 1888)	Luzindole (L 2407) Prazosin (P 7791) N-Acetyltryptamine (A 7342) 4P-CADOT
Signal Transduction Mechanisms	G_i (cAMP) modulation) $G_{q/11}$ (increase IP_3/DAG)	G^i (cAMP) modulation) cGMP modulation	$G_{q/11}$ (increase IP_3/DAG)
Radioligands of choice	2-[^{125}I]-Iodomelatonin [^{3}H]-Melatonin	2-[^{125}I]-Iodomelatonin [^{3}H]-Melatonin	2-[^{125}I]-Iodomelatonin 2-[^{125}I]-MCA-NAT

MT1

The diversity of melatonin's response within the body may be attributed to the fact that its receptors are expressed in a wide variety of tissues. The MT1 melatonin receptor (MT1R) which is expressed in the suprachiasmatic nucleus of the hypothalamus (SCN) and cardiac vessels is involved in modulating circadian rhythms (Liu et al., 1997; Dubocovich et al., 1998) and constricting cardiac vessels (Doolen et al., 1998). Besides these specific regions, the MT1R is expressed in other regions of the brain and peripheral tissues (Von et al., 2002; Masana et al., 2001). Elucidating the physiological role of MT1Rs in many of these tissues is still under investigation, owever, the result from these studies are beginning to reveal some interesting results.

Besides its vast tissue distribution, the fact that MT1Rs can couple to a wide variety of G-proteins including $Gi_{\alpha 2}$, $Gi_{\alpha 3}$ and $G_{\alpha q}$ (Brydon et al., 1999; Witt-Enderby et al., 2001) $G_{\alpha s}$, $G_{\alpha z}$ and $G_{\alpha 16}$ (Chan et al., 2002) may also explain its diversity of response within the body. Even at the level of the cell, melatonin, acting through MT1Rs, can produce multiple cellular responses. As shown in numerous studies, MT1Rs have been shown to produce inhibitory responses on the cAMP signal transduction cascade, resulting in decreases in PKA activity (Morgan et al., 1994; Witt-Enderby et al., 1998) and decreases in CREB phosphorylation (Mcnalty et al., 1994; Witt-Enderby et al., 1998, 2003). This is a more generalized signaling mechanism proposed for MT1Rs. However, in COS-7 cells, MT1Rs have also been shown to stimulate cAMP probably through $G_{\alpha s}$ (Chan et al., 2002). Whether or not this is an artifact of the system needs to be supported or refuted by further investigations in other cell models and/or tissues. Besides the cAMP-dependent cascade, MT1Rs can couple to stimulation of PLC-dependent signal transduction cascades directly (Brydon et al., 1999) or indirectly via $G_{\beta\gamma}$ subunits (Godson et al., 1997) and can activate PKC (Witt-Enderby et al., 2003). MT1Rs can couple to calcium activated potassium ($BKCa_{2+}$) channels and G-protein-activated inward rectifier potassium (GIRK Kir 3) channels (Geary et al., 1997, 1998). These receptors can also modulate the formation of arachidonic acid (Godson et al., 1997), can stimulate c-Jun N-terminal kinase (JNK) activity and also modulate MAP kinases (Chan et al., 2002). As stated above, MT1Rs can modulate the nuclear factor, CREB. However, others have shown that activated MT1Rs can also inhibit the induction of c-fos and jun-B mRNA and c-fos translation induced by forskolin (Ross et al., 1996).

Activator protein-1 (AP-1), a transcription factor formed by the immediate-early gene products c-fos and c-jun, has been shown to be extensively regulated through the MAP Kinase pathway, specifically ERK1 and ERK2, and JNK (Whitmarsh and Davis, 1996). Thus, taken together, the findings that melatonin can regulate these factors may provide a mechanism underlying melatonin's ability to induce differentiations of certain cells (Witt-Enderby et al., 2003).

MT2

The role of MT2 melatonin receptors (MT2R) in mammalian physiology as well as its signaling properties is now becoming clearer with the recent development of MT2-selective ligands (Dubocovich et al., 1997; Witt-Enderby et al., 2003).

To date what is known is that MT2Rs are involved in retinal physiology, in modulating circadian rhythms, in dilating cardiac vessels and are involved in inflammatory responses in the microcirculation (Witt-Enderby et al., 1998; Doolen et al., 1998; Dubocovich et al., 1998). Unlike the MT1Rs, these receptors are more restricted in their localization which includes the cerebellum, SCN of the hypothalamus, the retina, kidney, ovary, cardiac vessels and various cancerous cell lines (Von-Gall et al., 2002; Masano et al., 2001). Similar to the MT1R, MT2Rs couple to an inhibition of cAMP formation in various transfected models and to a stimulation of PI hydrolysis (Mackenzie et al., 2002; Brydon et al., 1999). However, unlike the MT1R, activation of the MT2Rs expressed in HEK293 cells can also result in decreases in cGMP (Peti et al., 1999). Perhaps the subtle differences in pharmacology and signaling properties between the MT1R and MT2R may prove to be an essential component underlying melatonin's effects on specific physiological processes.

MT3

Recently, a protein that displays a binding profile similar to that of the ML2 receptor now denoted MT3 was affinity-purified from a Syrian hamster kidney (Nosjean et al., 2000). It was shown that this protein shares 95% homology to the human quinone reductase 2, an enzyme involved in detoxification. This protein and its associated activity, as revealed through radioligand binding and enzymatic assays, shows that it is expressed in the liver, kidney, brain, heart, brown adipose tissue, skeletal muscle, lung, intestine, testis and the spleen of a hamster, mouse, dog and

monkey (Nosjean et al., 2001). Very recently, though, it was shown that the MT3 protein may be involved in the regulation of intraocular pressure in rabbits and in inflammatory responses in the microvasculature (Witt-Enderby et al., 2003). Further investigation into this protein may reveal other unique properties as well.

Regulation of Melatonin Receptors

Melatonin is released from the pineal gland in a circadian manner where melatonin levels peak at night and persist for about 8 hours. During the day, melatonin levels decrease by 10-fold and persist for about 16 hours (Reiter et al., 1991). Because melatonin receptors are exposed daily to melatonin for prolonged periods of time, desensitization is thought to be an essential component underlying the functional effects of melatonin within the body. Prolonged exposure of MT1R to melatonin results in the desensitization of both endogenous and recombinant MT1Rs (Hazlerigg et al., 1993; Witt-Enderby et al., 1998). Additionally, prolonged exposure of recombinant MT2Rs to melatonin results in its desensitization (Mackenzie et al., 2002). However, others have shown in vivo that following a brief exposure (V 1 hour) to pharmacological levels of melatonin, no desensitization of the receptor occurs (Ying et al., 1998). Perhaps, desensitization of the receptor to melatonin occurs only following a prolonged exposure to melatonin (5 hours). This longer exposure paradigm is probably more reflective of the in vivo condition being that the release of melatonin persists for approximately 8 hours each night.

Little is known about the mechanisms underlying MT2R regulation. However, chronic exposure of MT2R expressed in CHO or NIH3T3 cells to both pharmacological and physiological levels of melatonin results in a desensitization of these receptors. This is manifested by a reduced potency of melatonin for the receptor (Mackenzie et al., 2002).

The mechanisms underlying melatonin receptor desensitization, the process by which receptors become refractory to their agonist, are unknown. Melatonin receptors can be regulated in a homologous manner, that is, by melatonin itself, or in a heterologous manner, that is by other stimuli such as the photoperiod or estradiol. As for homologous regulation of melatonin receptors, G-protein coupling and receptor down-regulation appear to play a role. The studies performed to date determining the

mechanisms underlying heterologous regulation of melatonin receptors, however, have primarily focused on changes in melatonin receptor density. Analysis of the functional status of the melatonin receptor following exposure to stimuli other than melatonin, may reveal that G-protein uncoupling, internalization, down-regulation as well as other mechanisms also participate in melatonin receptor regulation (Witt-Enderby et al., 2003).

A daily fluctuation in melatonin receptor mRNA and melatonin receptor protein in the SCN and pars tuberalis (PT) has been shown (Ross et al., 1996). In PT cells, the levels of melatonin receptor mRNA are increased following an increase in cAMP. During the daylight, when melatonin levels are very low, there is an increase in cAMP and subsequently an increase in melatonin receptor mRNA. During the night when melatonin levels begin to rise, melatonin can act on its receptor and cause a decrease in cAMP and thereby prevent any further melatonin receptor expression. Melatonin has also been shown to cause both desensitization and down-regulation of its own receptor by regulating its phosphorylation by PKC and PKA (Barrett et al., 1999; Ross et al., 1996).

Interaction between melatonin and other neurotransmitters

Melatonin as a free radical scavenger and neural antioxidant

A variety of oxygen free radical molecules are generated in the brain (Figure 2-12). The damage that these species inflict is referred as oxidative stress (Sies, 1991). Melatonin has proven to be to be highly protective of the brain against oxidative deterioration (Reiter 1995, 1999; Reiter et al., 1996, 1998). The action of melatonin is noteworthy because the ·OH is very reactive and indiscriminately damage any molecule in the vicinity of where it is produced. It is estimated that 50% of the total oxidative damage that organisms sustain is the consequence of the unceasing plundering by the ·OH. Melatonin scavenges this molecule with a calculated rate constant of 2.7×10^{10} M^{-1} s^{-1} which is greater than that of most antioxidants (Pappolar et al., 2000).

The brain is highly susceptible to oxidative damage for several reasons. Particularly noteworthy are the high concentrations of polyunsaturated fatty acid (PUFA) in the CNS. These molecules are easily oxidized in the process of lipid peroxidation. The peroxidation of lipids is as especially damaging as much as one of

the products generated during the breakdown of lipid, i.e., the peroxyl radical (LOO·), which is sufficiently toxic to propagate lipid peroxidation (Hall, 1997). Because of the result of the chain reaction, the damage from the lipid peroxidation process is dramatically greater than those of other free radicals (Pappolla et al., 2000).

The major chain breaking antioxidant is vitamin E (α-tocopherol). Some evidence suggests that melatonin may function as a chain breaking antioxidant as well due to its ability to detoxify the LOO·. While it has been suggested that melatonin is a more efficient scavenger of the LOO· than vitamin E (Peiri et al., 1994), this is not supported by all findings. Melatonin has one distinct advantage over vitamin E in protecting the brain against lipid peroxidation; which is that melatonin readily cross the blood brain barrier (Menendez-Pelaez et al., 1993). This contrasts with vitamin E which transverses this barrier much less easily (Pappolla et al., 2000).

Besides the evidence that illustrates its ability to direct scavenge reactive oxygen intermediates, melatonin also acts as indirect antioxidant in that it stimulates several important antioxidative enzymes. These enzymes provide protection against free radical damage by metabolizing free radical precursors as well as reactive oxygen intermediates to non-toxic products. Major anti-oxidative enzymes in the brain include a family of enzymes the superoxide dismutase, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase (Figure 2-12). Both pharmacological and physiological levels of melatonin have been shown to stimulate either mRNA levels or the activities of these antioxidative agents (Barlow-Walden et al., 1995; Pappolla et al., 2000; Reiter et al., 2000).

The protecting effect of melatonin to oxidative damage in the brain has been abundantly demonstrated (Reiter et al., 1998). In numerous models of experimentally-induced oxidative destruction of the brain, melatonin has proven effective in significantly reducing the damage. Melatonin reduced macromolecular damage caused by processes which works via free radical mechanisms in ischemia reperfusion, in vivo excitotoxicity, traumatic brain injury, hypoxia, and model of Parkinson's disease (Kilic et al., 1999; Jin et al., 1998; Mayo et al., 1998; Pappolla et al., 2000). Also melatonin has been shown effective in reducing amyloid β-induced lipid peroxidation in vitro (Pappolla et al., 2000; Daniels et al., 1998). To date there are no published reports in which melatonin has failed to protect the CNS from destructive processes which involve free radicals and reactive oxygen intermediates.

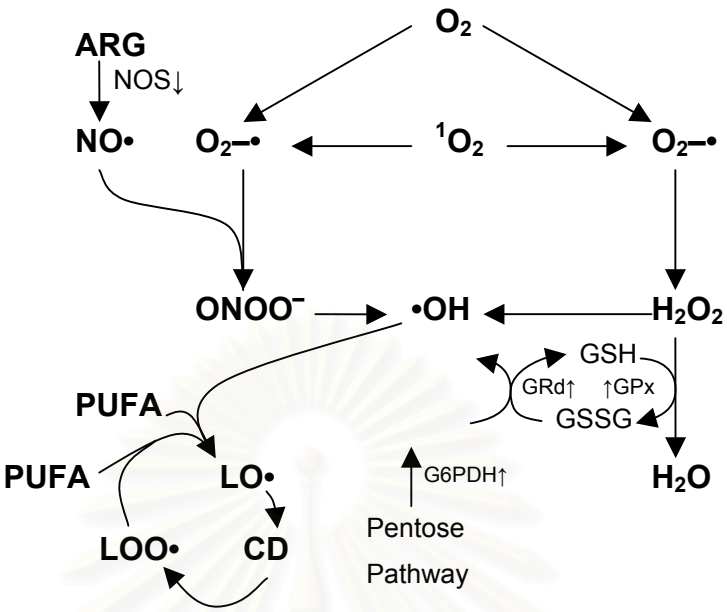


Figure 2-12. A summary of the products that are formed when oxygen (O_2) undergoes successive one electron reduction within cells. The most reative and toxic of the products formed is the hydroxyl radical (·OH). Other species however, also destroy neuronal elements including the peroxynitrite anion ($ONOO^-$), nitric oxide (NO), single oxygen ($^1O_2$), and the peroxyl radical (LOO). Much of the superoxide anion radical (O_2^-) generated in cells undergoes dismutation due to the catalytic action of a family of a super oxide dismutase (SOD). The resulting product, hydrogen peroxide (H_2O_2) as well as hydroperoxidase, can be enzymatically removed from cells by the action of two enzymes, catalase (CAT) and glutathione peroxidase (GPx). GPx oxidizes glutathione (GSH) to its disulfide form (GSSG) which is recycled back to GSH in the presence of glutathione reductase (GRd). The enzyme required to generate the co-factor (NADPH) for reduction of GSSG is glucose-6-phosphate dehydrogenase (G6PD). Nitric oxide synthase (NOS) is potentially a proxidative enzyme since NO can have inherent toxicity in addition to combinding with O_2^- to form $ONOO^-$. Melatonin anti oxidative actions include direct scavenging of the ·OH, $ONOO^-$, $^1O_2$ and possibly O_2 and the peroxyl radical (LOO). Additionally, melatonin may stimulate important antioxidative enzyme in the brain including SOD, GPx, GRd and G6PD while inhibiting the peroxidative enzyme, NOS. Thus melatonin has a number of means by which it reduces oxidative destruction of essential neural elements (Pappilloma et al., 2000).

Melatonin & NO

Besides detoxifying the OH, melatonin also neutralizes other reactive oxygen intermediates that damage essential neuronal molecules. Melatonin scavenges the highly reactive $ONOO^-$ (Gilad et al., 1997; Cuzzocrea et al., 1997) and reportedly scavenges one of its precursor molecules (nitric oxide, NO) (Noda et al., 1999).

Recent biochemical evidence also indicated that melatonin treatment could significantly reduce the NOS activity in a dose-dependent manner in the rat cerebellum as well as in the hypothalamus (Bettahai et al., 1996). Although the detailed mechanisms of melatonin in the prevention of NO mediated neurological deficits remain to be explored, it is likely that the neuroprotective effect of melatonin may be attributed to its antioxidant properties and its free radical scavenging ability. It is known that there are no morphophysiological barriers to melatonin (Reiter et al., 1999), and the melatonin-induced suppression of NOS activity is believed to be a consequence of the binding of calmodulin by this indole. It has been suggested that intracellular melatonin can interact with calmodulin and modify the binding of the peptide to the synthetic NOS peptide encompassing the calmodulin-binding domain of constitutive NOS, the natural mechanism by which calmodulin activates this enzyme. Besides reducing NO formation by restricting the activation of NOS and thereby limiting the secondary cytotoxicity caused by this free radical, melatonin has recently shown to directly scavenge the highly toxic peroxynitrite anion (ONOO-) as well. Thus, with a drop in NO synthesis, melatonin can also protect neurons by its ability to scavenge ONOO- and associated oxidants and the potential oxidative damage resulting from this latter molecule will be averted (Pozo et al., 1997; Chang et al., 2000).

With the antioxidative effect, especially to NO, it may imply that melatonin could play some role in migraine pathophysiology which being involved with NO.

Melatonin and GABAergic transmission

Melatonin has been shown to bind directly to the $GABA_A$ receptor (Coloma and Niles 1988) increasing allosterically its affinity to the agonist (Wu et al., 1999). The allosteric interaction, which occurred at micromolar concentrations, was shown not to be due to binding of melatonin at the steroid or benzodiazepine binding sites (Wu et al., 1999). In amacrine-like cells, melatonin led to a decrease in the amplitude and desensitization kinetics of $GABA_A$ mediated currents (Li et al., 2001). However

in the rod-dominant bipolar cell melatonin led to a desensitization of the $GABA_A$ mediated current, only (Li et al., 2001). These differential sensitivities were postulated to be due to expression of different $GABA_A$ receptors subunits in each preparation (Li et al., 2001).

While the studies described above are considered non-receptor mediated actions of melatonin, other studies have shown melatonin receptor-mediated actions on $GABA_A$ receptors. In the SCN, MT1 receptor activation causes an enhancement of $GABA_A$ currents and in the hippocampus activation of MT2 receptors leads to an attenuation of $GABA_A$ receptor mediated currents (Wan et al., 1999). Furthermore when MT1 and MT2 receptors were expressed in HEK 293 cells (Human embryonic kidney 293) there was either an increase (MT1), or a decrease (MT2) in GABA mediated currents. This study in particular implicates the possibility of different pathways activated by MT1 and MT2 melatonin receptors. Because activation of either melatonin receptor has been shown to lead to a decrease in cAMP, this decrease cannot account for the reduction in GABA currents when MT2 receptors were expressed vs. the increase in GABA currents when MT1 receptors were expressed (Wan et al., 1999).

Melatonin and Glutamatergic transmission

Glutamate is the major excitatory neurotransmitter in the brain. Glutamate leads to depolarization of neurons by activating ionotropic (AMPA, kainate, NMDA) or metabotropic (mGluR1, mGluR2, or mGluR3) glutamate receptors (Ireland and Abraham 2002; Semyanov and Kullmann 2001). In the case of ischemia/hypoxia and severe seizures, prolonged activation of glutamate receptors can lead to cell death (Rothman and Olney 1986; Olney et al., 1986). Melatonin has been shown to protect against damage caused by enhanced glutamate transmission (Cazevieille et al., 1997; Cazevieille and Osborne 1997; Cabrera et al., 2000; Skaper et al., 1998) by interacting with toxic oxygen species generated by glutamate-induced hyperexcitability (Avshalumov and Rice, 2002).

Melatonin has also been shown to influence glutamate-mediated transmission. In rat striatum neurons, melatonin significantly attenuated glutamate mediated responses to sensorymotor cortical stimulation (Leon et al., 1998). Hogan et al. demonstrated that 1mM melatonin attenuated hippocampal evoked-potentials, generated by stimulation of the glutamatergic synapses (Hogan et al., 2001). The

response of hippocampal, evoked-potentials to melatonin were later shown to be mediated by melatonin receptor activation (El-Sherif et al., 2002). While these studies demonstrate a receptor-mediated action of melatonin on glutamate-mediated neurotransmission, they did not determine the specific mechanism of action. Glutamate release, uptake and receptors can all be possible sites for melatonin action. Indeed studies on the golden hamster retina have shown that melatonin can increase [3H]-glutamate uptake and release (Faillace et al., 1996).

Melatonin and Ion Channels

Activation of ion channels is the major way of cell to cell signaling in the brain. The changes in permeability of voltage-gated Na^+, K^+ and Ca^{2+} channels are responsible for the generation of the action potential (AP) and neurotransmitter release. A few neurotransmitters have been shown to modify voltage-gated channels leading to a variety of changes including an increase/decrease in current (Brown et al., 2002; Sun et al., 2001; Imendra et al., 2000; Cantrell et al., 1999), shift in activation/inactivation curves (El-Sherif et al., 2001; Neusch et al., 2000) as well as a change in the time constants of channel activation/inactivation (White et al., 1994).

In mouse ocular tissue, melatonin enhances the activation and inactivation kinetics of TTX-insensitive voltage-gated Na^+ channels (Rich et al., 1999), at concentrations similar to the K_d of the cloned melatonin receptor. At higher concentrations of melatonin (1mM), considered pharmacological, there was an enhancement of the delayed rectifier K^+ channel (Rich et al., 1999). In a study by Huan (Huan et al., 2001), melatonin (1-100μM) was able to reversibly enhance the K^+ current in rat cerebellar granule cells. The effect of melatonin appeared to be receptor mediated, as it was mimicked by lower concentrations of iodo-melatonin, blocked by pre-incubation of cells with pertussis toxin, and inclusion of GTP-γ-S led to a non-reversible enhancement of the delayed rectifier K^+ channel in the presence of melatonin. Melatonin also blocks specific K^+ channels (composed of the Kv1.3 subunit), by directly interacting with the channel (Varga et al., 2001).

Melatonin and Intracellular Molecules

Melatonin has been shown to directly bind to calmodulin in vitro, via a non-receptor mediated process (Benitez-King et al., 1991, 1993; Benitez-King and Anton-Tay 1993). Melatonin's binding antagonizes calmodulin's normal physiological

effects (Romero et al., 1998), which include activation of Ca2+/calmodulin kinase II (Benitez-King et al., 1996), modulation of nitric oxide synthetase (Leon et al., 2000), and tubulin polymerization (Benitez-King and Anton-Tay, 1993). Melatonin also binds to an orphan receptor family, the RZR/ROR nuclear receptor (Wiesenberg et al., 1995) leading to apoptosis of cancer cells (Winczyk et al., 2001; Ciesla, 2001).

Roles of melatonin

There is now evidence that melatonin plays an important role in the biologic regulation of circadian rhythms, sleep, mood and perhaps reproduction, tumor growth and aging (Table 2-4).

Table 2-4. Biological function of melatonin.

Function or process	Effect	Suggested mechanism	Type of evidence
Sleep	Hypnotic effect and increased propensity for sleep	Hypothermic effect (at pharmacologic doses) Receptor-mediated action on limbic system	Placebo-controlled clinical trials
Circadian rhythm	Control of circadian rhythms and entrainment to light-dark cycle	Secretion of melatonin in response to neural input from the eyes and suprachiasmatic nucleus Receptor-mediated effects on neural and peripheral tissues Thermoregulation	Studies in animals and in humans on the effect of light and the light-dark cycle on the pattern of melatonin secretion
Mood	Possible role in cyclic mood disorders (seasonal affective disorder depression)	Unknown	Comparative clinical studies of the pattern of melatonin secretion and studies of phototherapy for mood disorders
Sexual maturation and reproduction	Inhibition of reproductive process	Inhibition of hypothalamic-pituitary gonadal axis Effect on ovarian steroidogenesis	Studies in animals and comparative clinical studies of the pattern of melatonin secretion (during puberty and in women with amenorrhea)
Cancer	Antiproliferative effects	Direct antiproliferative effect Enhanced immune response Scavenging of free	In vitro and in vivo studies in animals, in vitro studies of human neoplastic cells and cell lines,

		radicals	and a few small clinical studies
Immune response	Enhanced immune response	Increased interleukin production by T-helper lymphocytes	Studies in animals and a few uncontrolled studies in humans
Aging	Possible protective effects and decreased cell damage	Scavenging of free radicals	In vitro and in vivo studies in animals

Besides these processes, several studies in both clinical and experimental animal models have demonstrated that melatonin also plays a role in several physiological and pathological processes. The interesting biological properties of melatonin are listed as follow;

Effects of melatonin on blood vessels

Studies in the distribution of melatonin receptors demonstrated that the receptor for this hormone distributed in several important tissues and organs including vessels (Dubokovich, 1998; Mahle et al., 1997; Doolan et al., 1998; Wu et al., 1998; Ting et al., 1999). Melatonin receptors have been demonstrated in the cerebral and caudal arteries of rats (Regringny et al., 1998) and the vertebral spinal arteries, inferior cerebral and acoustic arteries, and circle of Willis of Old World primates (Viswanathan et al., 1993). The presence of melatonin receptors in the blood vessels correlate well with the hypertension developed following pineal removal (Zonoboni et al., 1967; Karppanen et al., 1970). Furthermore the density of vascular melatonin binding sites in rats was reported to differ in genetically hypertensive animals, and to vary with age and stage of the female estrous cycle (Viswanathan et al., 1992; Seltzer et al., 1992). These variations in the density of melatonin receptors suggest physiological and pathological regulation of melatonin in the vascular system.

The role of melatonin on cerebrovascular modulation was first suggested when binding sites of melatonin were identified in the median layer of cerebral arteries from rats and primates using 2-[^{125}I] iodomelatonin autoradiography (Viswanathan et al., 1993). In 1997, based on the results of their *in vitro* experiments, Geary et al. showed

that melatonin is a direct vasoconstrictor of cerebral arteries. Its vasoconstrictor effect was abolished by pretreatment with pertussis toxin, therefore, G_i or G_o should be involved in the process. An elevation of extracellular potassium concentration as well as administration of the blocker of the large conductance Ca^{2+} activated potassium (BK_{Ca}) channel also diminished the melatonin-induced vasoconstriction. These results suggest that activation of melatonin receptors on cerebral arteries increases vascular tone through G_i or G_o protein–mediated inhibition of BK_{Ca} channels (Geary et al., 1997). This proposed mechanism is further confirmed by a subsequent *in vivo* study. In 1999, Regrigny et al. performed an experiment to investigate the mechanism of melatonin-induced cerebral vasoconstriction by using an open skull preparation. In this study, cerebral arterioles were exposed to two doses of melatonin in the presence and absence of mt1 and/or MT2 receptor antagonist, luzindole and the BK_{Ca} channel blocker, TEA. They demonstrated that the melatonin-induced vasoconstriction was abolished in the presence of luzindole. The results also showed that administration of melatonin did not produce any additional effect on TEA-induced vasoconstriction. The absence of vasoconstriction in cerebral arterioles preconstricted with TEA was specific for melatonin as L-NAME in the presence of TEA induced an additional, significant vasoconstrictor response (Regrigny et al., 1999). These results confirmed the vasoconstrictor effect of melatonin. This effect is mediated by inhibition of BK_{Ca} channels following activation of mt1 and/or MT2 receptors.

While others have shown that melatonin alone has no vasoconstrictor activity in cerebral arteries (Evan et al., 1992; Wu et al., 1998). However, when the cerebral arteries are pre-activated by vasopressin, phenylephrine, norepinephrine, high K^+, electrical or adrenergic nerve stimulation, melatonin causes vasoconstriction at a dose rang from 10^{-11} to 10^{-7} M (Ting et al., 1999; Doolen 1998; Kruse et al., 1995; Doolen et al., 1999; Bucher et al., 1999; Lew et al., 1999).

The vasoconstrictive effect of melatonin on these cerebral vessels indicates the role of this indole in regulation of cerebral circulation.

Melatonin and anti-inflammatory effect

Melatonin has been shown to possess marked anti-inflammatory effects in many inflammatory models (Bilici et al., 2002; El-Shenawy et al., 2002). The carrageenan induced paw edema assay is a widely used model for the investigation of anti-inflammatory agents. The edema and secondary hyperalgesia in this model

involve both cyclooxygenase-dependent and -independent mechanisms, being the result of the local release of such inflammatory mediators as prostaglandins, nitric oxide, and substance P (Garcia-Perganeda et al., 1999; Maestroni et al., 1994; Di Stefano et al., 1994). Both regular NSAIDs and also selective COX-2 inhibitors are effective in relieving edema and pain in this model (Moshage et al., 1995).

It appears that the early phase of the carrageenan edema is related to the production of histamine, leukotrienes, platelet-activating factor and possibly cyclooxygenase products (prostacyclin), while the delayed phase of the carrageenan-induced inflammatory response has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide and OH. radicals, as well as to the release of other neutrophil-derived mediators (Dawson et al., 1991; Peskar et al., 1991).

n the recent years, the NO pathway has been proposed to play an important role in the carrageenan induced inflammatory response (Salvemini et al., 1996). Pharmacological inhibitors of NOS and ablation of the gene for iNOS have been shown to reduce the development of the carrageenan-induced inflammatory response (Salvemini et al., 1996). The systemic inflammatory response is also associated with the production of oxygen-derived free radicals and there is now substantial evidence that most of the cytotoxicity is due to a concerted action of oxygen and nitrogen-derived free radicals and oxidants (Cuzzocrea et al., 1999). An important part of the oxidative injury associated with simultaneous production of NO and oxygen free radicals may be mediated by $ONOO^-$, a toxic oxidant formed from the reaction of NO and superoxide (Beckman et al., 1990). In a number of pathophysiological conditions, $ONOO^-$ has been proposed as an important mediator of cell damage under condition of inflammation and oxidative stress (Cuzzocrea et al., 1997). The ONOO- is cytotoxic via a number of independent mechanisms. Its cytotoxic effects include the initiation of lipid peroxidation, inactivation of a variety of enzymes (most notably, mitochondrial respiratory enzymes), and glutathione depletion (Phelps et al., 1995).

Melatonin has been reported to be a free radical scavenger and antioxidant (Ebstein et al., 1997; Ebstein et al., 1997; Reiter et al., 1994; Phelps et al., 1995). It reduces oxidative stress by direct scavenging the free radical molecules and stimulation of several anti-oxidative enzymes like superoxide dismutase, glutathione peroxidase and glutathione reductase (Ebstein et al., 1997; Reiter et al., 1995; Pieri et al., 1994).

Furthermore melatonin's protective role in some inflammatory models has been demonstrated to mediate by the suppressing pro-inflammatory cytokines, prostaglandins and NO production (Sacco et al., 1998; Raghavendra et al., 2000).

More recently the novel role of melatonin as an immuno-modulator has also been proposed. Melatonin was known to modulate immune functions (Rubbo et al., 1994) and it was also demonstrated that melatonin binding sites are found on lymphocytes and macrophages (Garcia-Perganeda et al., 1999). Melatonin may exert certain biologic effects by augmenting the immune response. Studies in mice have shown that melatonin stimulates the production of interleukin-4 in bone marrow, T helper cells and of the granulocyte–macrophage colony-stimulating factor in stomal cells (Maestroni et al., 1994), as well as protecting bone marrow cells from apoptosis induced by cytotoxic compounds (Maestroni et al., 1994). In vitro studies on human peripheral blood mononuclear cells have shown that melatonin inhibits the production of tumor necrosis factor (TNF), a pathogenic mediator of various infective and inflammatory diseases, and interferon-gamma (Di Stefano et al., 1994; Raghavendra et al., 2000). Several studies demonstrated that melatonin inhibits the TNF production through its antioxidant activity (Peristeris et al., 1992). Interestingly, melatonin also inhibits the transcription factor, nuclear factor-κβ (NF κβ) as do other antioxidants (Sacco et al., 1998). The NF κβ induces many inflammatory genes that encode for pro-inflammatory cytokines, chemokines that selectively induce the inflammatory enzymes, such as inducible NO synthase, cyclooxygenase-2, adhesion molecules and inflammatory receptors (e.g. interleukin-2 receptors) (Raghavendra et al., 2000). Thus inhibition of this transcription factor together with the cumulative data mentioned above suggests that melatonin exerts potent anti-inflammatory effects. Parts of these anti-inflammatory effects are related to a reduction of several inflammatory mediators and the anti oxidative effect of melatonin (Cuzzocrea et al., 1997; El-Shenawy et al., 2002).

Role of melatonin in pain

The occurrence of systematic diurnal variation in pain thresholds has been documented in humans and various rodent species (Lakin et al., 1981). These fluctuations appear to be mediated primarily by changes in endogenous opioid activity (Kavaliliers, 1989). The synthesis and secretory patterns of the most potent and abundant endogenous opioid, β-endorphin (β-END), are marked by diurnal

fluctuations in humans and rats. Rhythmic changes in plasma β-END levels (Hamra et al., 1993) and total brain opioid levels are correlated with changes in nociceptive sensitivity in mammals. Furthermore when applied intravenously β-END produces potent antinociceptive effects in mice as determine with a tail flick and hot plate tests (Tseng, 1976). Moreover, both the nonspecific opioid antagonist, naloxone, and the highly selective, non-reversible μ receptor antagonist, β-funatrexamine disrupt the pain threshold in mice (Kavaliliers, 1989).

pivotal role of the pineal gland and melatonin in modulating both opioid rhythumic and pain perception is suggested by several findings. The diurnal variation of the pain threshold in rat testing using hot plate technique is attenuated by surgical pinealectomy (SPX) (Lakin et al., 1981) and light induced functional pinealectomy (LFPX). Furthermore, exogenous melatonin produces dose –dependent analgesia in mice and rat (Golombek, 1991; Sugden, 1983) and augments photo phase morphine analgesia (Lakin et al., 1981). Moreover, inhibition of melatonin synthesis by either acute light- pulses or administration of benzarazide, produce a significant decrease in nocturnal hotplate response latency in mice, effects which are, in part, melatonin reversible. Finally melatonin replacement (at physiological levels) restores the diurnal variation in pain threshold in LFPX rats (John et al., 1994). Unfortunately, the effectiveness of melatonin suppression was not confirmed in these studies. Nonetheless, these finding point to melatonin as a major candidate for controlling variations in pain sensitivity. In the case of opioid rhythmic, exposure to a short photoperiod (2L: 22D) decreased the level of hypothalamic and serum met-enkephalin and β-END like immunoreactivity in hamsters (Kumar, 1982). LFPX effectively eliminated the diurnal rhythm of β-END in the rat hypothalamus, even though, the suppression of melatonin was not confirmed. These data do, however, provide evidence that the circadian variation in β-END levels and pain threshold are contingent on the melatonin rhythm (Barret et al., 1999).

Several findings have fuelled speculation that melatonin affects pain sensitivity via an opiaternagic mechanism. For example, naloxone is capable of reversing the analgesic effects of the melatonin in the mouse and rats (Yu et al., 2000) and SPX abolishes the diurnal rhythm of morphine–induced analgesia. Given that both naloxone and morphine act on μ-opioid receptors, and that β-END is a potent

ligand for these receptors (Akil et al., 1984), it is conceivable that melatonin may modulate variations in pain perception by affecting the activity of β-END.

The mechanism underlying the putative action of melatonin on the opioid system remains speculative. However, the localization of melatonin receptors in the median eminence (Stankov and Reiter, 1990) and pars tuberalis (Morgan and Williams, 1989) of the anterior pituitary gland, the primary site of β-END synthesis and release, is consistent with a direct action of the melatonin at these sites. Whether melatonin acts on these sites to alter concentration of β-END in plasma remains to be established.

It can be concluded that melatonin has an analgesic effect and the CNS may be the primary site for melatonin to exert it. The promotion of the release of β-endorphin in the brain may be one of the mechanisms of action of melatonin.

2.3 Animal model of migraine

Migraine and other forms of primary headaches have become a major interest to neuroscientists. To search for a new approach of treatment, the precise anatomical and physiological basis for migraine needs to be clarified. However it is difficult to perform the experiment in patients during a migraine attack. Therefore an animal model of migraine has been developed. Even though there is no ideal animal model of migraine since nobody knows whether animals do experience migraine headaches. However a model has the advantage that the complex mechanism that underlines the migraine attack can be studied in controlled condition. Several models have been used in recent years (Table 2-5) (Goadsby, 2000).

Table 2-5. Current animal models of migraine.

Site	Measurement
Trigeminal ganglion stimulation	cerebral blood flow plasma protein extravasation
Meningeal irritation and c-fos	Blood other chemical
Cranial vessel stimulation	neuropeptides c-fos cerebral blood flow trigeminal nucleus recordings thalamic neuron recordings
Cranual and other vascular dynamics	Arteriovenous anastomoses Saphenous vein studies

To achieve a reasonable model of migraine it is essential to activate a structure that expects to be involved in the process. Since migraine is generally agreed, at the very least, to involve the trigeminally-inervated structure that should be dura or intracranial large vessels, a base is provided for selection (Table 2-6) (Goadsby, 2000).

Table 2-6. Structure stimulated in models of migraine.

Site	
Blood vessels/dura mater	superior sagittal sinus middle meningeal artery direct dural stimulation extracranial vessel stimulation
Trigeminal ganglion	
Greater occipital nerve	
Stimulation means	Electrical mechanical chemical

Most animal models published so far are the model of stimulation of the trigeminovascular system. The trigeminovascular system consists of the intracranial vasculature in the dura mater and the subarachnoid space that are innervated by

afferents of the trigeminal system. Anatomical studies have shown that the meningeal vasculature is innervated by the small unmyelinated sensory fibers which originated in the trigeminal ganglion. Animal models of trigeminovascular stimulation are based on electrical, mechanical or chemical stimulation of the trigeminovascular system. Upon activation, the trigeminal afferents transmit impulses orthodromically to synaptic nerve endings within lamina I and II of the trigeminocervical complex (TNC, C1, and C2) (Goadsby, 1996). This is the relay station for the nociceptive information of the trigeminovascular system. From the trigeminocervical complex the nociceptive signal is transmitted to the cortical area where the pain is sensed. Nociceptive stimulation of trigeminal afferent, not only causes orthodromic conduction but also causes antidromic conduction. This will lead to the release of several neuropeptides at the perivascular nerve terminal. These neuropeptides will cause the changes in cerebral vessels, vasodilatation and cerebral blood flow (Figure 2-13). Therefore changes in cerebral circulations are assessed as a marker for antidromic activity in the trigeminovascular system. c-fos expression in area lamina I and II of the trigeminocervical complex is the most frequently used as the marker for orthodromic activity in the trigeminovascular system.

In this study, activation of the trigeminovascular system by CSD and NO-donor (GTN) were employed. The regional cerebral blood flow (rCBF) changes and dilatation of cerebral arterioles were detected as a marker for antidromic activity in the trigeminovascular system. c-fos and NOS expression in laminar I and II of the cervical spinal cord at the level C1 and C2 were detected as marker for orthodromic activity in the trigeminovascular system.

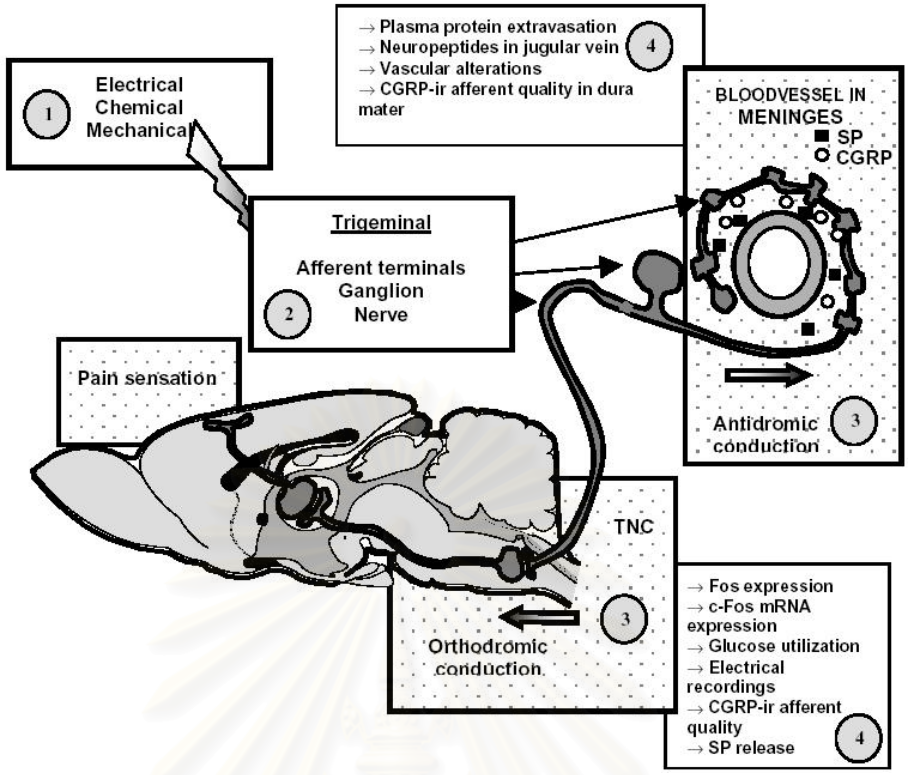


Figure 2-13. Schematic representation of the various animal models of trigeminovascular activation. Stimulation (1) of various parts of the trigeminovascular system (2) causes orthodromic and antidromic conduction to the trigeminal nucleus caudalis (TNC) and the perivascular afferent terminal respectively (3). As the result of antidromic activation several substances (SP, CGRP) are released at the afferent terminal. To assess the orthodromic and antidromic activity of trigeminovascular afferents, various parameters are measured.

NTG model

Several years ago the research in the molecular mechanism of migraine pain focused on the study of headache induced experimentally by GTN or histamine. By itself, GTN has no known action in the human body but acts via liberation of NO and it thus generally regarded as an NO donor. It is the most suitable substance for experimental studies of NO-induced headache since it is well tolerated and diffused freely across membrane due to its lipid solubility. It may thus deliver the NO to vascular tissues including those protected by the blood brain barrier. Histamine also seems to induce headache via NO. In human cerebral blood vessels it stimulates an endothelial H1 receptor that probably activates NOS (Toda, 1990). Most studies supporting a role of NO in migraine pathophysiology have used GTN since GTN

delivers NO directly while histamine stimulates the endogenous formation of NO (Sicuteri, 1987; Iversen and Olesen, 1996; Thomsen et al., 1994; Thomsen et al., 1993; Olesen et al., 1993).

The most prominent of GTN on the vascular system is vasodilatation. The mechanism underlying relaxation of vascular smooth muscles induced by nitroglycerin has been studied for the most part in vitro. The classic view is the nitrates act directly on the vascular smooth muscle to generate NO, then activates soluble guanylate cyclase and thus increase cGMP and c-GMP-dependent protein kinase with resultant smooth muscle relaxation (Tassorelli et al., 1999) (Figure 2-14). In human, GTN, single sublingual dose, induce headache in patients and subjects. Three different forms of headache are reported. One is immediate bifrontal or bitemperal, throbbing, and last for 20-60 minutes (Peter, 1953; Sicuteri et al., 1987). The other is delayed several hours and is described as occurring only in migraine patients and subjects with a family history of migraine. It often resembles previous migraine attacks. The third is clustered headache attacks which can be induced during cluster periods, but not at other times. A surprisingly long, unexplained and relatively constant latency period of 30-50 minutes is interposed between sublingual NTG and the onset of the attack (Gruetter et al., 1981).

Furthermore i.v. injection of GTN into normal subjects produced a reproducible moderate, throbbing and bifrontal headache (Figure 2-15). They also found that constant infusion of 0.5 μg/kg/minute is suitable for studies of changes of cerebral circulation. According to these data they have concluded that this NTG model is suitable as an experimental headache model (Iversen et al., 1989; Olesen et al.; 1993; Olesen et al., 1997).

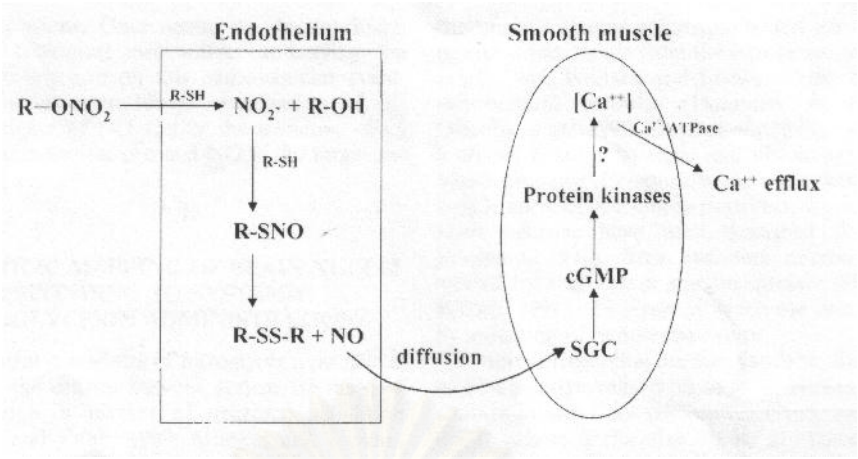


Figure 2-14. Schematic illustration of the proposed mechanism of vascular smooth muscle relaxation elicited by nitrovasodilators. In the presence of free thiols (R-SH) and sulphydryl groups, organic nitrate esters ($R\text{-}ONO_2$) form nitrate esters (R-OH) and inorganic nitrite (NO_{2^-}). Nitrite rapidly generates nitric oxide (NO) by forming the labile intermediate S-nitrosothiol (R-SNO). NO diffuses from endothelial cells to smooth muscle cells and activates the soluble guanylate cyclase (sGC), which in turn leads to an increased cyclic guanosine monophosphate (cGMP). The activation of protein kinases and subsequent poorly understood intermediary processes stimulate the membrane bound Ca^{2+} -ATPase. Ultimately Ca^{2+} exits from the cells, causing smooth muscle relaxation and vasodilation.

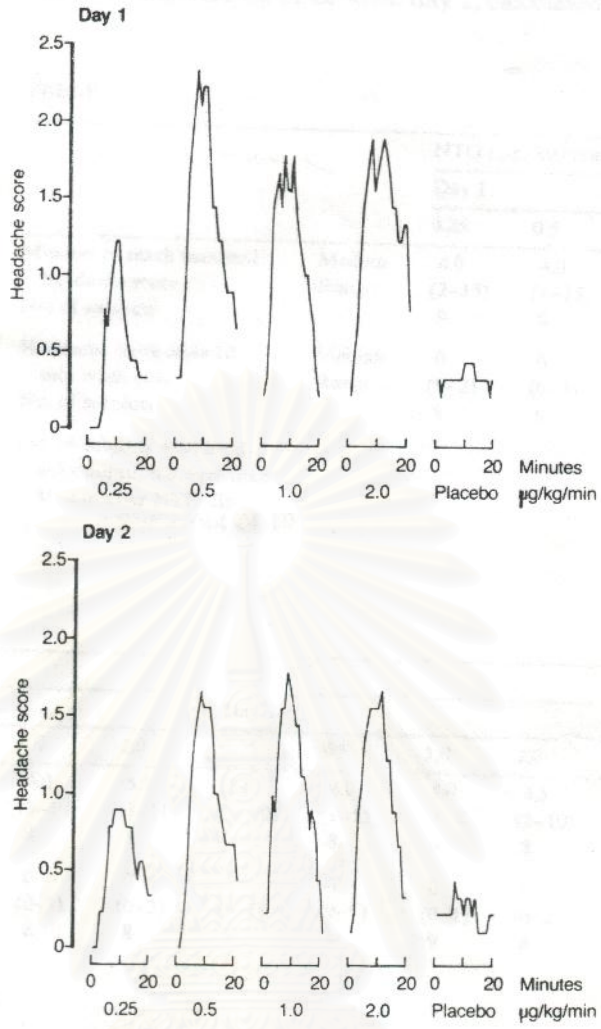


Figure 2-15. Mean headache scores (0 to 10 scale) during and after four doses of intravenous glyceryl trinitrate in normal headache-free subjects on day 1 of 2 separate study days. Glyceryl trinitrate was infused for 10 minutes, and during this period a rapid increase in headache was observed. This was followed by a 10-minute washout period, which resulted in a rapid decrease in headache. There was a relatively low-to-day variation and effect at approximately 0.5 µg/kg/min. from Iverson et al., 1989.

In animal, infusion of GTN causes an increase beading of a NOS-immunoreactive nerve fiber in the dura (Csillik et al., 1998). Structural alterations of NO-ergic axons that innervate blood vessels of the dura suggest an action on the primary peripheral afferent (Csillik et al., 1998).

Several studies have revealed the effect of GTN infusion on neural elements in the central nervous system. The results show the dramatic array of c-fos-immunoreactivity in a variety of nuclear complexes (Figure 2-16) (Tassorelli and Joseph, 1995).

In spinal trigeminal nucleus caudalis (TNC), the majority of c-fos positive cells were seen in lamina I which receives input from the nociceptive fibers, and in lamina II, where the nociceptive inputs are transmitted to rostal centers (Yong 1990). These findings suggest a possible activation of neurons which is involved in pain sensation from the face, cerebral vessels, or mininges (Kaube et al., 1993; Yong, 1990). The activation of TNC by GTN infusion was recently confirmed by Parduzt et al. who demonstrated that systemic a very large dose of GTN produced a significant increase in NOS and c-fos immunoreactive neurons in the cervical part of the nucleus caudalis.

Activation of both peripheral afferent which innervated cerebral vessels and second order neurons of trigeminal nerve which located in trigeminocervical complex can confirm that the systemic infusion of GTN can activate the trigeminovascular system. So this model can be used as a good animal model for migraine study.

In this study the changes of the cerebral arteriolar diameter and endothelial cells in cerebral capillary and arterioles were assessed as a marker for antidromic activity in the trigeminovascular system. c-fos and NOS expression in laminar I and II of the cervical spinal cord at the level C1 and C2 were detected as marker for orthodromic activity in the trigeminovascular system.

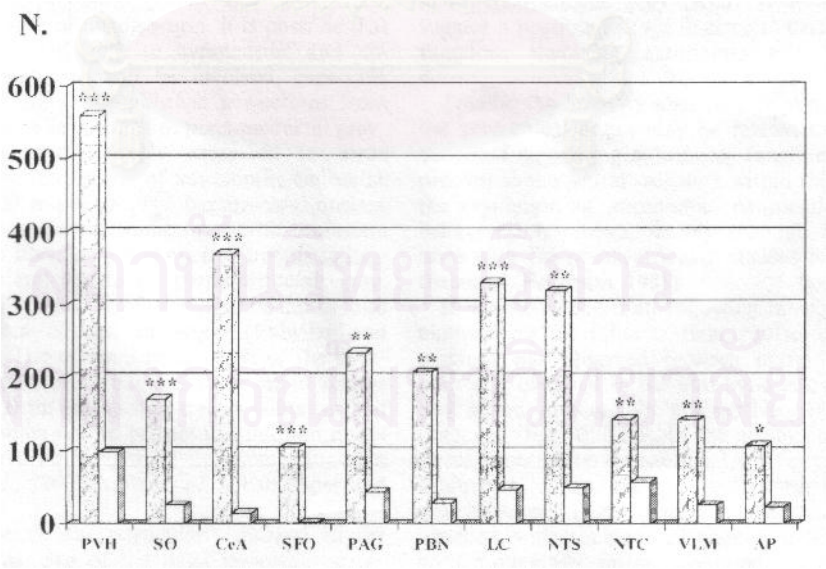


Figure 2-16. Bar graphs summarizes the expression of c-fos protein in brain nuclei of rats injected s.c. with nitroglycerin at a dose of 10 mg kg-1 (dashed bars) or vehicle (white bars), 4 hr after the injection. Data are presented as the mean number of c-fos-immunoreactive neurons within a given nucleus. Cell

counts of individual nuclei were made from sections 250 μm apart throughout the rostrocaudal extent of the nucleus itself. Statistical analysis (nitroglycerin vs. vihicle): *P o.o1, **P 0.0001. PVH, Paraventricular nucleus of the hypothalamus; SO, supraoptic nucleus of the hypothalamus; CeA, central nucleus of the amygdala; SFO, subfornical organ, PAG, ventrolateral column of the periaqueductal gray; PBN, parabrachial nucleus; LC, locus coeruleus; NTS, nucleus tractus solitarius; NTC, nucleus trigeminalis caudalis, VLM, ventrolateral medulla; AP, area postrema.

CSD model

This model is based on the CDS theory, migraine attack is initiated by a CSD (Lauritzen et al., 1987; Moskowitz et al., 1993).

The cortical spreading depression has been induced in most gray matter regions studied so far, e.g. in the cortex, the hippocampus and the cerebellum of a variety of species (Bures et al., 1974). It has been observed in human cortical tissues in vitro (Avoli et al., 1991), and in human hippocampus and istriatum in vivo (Sramka et al., 1998). Thus, the human cortical tissue do support the development of CSD, however the recording of CSD from human neocortex in vivo is still missing (Gloor, 1986). Therefore the researchers conduct the experiment in animal model in order to study pathophysiology of CSD.

The cortical spreading depression is associated with dramatic changes in the distribution of ions between the intra and extra cellular compartment. Na^+, Ca^{2+} and Cl^- enter together with water into the cell, while the K^+ and H^+ leave the cell, as the sized of the extra cellular decrease to half of the control values (Figure 2-17) (Lauritzen, 1994). A return to normal of most ion concentration and of the size of the extra cellular space occurs spontaneously after 30-60 seconds, whereas Ca^{2+} and pH usually take a few more minutes to recover. There is, at the moment, no satisfactory explanation of the spreading mechanism of CSD. The researchers suggest that the spread probably involves the diffusion of one or more chemical mediators, most likely K^+ and glutamate, into the extra cellular compartment (Nicholson, 1993). It has been suggested that a calcium wave in the glial cells underlines CSD, but this still remains to be proven. Lauritzen had tried to explain the spread of CSD (Figure 2-18) and regenerative process of CSD (Figure 2-19) in a simplistic scheme (Lauritzen, 1994).

Associated with the dramatic changes in ionic homeostasis, cortical blood flow is clearly changing following CSD in experimental animals. An initial transient increase in cerebral blood flow, followed by a long lasting decrease in flow, is

observed in animal models after induction of CSD (Piper et al., 1991; Duckrow, 1993). These changes in cerebral blood flow are consistent with changes observed in migraine patients (Olesen, 1992; Wood, 1994).

Besides the changes in cerebral blood flow, the results also demonstrate marked changes in diameter of pial arteries and arterioles which have been investigated regarding vasomotor aspects of CSD. CSD has been shown to induce transient vasodilatation of the pial circulation in a number of anesthetized animals (Shibata et al., 1990) and has also been shown to produce a change in cerebral blood flow (Piper, et al., 1991). Interestingly, CSD produces varying degrees of vasodilatation in different animal species. Differences in CSD-induced vasodilatation have also been found between pial arteries of different resting diameter in rabbits (Shibata et al., 1990).

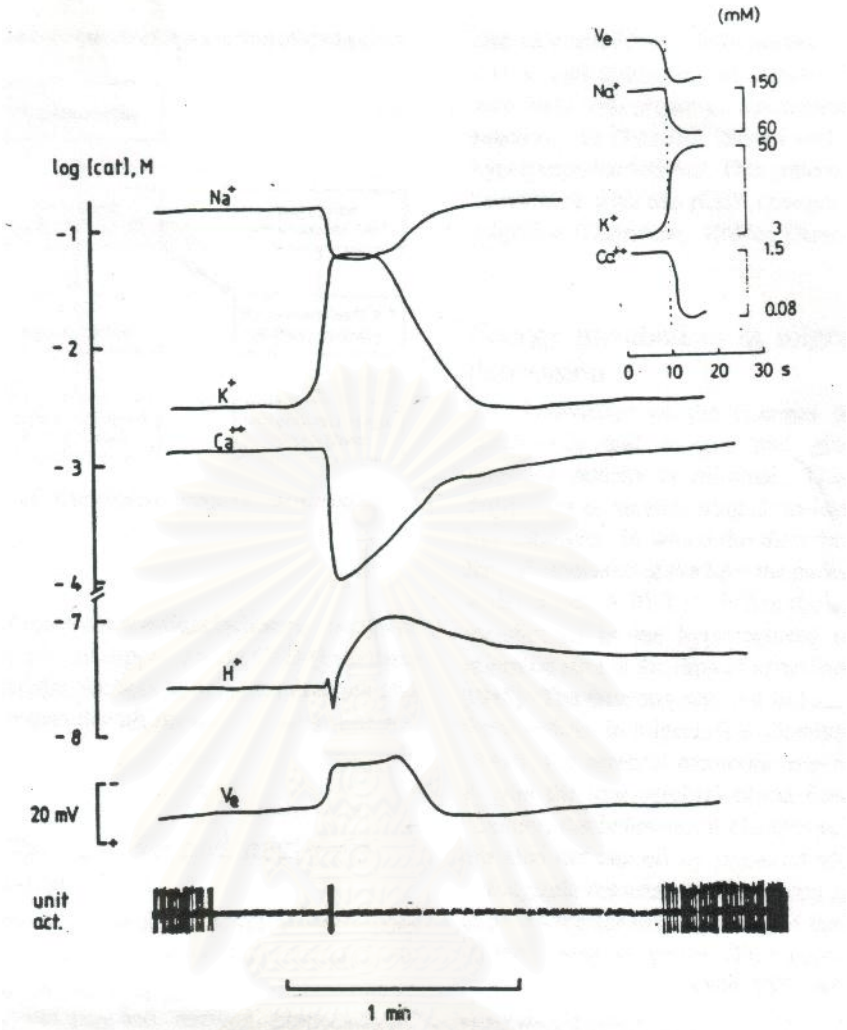


Figure 2-17. Electrophysiological changes accompanying cortical spreading depression in the rat brain. Interstitail ion concentrations of sodium, potassium, calcium and hydrogen were measured by ion-selective electrodes. The extra cellular potential (V_e) and the single unit activity were measured by single-barreled electrophysiological changes which were recorded in the parietal cortex.

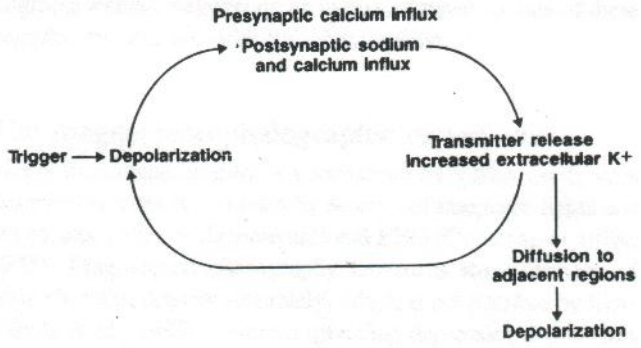


Figure 2-18. Simplistic scheme of autocatalytic cycle possibly occurring during CSD.

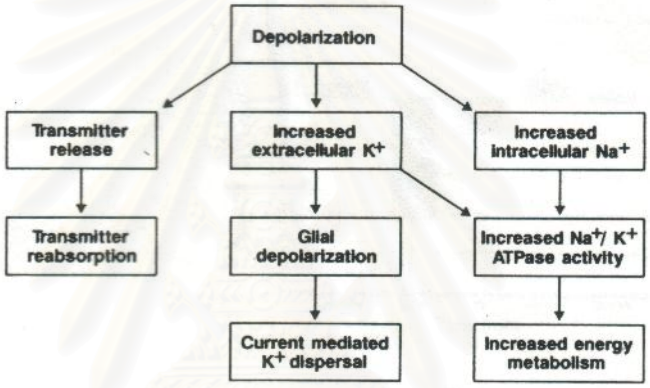


Figure 2-19. Simplistic scheme of regenerative processes assumed to occur during CSD.

Recent studies in rat models demonstrated the expression of c-fos, a marker of neuronal activity, in trigeminal neucleus caldalis (Bolay et al., 2002). This result confirms that second order neurons in trigeminal nucleus caudalis (TNC) are activated by CSD. According to these evidences it can be concluded that stimulation of the trigeminovascular system by CSD in animals can be used as an animal model for studying the pathophysiology of migraine headache.

CSD induction in animal

It is reasonable to assume that any disturbance of K^+ homeostasis would predispose the brain region to CSD (Grafstein, 1963). The clearance system of K^+ in the brain is largely depending on the capacity of the glia cell (Nicholson and Kraig, 1981). In human the ratio of glia cell/neuron cell is lowest in the primary visual

cortex. Therefore one would expect CSD to be initiated occipitally. As is well known, visual aura are indeed very frequent in migraine (Olesen et al., 1990; Lauritzen, 1994). Therefore in most animal experiments, CSD induction has been done at the occipital cortex.

Successful induction of CSD in experiments depends on the susceptibility of the tissue and the trigger factor involved. CSD can be induced in the animal brain by local electrical, mechanical or chemical stimulation. Among these techniques, mechanical and chemical stimulation are most widely used. In chemical stimulation, KCl is the key chemical using in this technique.

Mechanical stimulation

The common method used for elicitation CSD is blunt stabbing and pin pricking. Several research groups always choose pin prick to evoke CSD in the animal cortex, including cats, rats and rabbits (Piper et al., 1991; Ebersberger et al., 2001).

To activate CSD, a 26-30 gauge needle is inserted rapidly (1-2 mm depth) through the dipole and dura into cortical tissue and afterwards immediately withdrawn. The technique needs to avoid the pial vessal location. The location needs avoiding the pial vessels. However, this technique has been disagreed by some research groups. They suggested that this technique may link to the noxious stimulation (Read et al., 2000).

Chemical stimulation

As previously mentioned, KCl is the key chemical using in stimulating CSD. Application of KCl onto the cortical surface is the most widely used technique that has been chosen by several research groups. To induce CSD, KCl can be applied to surface of cortex in both liquid solution and solid crystal.

<u>KCl solution</u>

In order to induce CSD, a KCl solution is injected to the cortical area by a fused silica needle, catheter tube (PE-90) or glass micopipett. The concentration of the KCl solution can be vary from 300 mM to 3M depending on the species of the animal and the designed technique used in individual experiments (Ingvardsen et al., 1997; Moskowitz et al., 1993; Colonna et al., 1997; Kitahara et al., 2001; Choudhuri et al., 2002).

Solid KCl crystal.

In this technique, a solid crystal of KCl is directly applied onto the cortical surface. The amount of KCl (weight) and the duration of the cortical brain exposed to KCl can be varied. Some experiments apply 3mg solid KCl to the cortical surface for CSD activation throughout the experiment, however some experiments leave the solid KCl on the surface of the cortex just at the beginning of the experiment, the KCl crystal will be removed after inducing the CSD (Read et al., 2000; Parson 2000).

In most experimental designs for CSD activation, NaCl is usually applied on the brain of the control group for comparison.

In this study, I exploited the application of 3mg solid KCl to activate CSD in rats. The changes of cerebral blood flow and endothelial cells in cerebral capillary and arterioles were assessed as a marker for antidromic activity in the trigeminovascular system. With the same criteria in the NTG model, c-fos and NOS expression in laminar I and II of the cervical spinal cord at the level C1 and C2 were detected as a marker for orthodromic activity in the trigeminovascular system.

CHAPTER 3

Significance of problem and objectives

Based on this review, the pathophysiological basis of migraine is still controversy. The development of this disease may be attributing to both the neuronal and vascular mechanisms.

Furthermore some researchers have posited an environmental etiology since factors such as light, noise, smell, temperature, and humidity appear to contribute to the migraine onset (Gagnier, 2001). They suggest in addition, that periodicity and seasonal fluctuations support an environmental etiology to migraine.

The pineal gland is considered a photoneuroendocrine transducer that translates environmental information into neuroendocrine molecules (Brun et al., 1995; Toglia, 1986). Melatonin, the net result of this transduction, has been hypothesized to involve in migraine pathophysiology. Besides its role in the biologic regulation of circadian rhythms, sleep and mood, melatonin also demonstrates an anti-oxidative effect, anti-inflammation effect and analgesic effect (El-Shenawy et al., 2002). Furthermore melatonin can constrict blood vessels including cerebral vessels (Regringny et al., 1998 and 1999; Geary et al., 1998).

Since all of these properties are important in the trigeminovascular nociceptive control which is the major property of anti migraine agent, it is likely that this indole has a potential role in the treatment of migraine headache.

NO is the molecule involved in migraine in several levels. In the vascular endothelium, it appears to act as a large endocrine gland with a the potent vasodilatory role. NO acts as a pain mediator in the periphery. In the central role, NO is produced by neurons and glial cells and operates the process of neurogenic inflammation and activates the trigeminovascular system (Thomsen and Olesen, 2000).

The central role of NO in migraine pain does not only provide possible explanation as to the mechanisms of action of existing antimigraine drugs but it also likely offers further therapeutic possibilities. Thus, drugs that directly counteract the NO-activated cascade may be effective against migraine. Melatonin is known to be a strong anti-oxidative agent in the brain and several tissues. It can directly scavenge

NO, ONOO- or indirectly activates several anti-oxidative enzymes (Reiter et al., 2000). Besides being a highly effective direct free radical scavenger and indirect antioxidant, melatonin has several features that make it of clinical interest. Thus, melatonin is readily absorbed when it is administered via any route, it crosses all morphophysiological barrier and placenta. It seems to enter all part of every cell where it prevents all oxidative damage, it preserves the mitochondrial function, and it has a low toxicity (Reiter et al., 2000).

This anti-oxidative effect of melatonin leads this indole to become more interesting in using it as an anti-migraine drug. The effect of melatonin has been studied in several clinical studies. Alteration of melatonin level has been demonstrated in patients suffering from vascular headache. Furthermore attenuation of absence of nocturnal melatonin secretion was observed in migraine patients. The reduction of melatonin secretion is more pronounced during the headache period compared with the remission phase (Muraido et al., 1994). Leone et al. (1998) had tried to use melatonin as a preventive treatment in vascular headache. They found that four of the six patients reported a headache relief after starting melatonin infusion and the remaining two patients did so after the third night of the infusion. However the mechanism underlying the anti-migraine effect of melatonin has not been studied yet.

In order to investigate whether melatonin has a therapeutic role in migraine treatment, two headache animal models, cortical spreading depression (CSD) and NO-donor activation , were employed in this study. Both vascular and neural changes were measured in both animal models with and without melatonin pretreatment. The cortical blood flow (CBF) and changes in the ultrastructure of endothelial cells in cerebral arterioles were chosen to determine the change in the vascular compartment. Expression of c-fos and nitric oxide synthase (NOS) in the second order neurons of the trigeminovascular nociceptive system were chosen as a marker for the activation in the neural compartment.

The objectives of this study are:|

1. To study the effects of melatonin on the pial arteriolar diameter, cerebral blood flow (CBF) and trigeminovascular nociceptive pathway.
2. To study the effects of melatonin on rCBF changes induced by CSD.
3. To study the effects of melatonin on vasodilatation induced by the NO-donor.
4. To study the effects of melatonin on the nNOS expression in the trigeminovascular nociceptive pathway induced by CSD or the NO-donor.

5. To study the effects of melatonin on the c-fos expression in the trigeminal nociceptive pathway induced by CSD or the NO-donor.
6. To study the effects of melatonin on endothelial cell changes induced by CSD or the NO-donor.

CHAPTER 4
Material and methods

4.1 Animals

Wistar rats were supplied by the National Laboratory Animal Center of Mahidol University Salaya Campus.

4.2 Chemicals

A. Animal preparations

Pentobarbital sodium (Nembutal) was purchased from Sanofi (Thailand) Ltd. Glyceryl trinitrate (GTN) was purchased from DBL Thailand.

B. Substances used

Melatonin was purchased from Sigma Chemical company (USA). It was dissolved in artificial cerebro spinal fluid (aCSF) plus absolute ethanol (0.1% V/V) for the study of the effect of melatonin in cerebral vessels and was dissolved in 1% ethanol in NSS for the study of the effect of melatonin in changes of rCBF, diameter of cerebral vessels, nNOS expression, c-fos expression and endothelial cell changes. The melatonin solution was protected from light throughout the experiment.

C. Intravital Video-Microscopic study

Fluorescineisothiocyanate-dextran (MW 150D) (FITC) was purchased from Sigma USA. Sodium choride (NaCl) and Sodium carbonate ($NaCo_3$) were purchased from Merck. Temp-Bond was purchased from Kerr Corporation. Calcium choride (CaCl) and magnesium sulphate (MgSO4) were purchased from Riedel-de Hach, Germany.

D. Immunohistochemical study

Chemical reagents: Tissue freezing medium was purchased from Jung, Germany. Bovine serum albumin (BSA), 3,3 diaminobenzidine (DAB), Hydrochoric acic (HCl), paraformaldehyde, Potasssium phosphate (K_2PO_4) and sucrose were purchased from Sigma, USA. Hydrogen peroxide (H_2O_2), di-Sodium hydrogen phosphate ($Na2HPO_4$),

Sodium hydroxide (NaOH), and triton X-100 were purchased from Merk, USA. Gelatin was purchased from Boehringer Imgelheim Bioproducts partnership. LSB2 was purchased from DAKO, Thailand.

Antibody: Rabbit anti-neuronal NOS and Rabbit anti c-fos were purchased from Santacruz Biotechnology, USA.

4.3 Experimental animals

Adult male Wistar Furth rats weighing 250-300 grams were used in this study. The animals were housed five per cage in stainless-steel bottom cages. They were kept in a well-ventilated room with a 12/12 h light/dark cycle (light on from 06.00 to 18.00 h). The temperature was controlled at 28-32°C. All animals were allowed to have access to food (Purina Laboratory Chow, Premium quality feed, Zuellig Gold Coin Mills Pte., Ltd., Singapore) and tap water ad libitum. To limit the effect of nonspecific stress, all animals were accustomed to daily handing for at least 5 days before experimentation. All the protocols in this study were approved by the local ethics committee in Chulalongkorn University.

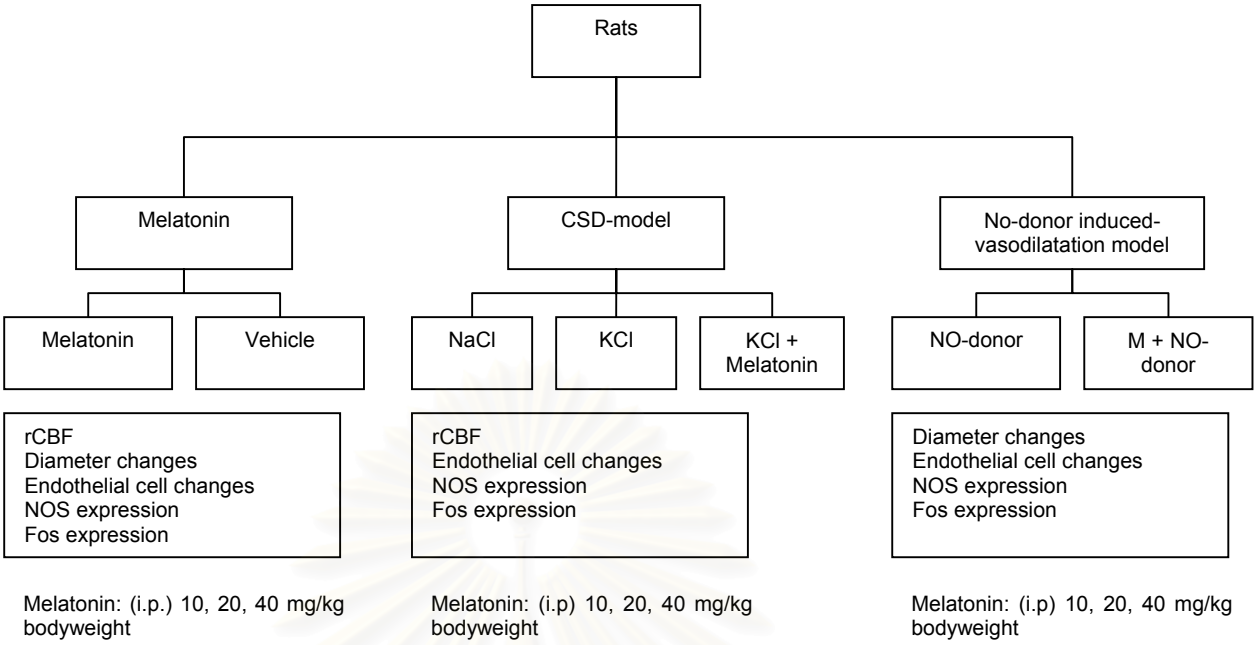
4.4 Study design

The experiment in this study was divided into three major parts as follows:

Part I : Study of the effect of melatonin on trigeminovascular nociceptive system.

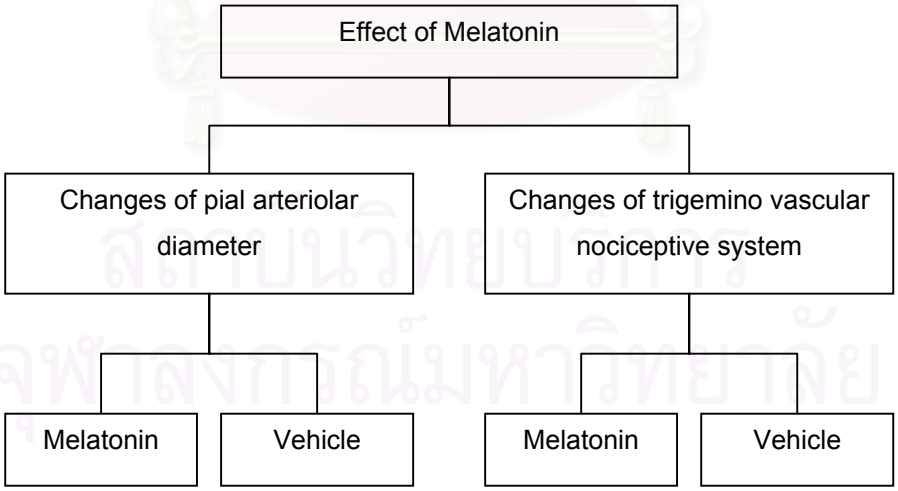
Part II : Study of the effect of melatonin on trigeminovascular nociceptive system in the CSD model.

Part III : Study of the effect of melatonin on trigeminovascular nociceptive system in the NO-donor induce vasodilatation model.



Part I: Study of the effect of melatonin on trigeminovascular nociceptive system

The experiment was conducted to investigate the effect of melatonin on trigeminovascular nociceptive system. This study comprised two experiments.



Study the effect of melatonin on the pial arteriolar diameter

In order to evaluate the effect of the i.p. administration of melatonin or suffusion of melatonin in the study of pial arteriolar diameter, the rats in this group were sub-divided into 2 subgroups:

A. Melatonin group

B. Vehicle group

In the melatonin group, the rats were divided into 2 groups of 5 each. The pial arterioles were superfused with aCSF plus melatonin in various concentrations (10^{-6}, 10^{-4} M). The arteriolar diameter were measured every 5 min during perfusion. After 30 min, melatonin perfusion was stopped, and then arterioles were superfused with aCSF until the diameter returned to the baseline.

In the vehicle group, the pial arterioles were superfused with a solvent, aCSF plus absolute ethanol (0.1% (VV^{-1}) for 20 min.. The arteriolar diameter was measured for 1 hour.

Study the effects of melatonin on trigeminovascular nociceptive system

In order to evaluate the effect of the i.p. administration of melatonin on the trigeminovascular nociceptive system, the rats in this group were sub-divided into 2 subgroups:

A. Melatonin group

B. Vehicle group

The rats in the melatonin group (A) were divided into 3 groups with 5 each. They were given i.p. injections of melatonin of the doses 10, 20, 40 mg/kg bodyweight respectively. Whereas the rats in the vehicle group (B) were given i.p. injection of 1% absolute ethanol in NSS 30 minutes after injections. The rCBF was measured for 2 hours then the rats were deeply anesthetized and perfused transcardially with 300 ml PBS, followed by 300 ml of 4% paraformaldehyde. After perfusion the brain and the spinal cord were removed and were processed for nNOS imunoreactivity, c-fos immunoreactivity and ultrastructural study of endothelial cells as protocols mentioned in the methods section.

Part II: Study of the effect of melatonin on trigeminovascular nociceptive system in the CSD model.

The cortical spreading depression model

In this experiment CSD was induced by application of 3 mg solid KCl. Two craniotomies were performed by drilling with a saline cooled drill. The measurements of the rCBF were performed at the anterior craniotomy and the CSD triggering was performed at the posterior one

In order to evaluate the effect of melatonin on the trigeminovascular nociceptive system in the CSD model, the rats in this group were sub-divided into into 3 sub-groups:

A. Control CSD group

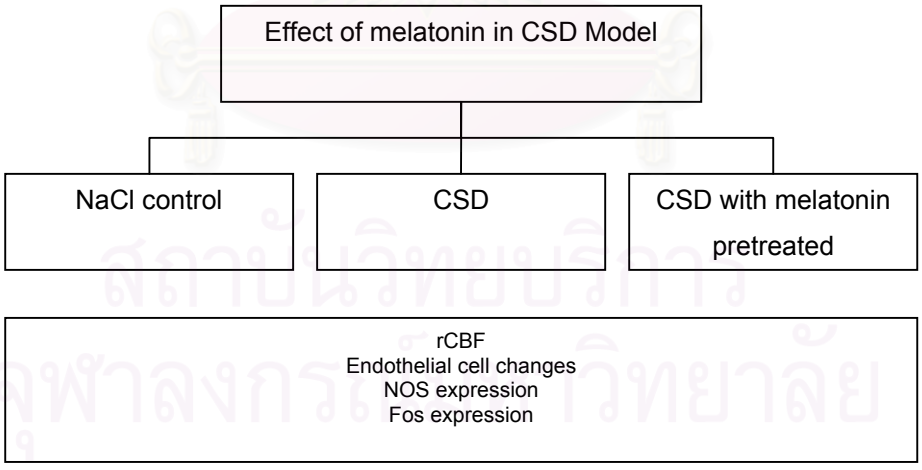
   NaCl was applied to the parietal craniotomy in this group (n=5).

B. CSD group

   KCl was applied to the parietal craniotomy in this group to produce CSD (n= 5).

C. Melatonin pretreated group

   The rats in this group were further divided into 3 groups of 5 each. They were injected intraperitoneally with various dosages of melatonin (10, 20, 40 mg/kg bodyweight) 30 minutes before the application with KCl.



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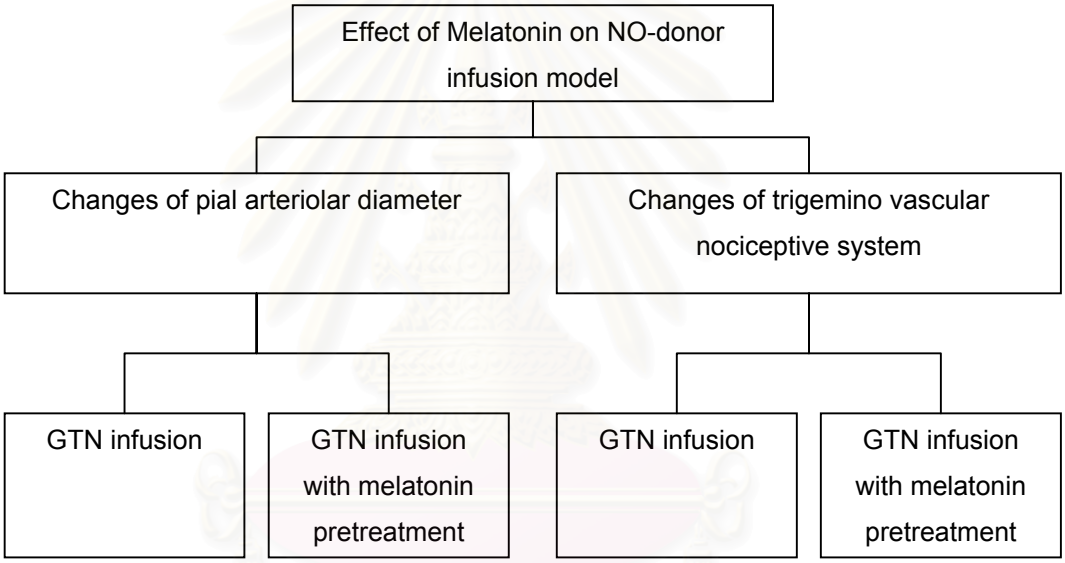
In every group the changes of the rCBF were continuously monitored for 2 hours. Then the rats were perfused and processed for detection of nNOS expression, c-fos expression and endothelial cell changes induced by CSD as the procedures mentioned in the methods section.

Part III: Study on the effect of melatonin on the trigeminovascular nociceptive system in the NO-donor induced vasodilatation model

This experiment was conducted to investigate the effect of melatonin on trigeminovascular nociceptive system in NO-donor infusion model. This study comprised two experiments.



Study the effects of melatonin on pial arteriolar dilatation induced by NO-donor

In order to assess the effect of melatonin on changes of trigeminovascular nociceptive system induced by GTN infusion, the rats were divided into 2 sub-groups:

A. NO-donor treated group (n = 5)
B. Melatonin pretreated group

   The rats in this group were further divided into 3 groups of 5 each. They were injected intraperitoneally with various dosages of melatonin (10, 20, 40 mg/kg bodyweight) 30 min before GTN administration.

In the NO-donor treated group, the exposed brain was continuously suffused with 30% GTN in aCSF for 30 minutes.

In the melatonin pretreated group the pial arterioles were suffused with 30% GTN after i.p. injections of melatonin in various dosages for 30 minutes.

The pial arterioles were continuously recorded by the videotape system throughout the experiment. The diameter of arterioles was measured from the digitized image with image analysis software as the procedures mentioned in the methods section.

Study the effects of melatonin on nNOS expression, c-fos expression and endothelial cell changes induced by the NO-donor

In order to assess the effect of melatonin on changes of trigeminovascular nociceptive system induced by i.v. injection of GTN , the rats were divided into 2 sub-groups:

A. NO-donor treated group (n = 5)

B. Melatonin pretreated group

The rats in this group were further divided into 3 groups of 5 each. They were injected intraperitoneally with various dosages of melatonin (10, 20, 40 mg/kg bodyweight) 30 min before GTN administration.

Intravenous injection of 10 mg/kg bodyweight GTN was administered to the rats in the NO-donor treated group. The rats in melatonin pretreated groups were i.v. injected with the same dose of NO-donor after 30 min of the i.p. injection of melatonin.

2 hours after GTN injection, the rats were processed for detection of nNOS expression, c-fos expression and endothelial cell changes with the same procedures mentioned below.

4.5 Methods

Surgical preparation

All experimental rats were anesthetized for the duration of the experiments by i.p. administration of 50 mg/kg of sodium pentobarbital. Additional doses of anesthetics were given as required to maintain surgical anesthesia bases on testing of the response to a tail pinch. After tracheotomy, the ventilation was assured by using a positive pressure ventilator (rodent ventilator model 683, Harvard Apparatus, USA).

A femoral artery and vein were cannulated to record the blood pressure and for intravenously infusion of the drug, respectively. Blood pressure was monitored throughout the experiments with pressure transducer (Nikon model TP-300T) which was recorded on the polygraph (Nikon RM 6000, Nikon Khoden, Japan). Arterial blood was collected periodically for determination of pH, PaO_2 and $PaCO_2$ by the pH/ blood gas analyzer (238 pH/blood gas analyzer, Ciba Corning Diagnostics, UK).

Measurement of the regional cerebral blood flow

The regional cerebral blood flow (rCBF) was 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 was performed by drilling with a dental drill in the frontal bone at 1 mm anteriorly and laterally from bregma and its diameter was about 7 mm. Care was taken not to damage the dura and excess heating was 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) (Modf ALF 21, Advance Co. Ltd., Japan) was placed perpendicularly to the cortical surface via the craniotomy opening. The probe was fixed 1-2 mm above the cortical surface of the brain. The results of blood flow were recorded on the polygraph (Nihon RM 6000 Nihon khoden, Japan) for 1 hour.

The Cortical spreading depression model

After tracheotomy and canulation, the head of the rats were fixed in a head holder. Two craniotomies were performed by drilling with a saline cooled drill. The anterior craniotomy was performed in the frontal bone at 1 mm anteriorly and laterally from bregma and its diameter was about 7 mm. The posterior craniotomy was performed in the parietal bone at 7 mm posteriorly and 1mm laterally from bregma and its diameter was about 2 mm. The measurements of the rCBF were performed at the anterior craniotomy and the CSD triggerings were performed at the posterior one. The frontal and parietal bones were thinned by drilling with a saline-cooled drill, until the blood vessels of the dura became visible. The dura was incised and the exposed brain was suffused with an artificial cerebrospinal fluid. The fiber optic needle probe of the laser Doppler flowmeter (wavelength 780 nm) (Modf ALF 21, Advance Co.

Ltd., Japan) was placed perpendicularly to the cortical surface via the frontal craniotomy opening. The probe was fixed 1-2 mm above the cortical surface of the brain. 3 mg solid KCl was applied to the parietal craniotomy opening to induce CSD. The rCBF was monitored for 2 hours after the KCl application. Then the rats were further processed for the NOS expression, the c-fos-expression and the endothelial cell changes.

Data collection of rCBF

The rCBF was measured by the laser Doppler flometry and was expressed as a percent change from baseline flow. Before drug administration, blood pressure, blood gas and the rCBF was measured until a steady state was reached. The flow average during 5 minute recording was determined as baseline flow and was used for further calculation for each rat. After that, the rCBF had been continuously recorded during drug administration and post infusion for 1 hour. The results were expressed as percentage change in the rCBF from the baseline flow.

After finishing the recording the rats were deeply anesthetized and perfused with 4% paraformaldehyde for further immunohistochemical and ultrastructural studies.

Measurement of the cerebral arteriols by the intravital fluorescein videomonitoring technique

After the craniotomy was performed in the same manner as in the rCBF study, a glass cranial window was placed over the craniotomy opening. An artificial cerebrospinal fluid was continuously infused into the intracranial space at the rate of 1 ml per hour, then the rat was placed under the fluorescent microscope (Optiphot 2, Nikon, Japan) fitted with the video monitoring system (SIT Camera, DAGE). To visualize the pial microvessels, the fluorescein-isothiocyanate labeled dextran (FITC-150, MW 150,000), a fluorescent plasma marker was intravenously injected (10 mg/200 μl). The images of the pial microvessels were recorded by the video system for further analysis.

All experiments started 30 minute after completion of the surgery which lasted about 2 hours. All drugs were added to the aCSF at their final concentration and superfused into the cranial window until a stable response was obtained. For each drug concentration, the diameter of the cerebral arterioles, the arterial blood pressure, the heart rate and the blood gases were measured prior to the infusion of drug and

after the stabilization of the diameter under drug infusion. Responses of cerebral arterioles were expressed as percentage change in diameter, using the diameter measured prior to drug infusion as baseline.

Measurement of the arteriolar diameter

The videotape segments of each experiment were played back frame by frame and 10 pial vessels were randomly selected from each experimental group. Images from selected vessels were digitized and the diameter of these vessels were determined by using the computer program "Global Lab Image". The diameter of the selected arteriole was assessed by the software indicated by the number of pixels (n). Then the software could convert the number of pixels to a distance (μm).

Perfusion and tissue preparation

The experimental rats which had to be further studied for the c-fos expression, the NOS expression and the ultrastructural changes 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 300 ml PBS, followed by 300 ml of 4% paraformaldehyde in a 0.1 M phosphate buffer, pH 7.4. After perfusion the brain was removed, a small portion of the anterior parietal cortex was taken out and cut into multiple 1 x 1 mm^2 cubes. Then these small pieces were immediately immersed in 3% glutaraldehyde in 0.1 M PBS, pH 7.4 for further transmission electron microscopy processing. The rest of the brain and the cervical spinal cord were immersed in 4% paraformaldehyde in a 0.1 M phosphate buffer (PBS) overnight at 4 ºC. Then the tissue was stored in a 30% sucrose solution in a phosphate buffer overnight for cryoprotection. The cervical spinal cord (C1-C2) was cut in a coronal plane by a cryostat microtome (Microm HM 50N) at 30 μm thickness and collected in a series of two in four sections. The first selected section was further processed for immunohistochemical staining of c-fos and the second one was further processed for immunohistochemical staining of nNOS. Sections were collected in 0.1 M a phosphate-buffered saline solution.

Immunohistochemistry study for the c-fos and the nNOS expression

The sections were rinsed in three changes of PBS. To block endogenous peroxidase, the sections were incubated with 3% hydrogen peroxide in 50% ethanol for 20 minutes. After repeated rinses in PBS, the non-specific binding of the antibody was blocked by incubating the tissues with a 5% normal horse serum (NHS), 1% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. After three rinses in PBS, the sections were incubated in the specific antibody solution (rabbit anti c-fos antiserum, and rabbit anti NOS antiserum) at a dilution of 1:1000 in the same solution at 4 ºC for 24 hours. After overnight incubation, the sections were then rinsed in PBS, incubated for 90 minutes with biotinylated mouse anti rabbit IgGs at a 1:200 dilution in PBS containing 2% NHS and 1% BSA, rinsed again in PBS, and incubated for 90 minutes in an avidin-biotin-peroxidase solution (Vectastain ABC kit, vector, Burlingham). This was followed by rinses in PBS and bound peroxidase was revealed by incubation of all sections in a solution containing 0.05% 3,3-diaminobenzidine (DAB), 0.005% hydrogen peroxide for 7 minutes. The reaction was stopped by repeated rinses in PBS. Following this reaction the tissue sections were washed and mounted onto gelatinized glass slides.

Measurement of the number of the c-fos immunoreactive cells

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 analysed. 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 lamina I and the lamina II area in both sides of each section were counted.

For all experimental rats in the CSD model, the Fos-IR cells were counted and reported as the number of immunoreactive cells in the dorsal horn ipsilateral to the KCl (CSD group) or to the NaCl (control group) application and contralateral side.

The data are reported as the mean and stardard diviation. The statistical difference was tested using the ANOVA test with a significance level of $p < 0.05$.

Measurement of the number of NOS immunoreactive cells

With the same criteria with c-fos, cell counts were carried out in sections processed for the NOS immunohistochemistry. A total of 10 sections were randomly

selected. Only cell profiles with a visible nucleus on the focal plane were analysed. The NOS immunoreactive cell (NOS-IR cell) were defined as those with a dark brown stain in their cytoplasm. With the same criteria with c-fos, the NOS-IR neurons in the lamina I and the lamina II area in both sides of each section were counted.

For all experimental rats in the CSD model, the NOS-IR cells were counted and reported as the number of immunoreactive cells in the dorsal horn ipsilateral to the KCl (CSD group) or to the NaCl (control group) application and contralateral side.

The data are reported as the mean and stardard diviation. The statistical difference was tested using the ANOVA test with a significance level of $p < 0.05$.

Ultrastructural study of endothelial cell changes

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 (500nm) and ultrathin sections (60-90 nm) which 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).

Quantitative study

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 equal or less than 10 μm. Vessels with a diameter about 15-30 μm were defined as arterioles. The morphometric parameter studied was the number of microvilli, the number of intracytoplasmic pinocytic vesicles.

The number of microvilli

The number of endothelium microvilli was randomly counted from 5 vessels of arterioles (15-30 μm in diameter) and 10 vessels of capillaries in every experimental rat. The data were reported as the number of microvilli per vessel.

The density of pinocytic vesicles.

The density of pinocytic vesicles was 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 grid was 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 was expressed as the number of pinocytic vesicle per square micrometer.

4.6 Statistical Analysis

All data were expressed as mean + standard deviation (SD). All data were analyzed for possible statistical significance using ANOVA with post hoc Bonferroni test. All statistical analyses were performed using computer software SPSS version 9 for windows (SPSS, Chicago, IL, USA). Probability values of less than 0.05 were considered to be statistically significant.

CHAPTER 5

Results

The results are divided into 3 major parts as follows:

Part I: Results from all experiments performed for studying the effect of melatonin on the trigeminovascular nociceptive system.

Part II: Results from all experiments performed for studying the effect of melatonin on the trigeminovascular nociceptive system in the CSD model.

Part III: Results from all experiments performed for studying the effect of melatonin on the trigeminovascular nociceptive system in the NO-donor induced vasodilatation model.

Part I: The effect of melatonin on the trigeminovascular nociceptive system.

The results in this part are subdivided into 5 parts as follows:

- The effects of melatonin on the cerebral arterioles.
- The effects of melatonin on the rCBF.
- The effects of melatonin on the nNOS expression in the trigeminovascular nociceptive pathway.
- The effects of melatonin on the Fos expression in the trigeminal nociceptive pathway.
- The effects of melatonin on the endothelial cell changes.

Part II: The effect of melatonin on the trigeminovascular nociceptive system in the CSD model.

The results in this part are subdivided into 4 parts as follows:

- The effects of melatonin on the rCBF changes induced by CSD.
- The effects of melatonin on the nNOS expression in the trigeminovascular nociceptive pathway induced by CSD.
- The effects of melatonin on the Fos expression in the trigeminal nociceptive pathway induced by CSD.
- The effects of melatonin on the endothelial cell changes induced by CSD.

Part III: The effect of melatonin on the trigeminovascular nociceptive system in the NO-donor induced vasodilatation model.

The results in this part are subdivided into 4 parts as follows:

- The effects of melatonin on the arteriolar dilatation induced by GTN.
- The effects of melatonin on the nNOS expression in the trigeminovascular nociceptive pathway induced by intravenously infusion of GTN.
- The effects of melatonin on the Fos expression in the trigeminal vascular nociceptive pathway induced by intravenously infusion of GTN.
- The effects of melatonin on the endothelial cell changes induced by intravenously infusion of GTN.

Part I: The effect of melatonin on the trigeminovascular nociceptive system

The effects of melatonin on cerebral arterioles

To test whether the solvent used in this experiment had effect on the cerebral arteriolar diameter, 0.1 % (V/V) ethanol in aCSF was superfused onto the pial arterioles. The results showed that superfusion with 0.1 % (V/V) ethanol in aCSF for 1 hour had no effect on the cerebral arteriolar diameter (Figure 5-1).

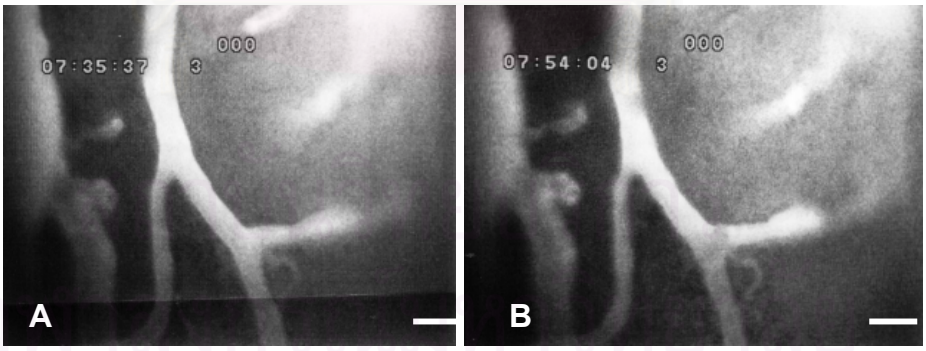


Figure 5-1. The intravital videomicroscope image of the cerebral arterioles superfused with aCSF (A) or with 0.1 % **(V/V)** ethanol in aCSF (B). No significant change of the pial arteriolar diameter is demonstrated. Bar = 100μm.

On the other hand, melatonin induced dose dependent constriction of the cerebral arterioles (Figure 5-2 and Figure 5-3). The constriction of the cerebral arterioles was maximam after 10 minute perfusion with aCSF plus melatonin (10^{-4} M). After the start of the infusion with melatonin for 10 minutes, the significant decrease in diameter of the arteriolar diameter was observed. Thirty minutes after the end of melatonin superfusion, the diameter of cerebral arterioles returned to the baseline value (Figure 5-2).

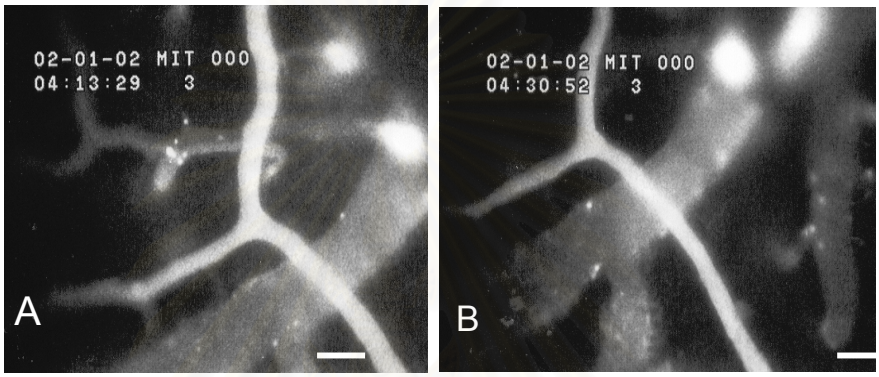


Figure 5-2. The intravital videomicroscope image of the cerebral arterioles superfused with aCSF (A) or with aCSF plus 10 $^{-6}$ M melatonin (B). Vasoconstriction effect of melatonin is demonstrated. Bar = 100μm.

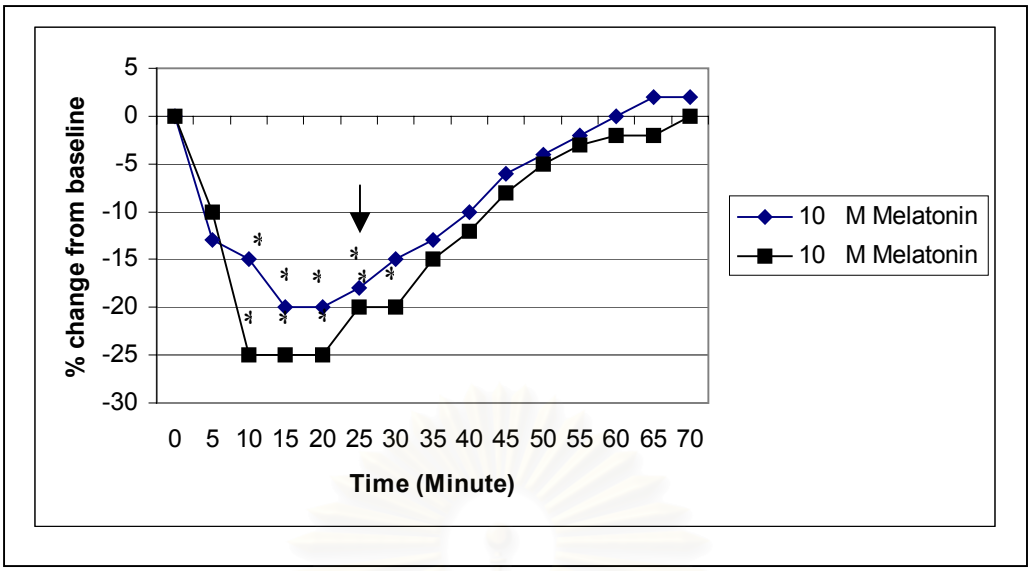


Figure 5-3. Effect of melatonin (10^{-6} M and 10^{-4} M) on the cerebral arteriolar diameter. Arrow shows the end of melatonin perfusion. Values are mean ± SD. *$p \leq 0.05$ vs. baseline.

The effects of intraperitoneal injection of melatonin on the rCBF

Intraperitoneal injection of melatonin doses of 10, 20 and 40 mg/kg bodyweight had no effect on the rCBF as compared with the rCBF obtained from the control group, the rats given i.p. injection of 5% ethanol in NSS (Figure 5-4).

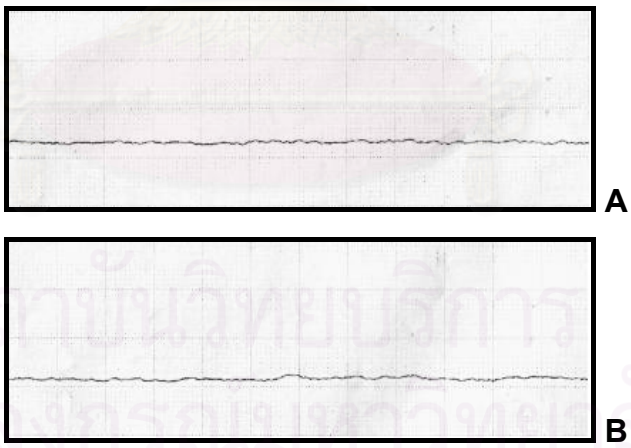


Figure 5-4. The tracing showing the rCBF in the control group (A) and the 40 mg/kg bodyweight melatonin treated group (B).

The effects of melatonin on the nNOS expression in the trigeminovascular nociceptive pathway

In this experiment the sections of C1 and C2 of the cervical spinal cord were studied under the light microscope.

The number of NOS-IR cells in the rats given i.p. injection of melatonin doses of 10, 20 and 40 mg/kg bodyweight did not show any significant difference as compared with the NOS-IR cells obtained from the control group, being the rats given i.p. injection of 5% (V/V)ethanol in NSS. The number of NOS-IR cells was 31 ± 4 cells per section in the control group and 32 ± 6, 31 ± 5 and 29 ± 5 cells per section for the 10, 20 and 40 mg/kg bodyweight melatonin treated group respectively (Table 5-1, Figure 5-5 and Figure 5-6).

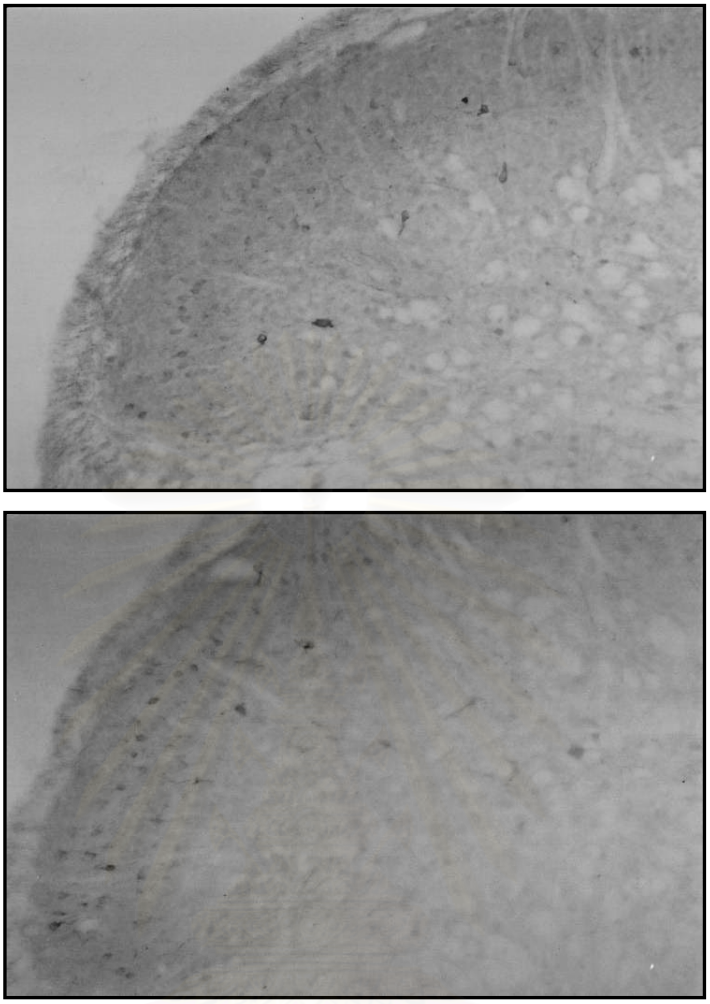


Figure 5-5. The photomicrograph showing the NOS immunoreactivity in the C1 and C2 cervical spinal cord sections of A) Control group, B) 40 mg/kg bodyweight melatonin treated group.

Table 5-1. The mean value ± SD of the NOS-IR cells in the C1 and C2 cervical spinal cord sections obtained from the control and the melatonin treated group**.**

Group	Number of NOS-IR cells
Control group	31 ± 4
10 mg/kg bodyweight melatonin treated	32 ± 6
20 mg/kg bodyweight melatonin treated	31 ± 5
40 mg/kg bodyweight melatonin treated	29 ± 5

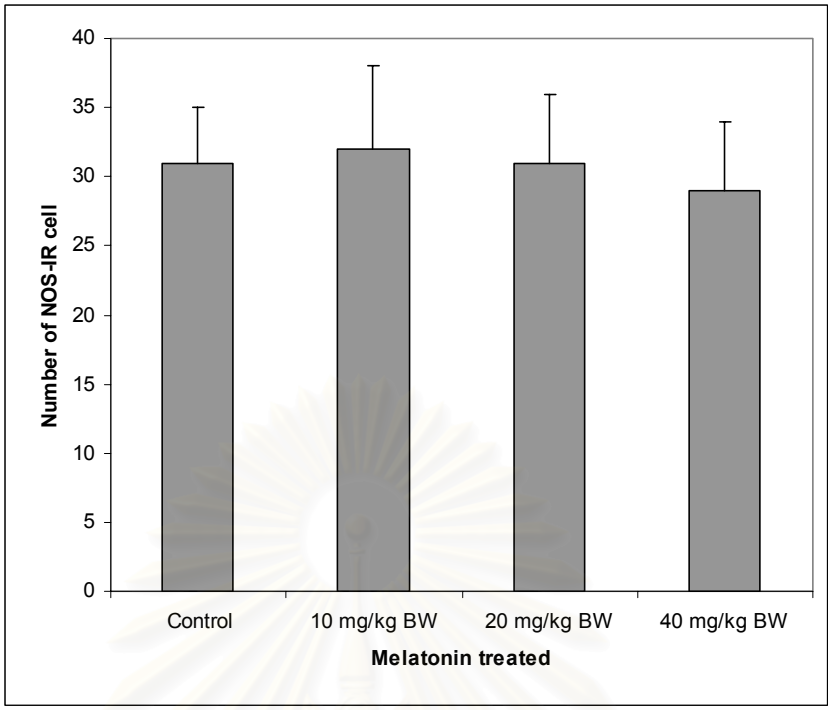


Figure 5-6. Bar graph showing the mean value ± SD of the NOS-IR cells in the C1 and C2 cervical spinal cord sections obtained from the control group and the melatonin treated group.

The effects of melatonin on the Fos expression in the trigeminovascular nociceptive pathway

Analysis of 10 randomly selected sections from each experimental group showed mean values of 15 ± 4, 15 ± 4, 16 ± 5 and 18 ± 6 Fos IR cells per section for the control group, 10, 20 and 40 mg/kg bodyweight melatonin treated group respectively. The number of Fos-IR cells did not show any significant difference between the control group and the melatonin treated group. The data are shown in Table 5-2, Figure 5-7 and Figure 5-8.

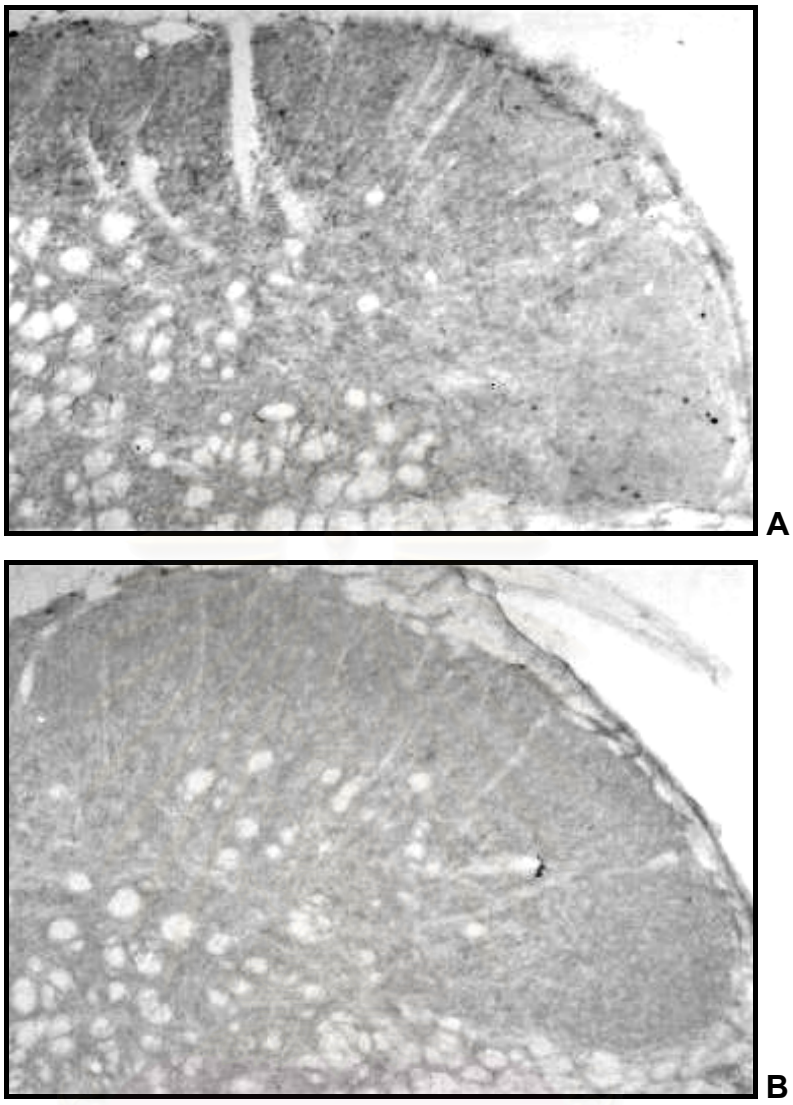


Figure 5-7. The photomicrograph showing the Fos immunoreactivity in the cervical spinal cord sections of A) Control group, B) 40 mg/kg bodyweight melatonin treated group.

Table 5-2. The mean value ± SD of the Fos-IR cells in the C1 and C2 cervical spinal cord sections obtained from the control and the melatonin treated group.

Group	Number of Fos-IR cells
Control group	15 ± 4
10 mg/kg bodyweight melatonin treated	15 ± 4
20 mg/kg bodyweight melatonin treated	16 ± 5
40 mg/kg bodyweight melatonin treated	18 ± 6

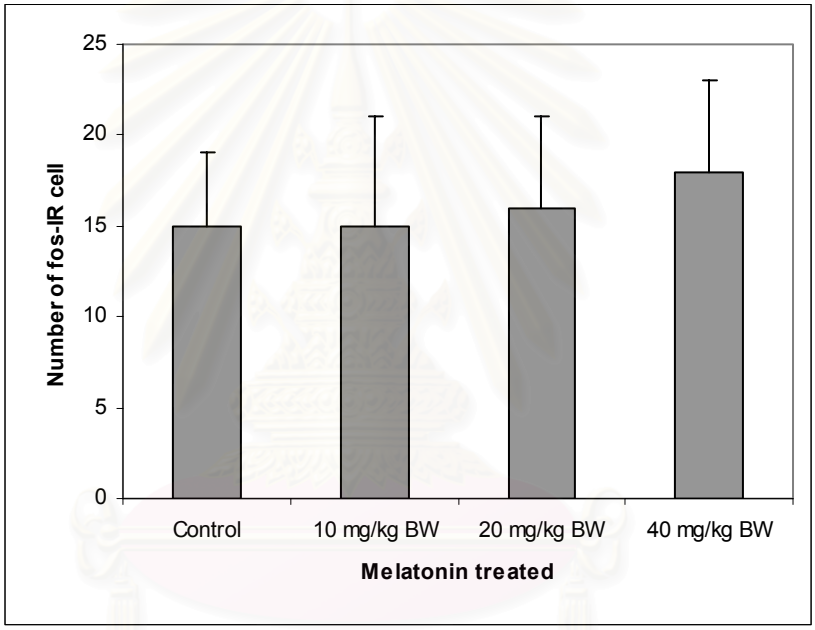


Figure 5-8. Bar graph showing the mean value ± SD of the Fos-IR cells in the C1 and C2 cervical spinal cord sections obtained from the control and the melatonin treated group.

The effects of melatonin on the endothelial cell changes

Analysis of 50 randomly selected capillaries and 25 arterioles obtained from the control group showed mean values of 0.5 ± 0.3 and 1.5 ± 0.5 microvilli per vessel for capillary and arteriole, respectively. The average density of pinocytic vesicle was 9.5 ± 2 vesicles per μm^2 for capillary and 10 ± 3 vesicles per μm^2 for arteriole, respectively.

The ultrastuctural study of arterioles and capillaries obtained from the melatonin treated group did not show any difference from those of the control group (Figure 5-9 and Figure 5-10).

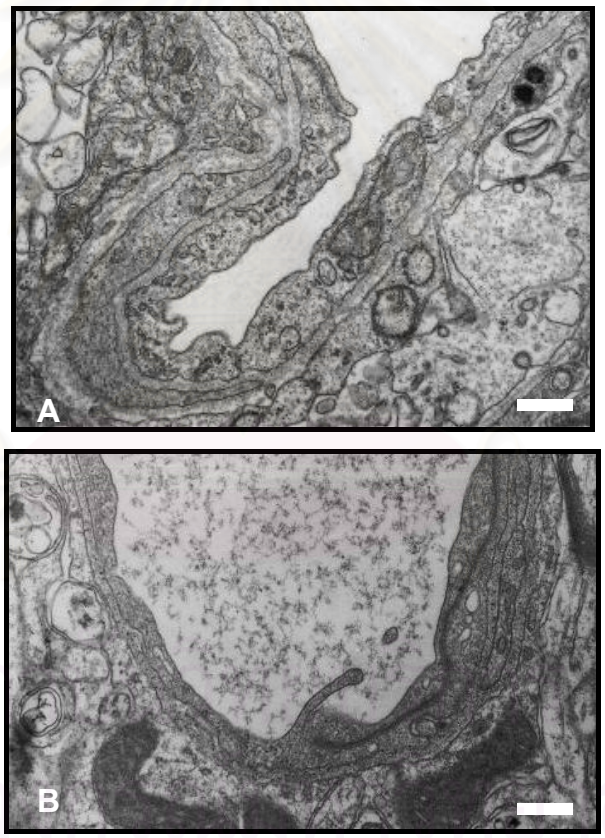


Figure 5-9. Electron micrograph of cerebral microvessels showing a few number of pinocytic vesicles and microvilli in capillary obtained from (A) the control group and (B) the 40 mg/kg bodyweight melatonin-treated group. (x 60000). Bar = 200 nm.

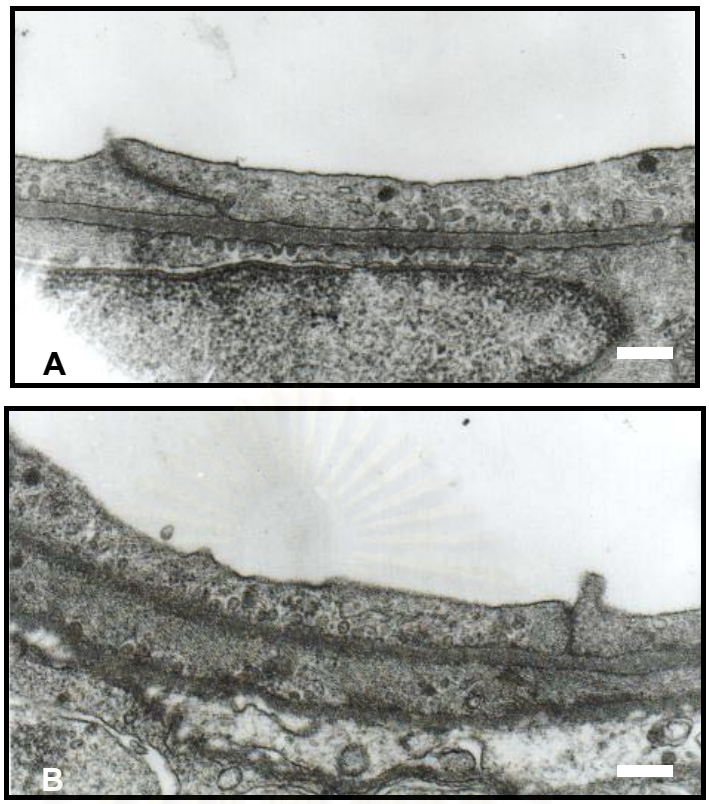


Figure 5-10. Electron micrograph of cerebral microvessels showing a few number of pinocytic vesicles and microvilli in arteriole obtained from (A) the control group and (B) the 40 mg/kg bodyweight melatonin-treated group. (x 60000)**.** Bar = 200 nm.

Part II: The effect of melatonin on the trigeminovascular nociceptive system in the CSD model. The effects of melatonin on rCBF changes induced by CSD.

rCBF changes induced by CSD

The rCBF changes were studied in the CSD group and the control group in order to evaluate the effect of CSD on rCBF changes.

In the CSD group, application of 3 mg solid KCl to the cortical parenchyma induced repetitive hyperemia after application of solid KCl for 4 minutes (Figure 5-11).

The amplitude of each peak was calculated as a percent change from the baseline flow (Figure 5-12). The magnitude was maximum in the first peak (220 % from the baseline). The median number of hyperemic cycles within one hour was 11

(varying from10 to14), the average duration of these cycles was 4.5 ± 0.7 minutes. On the other hand the application of solid NaCl had no effect on the rCBF. Statistical analysis demonstrated that CSD induce by KCl application induced significant change in the rCBF as compared with NaCl application ($p < 0.05$, ANOVA for repeated measurements). *

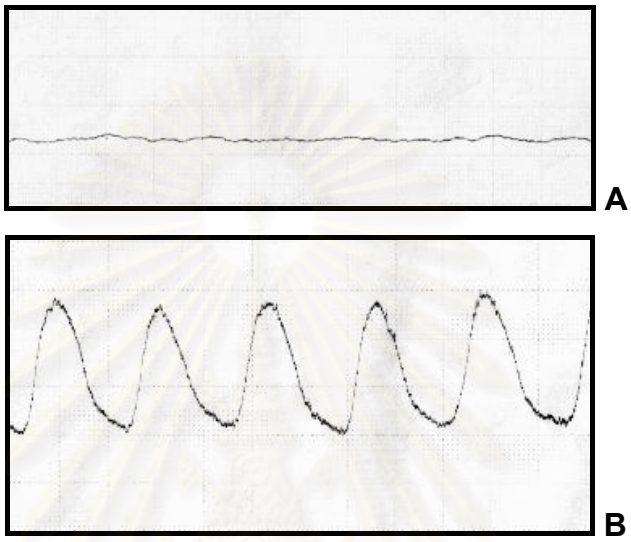


Figure 5-11. The tracing showing the rCBF changes in the NaCL application group (A) and the KCl application group (B).

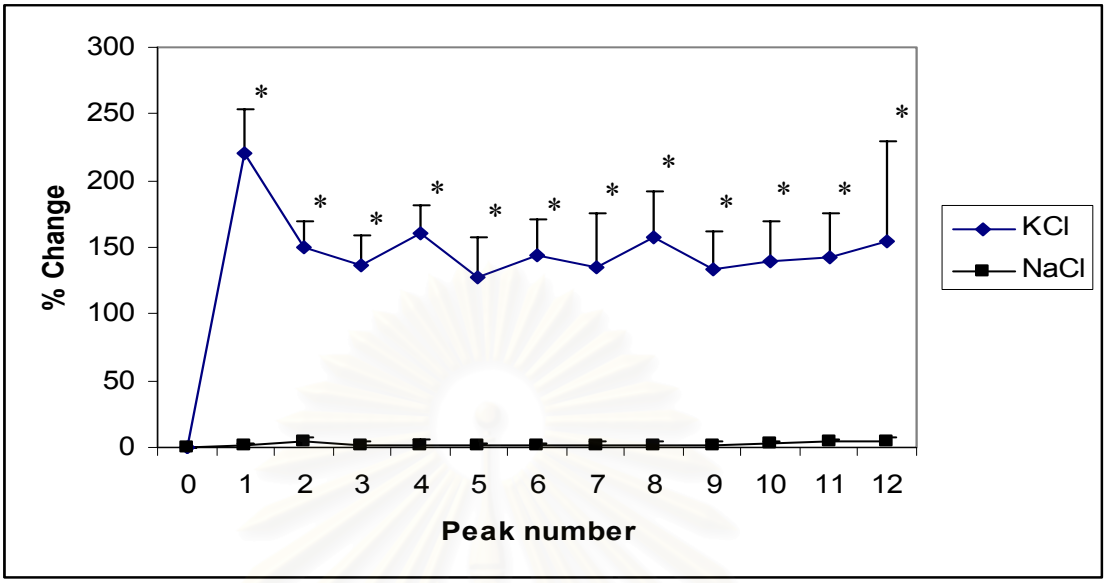


Figure 5-12. The percent change from the baseline flow in the rCBF of the NaCl and KCl application groups. Significant difference was assessed with ANOVA with the Bonferroni test. *p < 0.05 compared with NaCl control group.

The effects of melatonin on the rCBF changes induced by CSD.

To assess the effect of melatonin on the rCBF changes induced by CSD, the rCBF was studied in the rat given an i.p. injection of melatonin in varying doses (10, 20 and 40 mg/kg) for 30 minutes before KCl application.

The results demonstrated that melatonin pretreatment could significantly reduce the rCBF changes evoked by CSD. The repetitive hyperemia induced by KCl application was significantly reduced as compare with those of CSD group. Administrations of melatonin showed a significant reduction in the amplitude of the hyperemic peak as compared to those in the CSD group (Figure 5-13). The reduction of the amplitude of the hyperemic peaks by melatonin pretreatment was not dose dependent. (Figure 5-14).

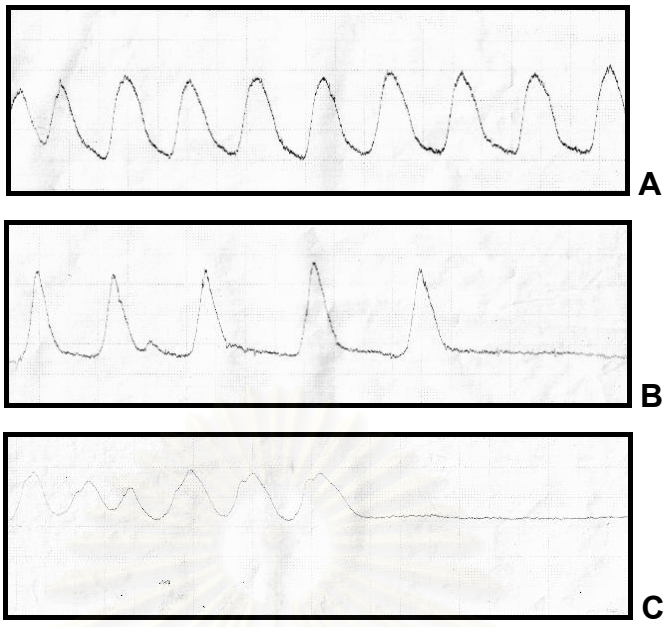


Figure 5-13. The tracing showing the rCBF changes in the CSD group (A) and the CSD with melatonin pretreated groups (B: 10 mg/kg bodyweight pretreated groups, C: 20 mg/kg bodyweight melatonin pretreated group).

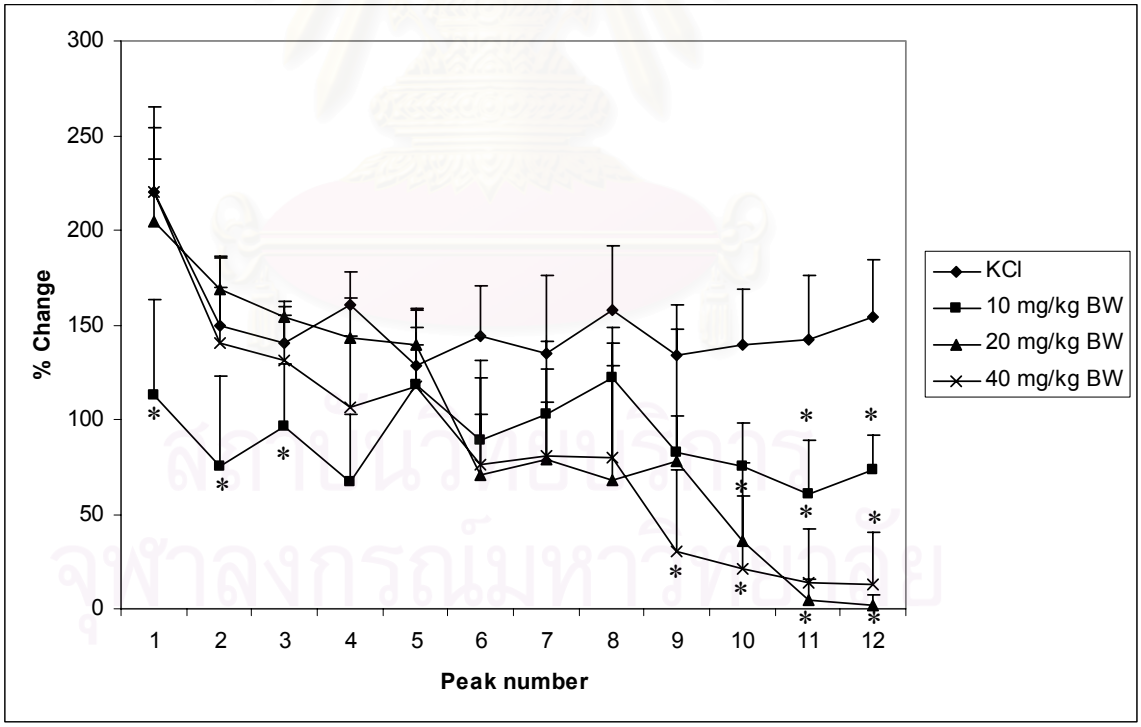


Figure 5-14. The percent change of the rCBF from the baseline flow in the CSD with and without melatonin pretreated group in various doses (10, 20 and 40 mg/kg). Significant differences were assessed with ANOVA with the Bonferroni test , *$p < 0.05$ compared with CSD group.

The effects of melatonin on the nNOS expression in the trigeminovascular nociceptive pathway induced by CSD.

The effect of CSD on the NOS expression

In order to determine the effect of CSD on the production of NO by the second order neuron in the trigeminocervical complex, the NOS expression in cervical spinal cord sections of the CSD group and the control group were studied. Ten sections were randomly selected from each rat and the NOS-IR cells were counted and reported as the number of immunorective cell in the dorsal horn ipsilateral to the KCl (CSD group) or to the NaCl (control group) application and contralateral side. The data are reported as the mean value and the standard deviation.

The application of KCl resulted in a significant increase in the number of NOS-IR neurons in the C1 and C2 cervical spinal cord. The expression of NOS-IR cells was mainly in the lamina I and II and the number of NOS-IR cells was most prominent in the side ipsilateral to the KCl application in the CSD group (40 ± 12 and 23 ± 4 cells /section for ipsilateral and contralateral side respectively) (Figure 5-15). The number of NOS-IR cells in the CSD group was higher than the number of NOS-IR cells in the NaCl control group. The number of NOS-IR cells was 40 ± 12 and 19 ± 5 cells/section for the CSD and the control group in the side ipsilateral to KCl application (CSD) or NaCl application (NaCl control) groups, respectively ($p < 0.05$). The number of NOS-IR cells in the contralateral side was not significant difference among these two groups. The data are shown in Table 5-3, Figure 5-16, and Figure 5-17.

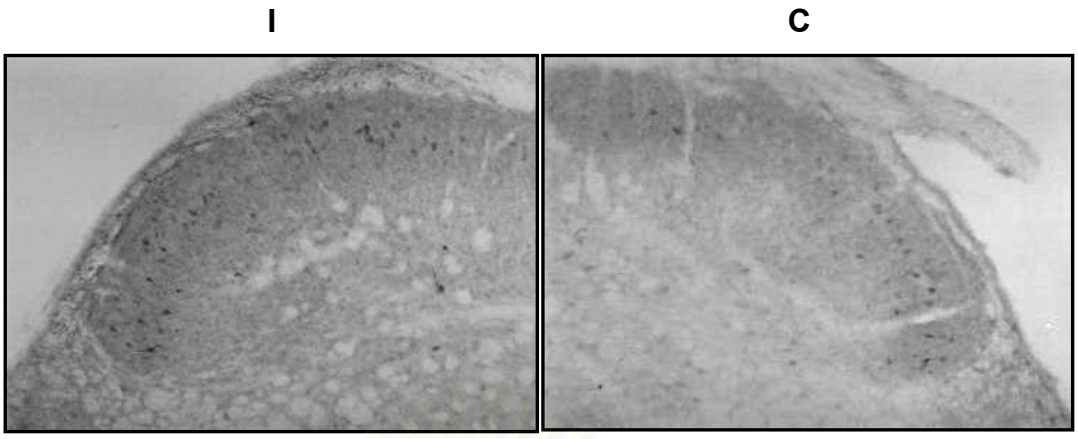


Figure 5-15. The photomicrograph showing the NOS immunoreactivity in the cervical spinal cord sections of the CSD (KCL application) group (I: ipsilateral side to KCl application, C: contralateral side to KCl application).

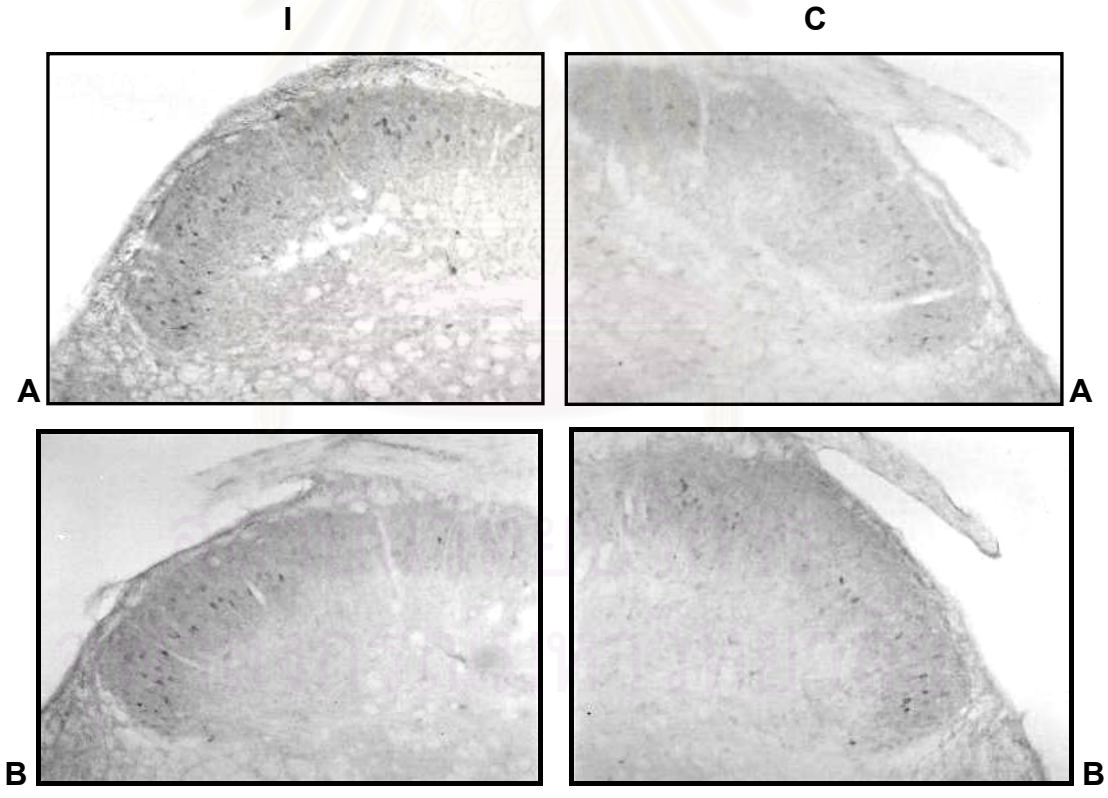


Figure 5-16. The photomicrograph showing the NOS immunoreactivity in the cervical spinal cord sections of A) the CSD (KCL application) group and B) the NaCl control group (I: ipsilateral side to KCl or NaCl application, C : contralateral side to KCl or NaCl application).

Table 5-3. The mean value ± SD of the number of NOS-IR cells in the C1 and C2 cervical spinal cord sections obtained from the CSD and the NaCl control group.

Group	Ipsilateral	Contralateral
NaCl	19 ± 5	17 ± 8
CSD	40 ± 12	23 ± 4

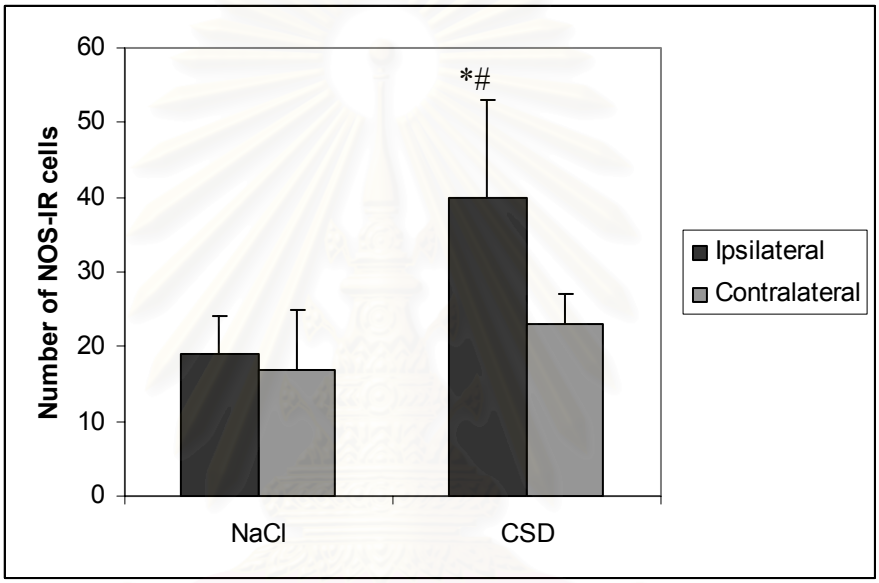


Figure 5-17. Bar graph showing the mean value ± SD of the number of NOS-IR cells in the C1 and C2 cervical spinal cord sections obtained from the CSD group and the NaCl control group. Significant difference was assessed with ANOVA with the Bonferroni test.*$p < 0.05$ compared with NaCl control group. # $p < 0.05$ compared with contralateral side.

The effects of melatonin on the nNOS expression induced by CSD

The effect of melatonin on the nNOS expression induced by CSD was tested by evaluating the number of NOS-IR cells in the melatonin pretreated group and in the CSD group.

The reduction in the number of NOS-IR cells in the lamina I and II was demonstrated in melatonin pretreated group. Even though the reduction of the number of NOS-IR cells in 10 mg/kg bodyweight melatonin pretreated group was not significant, pretreatment with melatonin with higher dose (20 and 40 mg/kg bodyweight) demonstrated the significant reduction in the number of NOS-IR cells in the lamina I and II as compared to the CSD group. The number of NOS-IR cells in the side ipsilateral to the KCl application was reduced from 40 ± 12 cells in the CSD group to 17 ± 5 cells and to 16 ± 9 cells respectively in the 20 and 40 mg/kg bodyweight melatonin pretreated groups. Furthermore the reduction of the number of NOS-IR cells by melatonin pretreatment was also demonstrated in the side contralateral to KCl application. The number of NOS-IR cells was reduced from 23 ± 5 cells in the CSD group to 12 ± 6 cells and to 12 ± 7 cells respectively in the 20 and 40 mg/kg bodyweight melatonin pretreated groups. The data are shown in Table 5-4, Figure 5-18, and Figure 5-19.

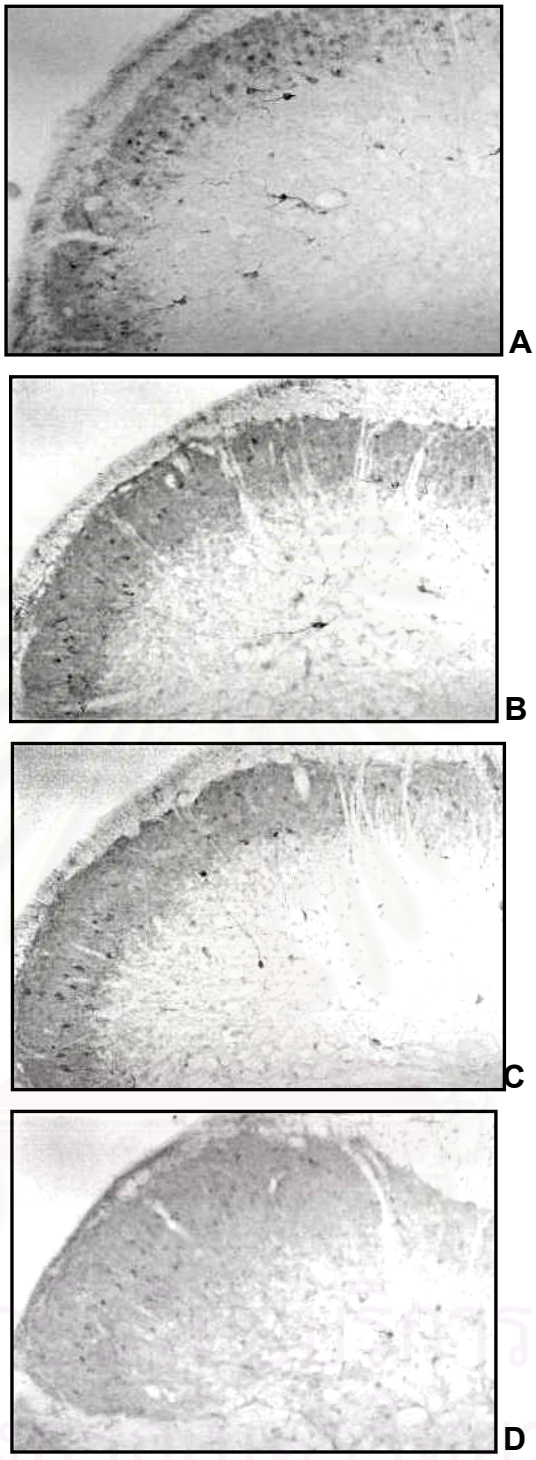


Figure 5-18. The photomicrograph showing the NOS immunoreactivity in the cervical spinal cord sections (ipsilateral side to KCl application) obtained from the CSD with and without melatonin pretreated groups A) the CSD group, B) the CSD with 10 mg/kg bodyweight melatonin pretreated group, C) the CSD with 20 mg/kg bodyweight melatonin pretreated group, and D) the CSD with 40 mg/kg bodyweight melatonin pretreated group.

Table 5-4. The mean value ± SD of the number of NOS-IR cells in the C1 and C2 cervical spinal cord sections obtained from the CSD with and without melatonin pretreated groups.

Group	Ipsilateral	Contralateral
NaCl	19 ± 5	17 ± 8
CSD	40 ±12	23 ± 4
10 mg/kg bodyweight melatonin pretreated	32 ± 8	23 ± 6
20 mg/kg bodyweight melatonin pretreated	17 ± 5	12 ± 4
40 mg/kg bodyweight melatonin pretreated	16 ± 9	12 ± 7

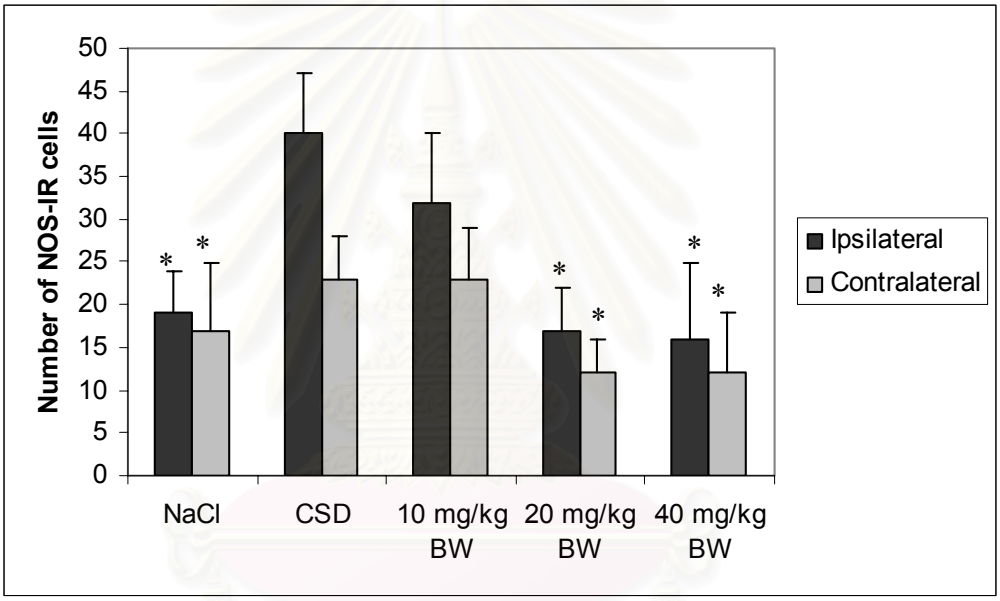


Figure 5-19. Bar graph showing the mean value ± SD of the number of NOS-IR cells in the C1 and C2 cervical spinal cord sections obtained from the CSD with and without melatonin pretreated groups. Significant difference was assessed with ANOVA with the Bonferroni test. *$p < 0.05$ compared with CSD group.

The effects of melatonin on the Fos expression in the trigeminal nociceptive pathway induced by CSD.

The effect of CSD on the Fos expression

To test whether the second order neuron in the cervical spinal cord is activated by CSD the expression of Fos, a surrogate marker for neuronal activation, was examined. Ten sections were randomly selected from each rat and the Fos-IR cells were counted and reported as the number of immunoreactive cells in the dorsal horn contralateral side and ipsilateral to the KCl application (in the CSD group) or to the NaCl application (the control group). The data are reported as the mean value and the standard deviation.

The Fos expression after CSD induction for 1 hour was mainly distributed in the lamina I and II of the cervical spinal cord sections on the side ipsilateral to the KCl application (Figure 5-20).

The greater number of the Fos IR cells was demonstrated in the side ipsilateral to the KCl application as compared to the contralateral side (34 ± 9 and 19 ± 7 cells /section for ipsilateral and contralateral side to KCl application, respectively, $p < 0.05$). The results showed the greater number of the Fos IR neurons in the CSD group as compare to the control group (34 ± 9 and 13 ± 5 cells/section for the CSD and the control group in the side ipsilateral to KCl application (CSD) or NaCl application (NaCl control) groups, respectively, and 19 ± 5 and 12 ± 4 cells/section for the CSD and the control group in the contralateral side, respectively, $p < 0.05$). The data are shown in Table 5-5, Figure 5-21 and Figure 5-22.

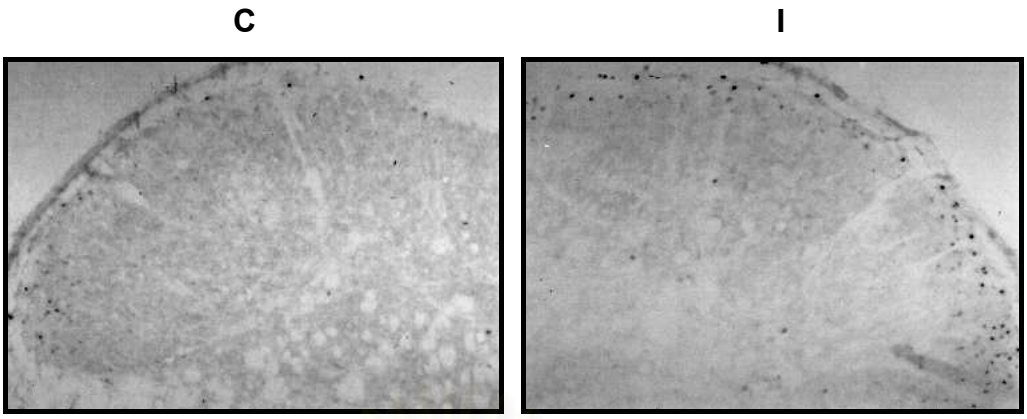


Figure 5-20. The photomicrograph showing the Fos immunoreactivity in the cervical spinal cord sections of the CSD (KCL application) group (I: ipsilateral side to KCl application, C: contralateral side to KCl application).

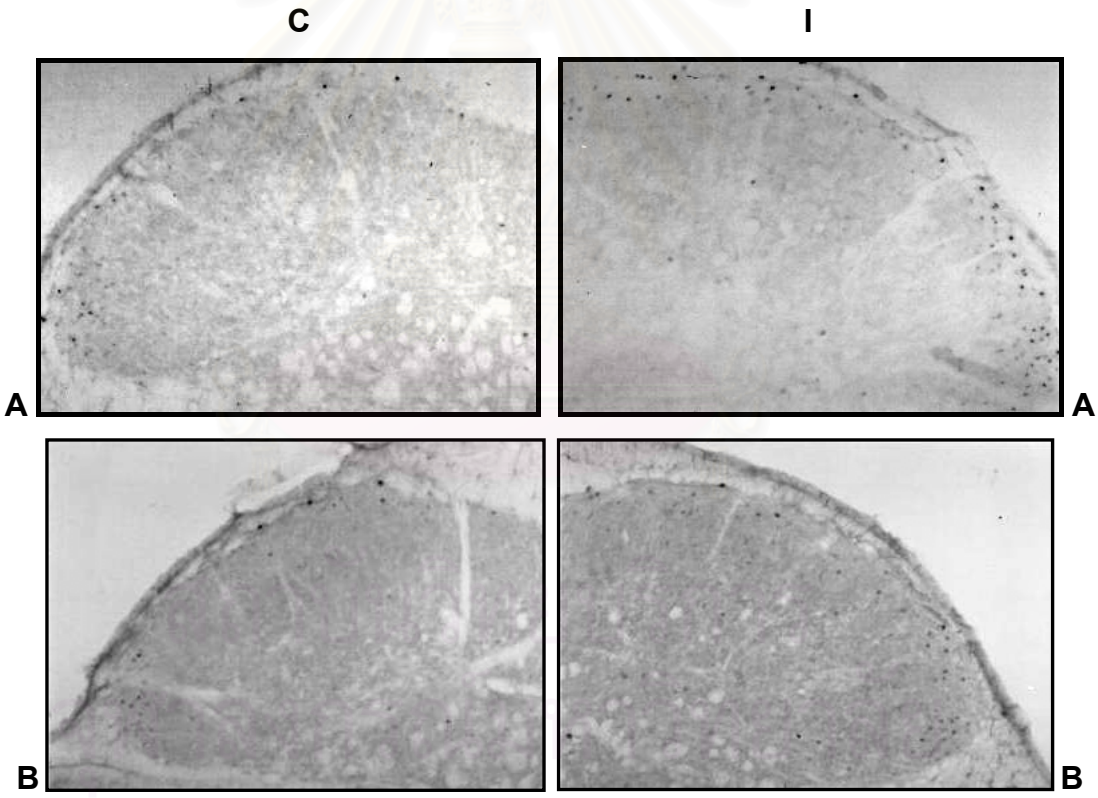


Figure 5-21. The photomicrograph showing the Fos immunoreactivity in the cervical spinal cord sections of A) the CSD (KCl application) group and B) the NaCl control group (I: ipsilateral side to KCl or NaCl application, C : contralateral side to KCl or NaCl application).

Table 5-5. The mean value ± SD of the number of Fos-IR cells in the C1 and C2 cervical spinal cord sections obtained from the CSD and the NaCl control group.

Group	Ipsilateral	Contralateral
NaCl	13 ± 5	12 ± 4
CSD	34 ± 9	19 ± 7

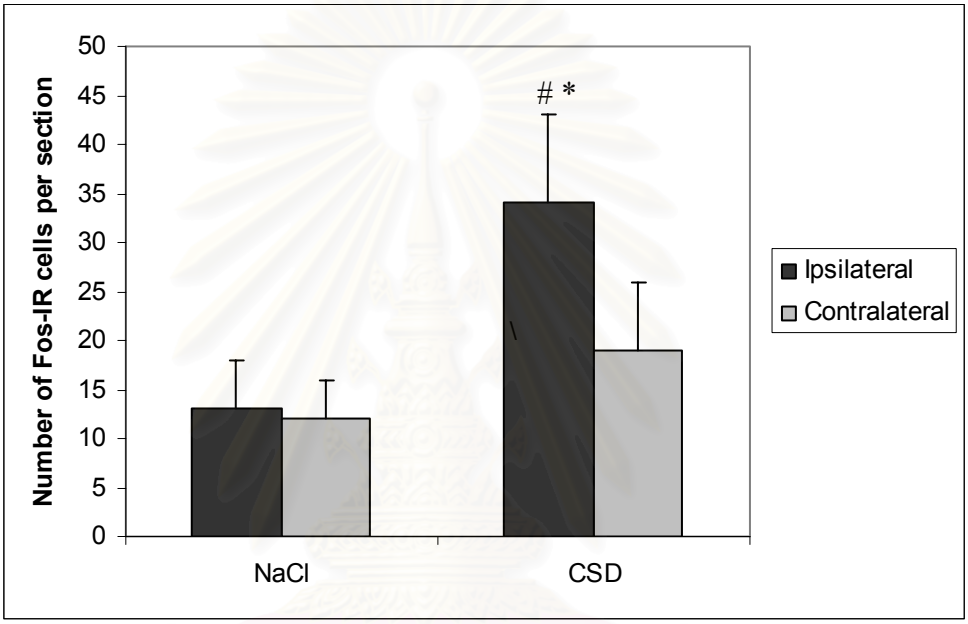


Figure 5-22. Bar graph showing the mean value ± SD of the number of Fos-IR cells in the C1 and C2 cervical spinal cord sections obtained from the CSD group and the NaCl control group. Significant difference was assessed with ANOVA with the Bonferroni test. * $p < 0.05$ compared with NaCl control. # $p < 0.05$ compare with contralateral side.

The effects of melatonin on the Fos expression induced by CSD

Similar to the observed decrease in the number of NOS-IR cells, melatonin (i.p. injection 30 minutes before KCl application) reduced dose dependently the number of the Fos-IR cells in the lamina I and II as compared to the CSD group. The number of Fos-IR cells in the side ipsilateral to the KCl application was reduced from 34 ± 9 cells in the CSD group to 25 ± 8 cells, to 21 ± 7 cells and to 17 ± 6 cells in 10, 20 and 40 mg/kg melatonin pretreated groups respectively. The reduction of the number of the Fos-IR cells in the side contralateral to KCl application did not demonstrate in CSD with 10 and 20 mg/kg bodyweight melatonin pretreatment. However in the CSD with 40 mg/kg bodyweight melatonin pretreatment, the number of the Fos-IR cells was significant reduced from 19 ± 4 cells in the CSD group to 13 ± 4 cells in the 40 mg/kg bodyweight melatonin pretreated group, $p< 0.05$. The data are shown in Table 5-6, Figure 5-23 and Figure 5-24.

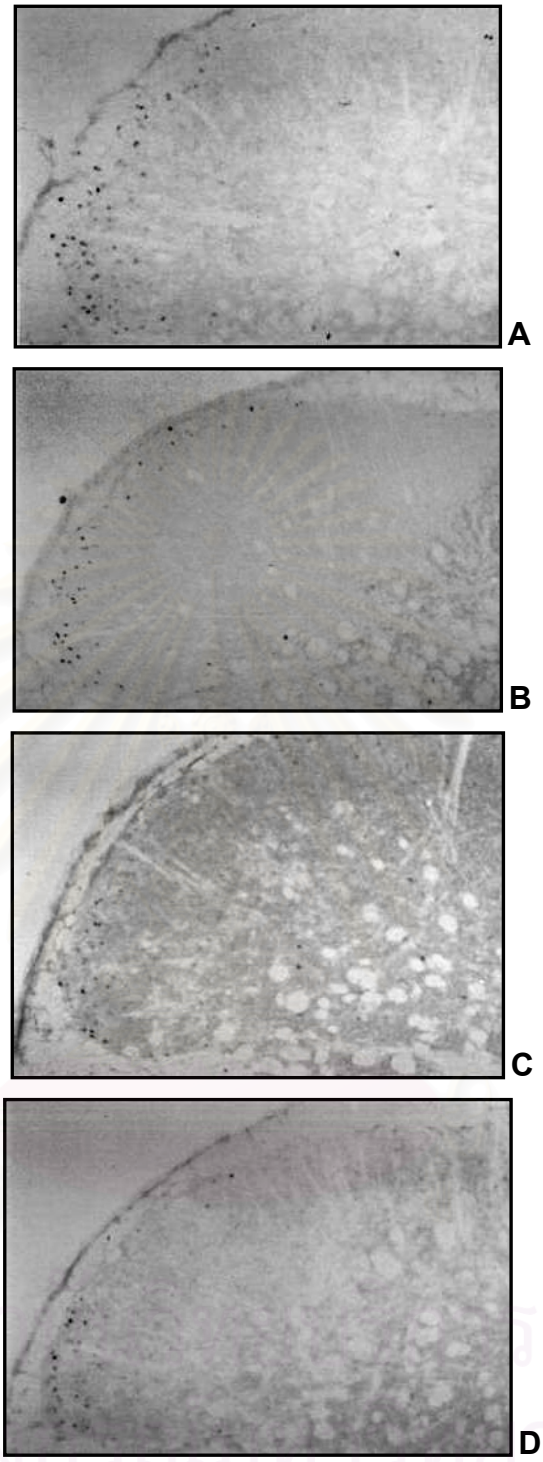


Figure 5-23. The photomicrograph showing the Fos immunoreactivity in the cervical spinal cord sections (ipsilateral side to KCl application) obtained from the CSD with and without melatonin pretreated groups A) the CSD group, B) the CSD with 10 mg/kg bodyweight melatonin pretreated group, C) the CSD with 20 mg/kg bodyweight melatonin pretreated group, and D) the CSD with 40 mg/kg bodyweight melatonin pretreated group.

Table 5-6. The mean value ± SD of the number of Fos-IR cells in the C1 and C2 cervical spinal cord sections obtained from the CSD with and without melatonin pretreated groups

Group	Ipsilateral side	Contralateral side
NaCl	13 ± 5	12 ± 4
CSD	34 ± 7	19 ± 4
10 mg/kg bodyweight melatonin pretreated	24 ± 8	16 ± 7
20 mg/kg bodyweight melatonin pretreated	21 ± 7	15 ± 6
40 mg/kg bodyweight melatonin pretreated	17 ± 6	13 ± 4

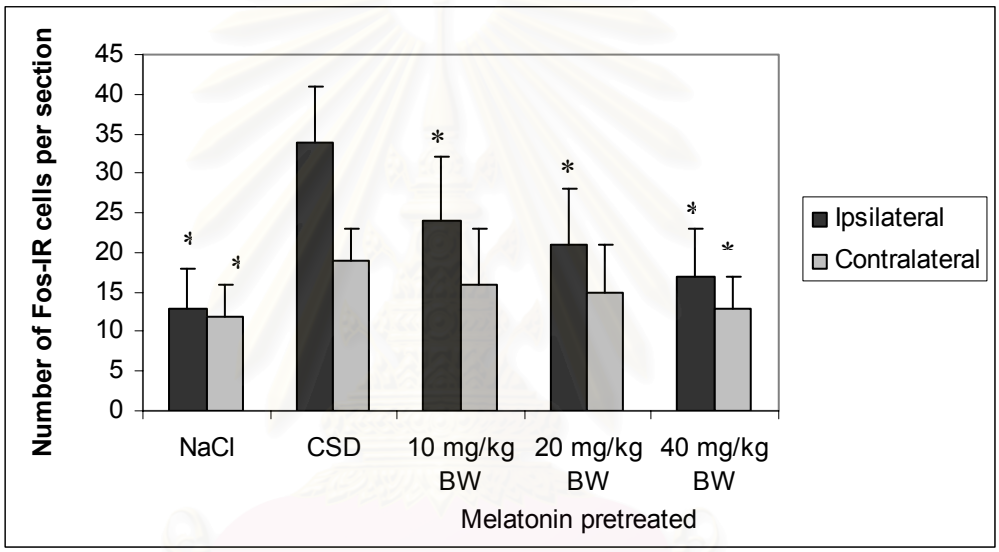


Figure 5-24. Bar graph showing the mean value ± SD of the number of Fos-IR cells in the C1 and C2 cervical spinal cord sections obtained from the CSD with and without melatonin pretreated groups. Significant difference was assessed with ANOVA with the Bonferroni test. $p < 0.05$ compared with CSD group.

5.2 The effects of melatonin on the endothelial cell changes induced by CSD

The effect of CSD on the ultrastructural changes of cerebral microvessels.

The ultrastructural study in endothelial cells ontained from CSD group demonstrated the increase in the number of pinocytic vesicles as compared with those obtained from the NaCl control group. The number of pinocytic vesicles in both arterioles and capillaries. Pinocytic vesicles were observed both luminal and abluminal surfaces (Figure 5-25). In the NaCl control group, only a few number of pinocytic vesicles were observed in the endothelial cells of the cerebral microvessels. These vesicles become more abundant in the endothelial cells of the cerebral microvessels obtained from the CSD group. The average density of pinocytic vesicles in capillaries was significant increased from 15.5 ± 7 vesicle/μm^2 in the NaCl control group to 33 ± 10 vesicle/μm^2 in the CSD group, $p< 0.05$. In arterioles, the average density of pinocytic vesicles was 18 ± 7 and 43 ± 10 vesicle/μm^2, for the NaCl control and the CSD group respectively, $p< 0.05$. However the increase in the number of microvilli of the endothelial cell obtained from the CSD group was not significant. The data are shown in Table 5-7, Figure 5-26, Table 5-8, and Figure 5-27.

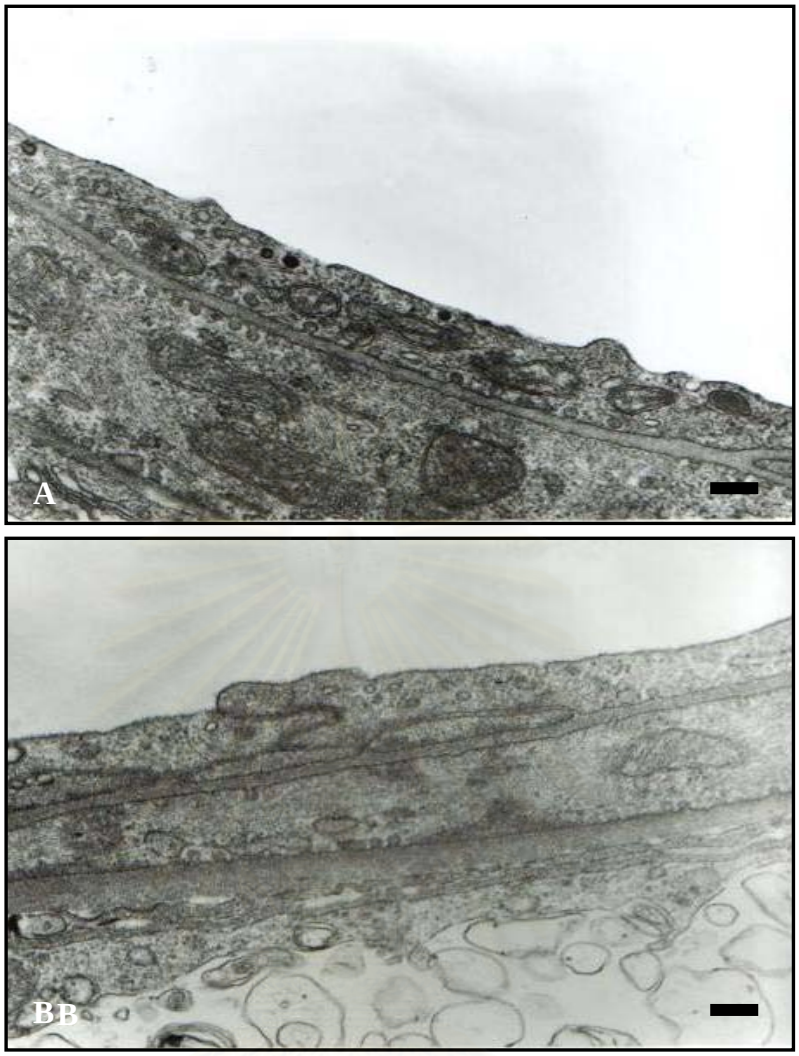


Figure 5-25. Electron micrograph of cerebral microvessels showing a few number of pinocytic vesicles in an endothelial cell obtained from (A) the NaCl control group and showing an increase in the number of pinocytic vesicles in endothelial cell obtained from (B) the CSD group. (x 60000). Bar = 200 nm.

Table 5-7. The mean value ± SD of the number of microvilli per vessel in the endothelial cell of cerebral capillaries and arterioles obtained from the NaCl control group and the CSD group.

Group	Number of microvilli / vessel	
	Capillary	Arteriole
NaCl	1 ± 0.7	9.8 ± 4
CSD	1.4 ± 1.3	14.7 ± 5

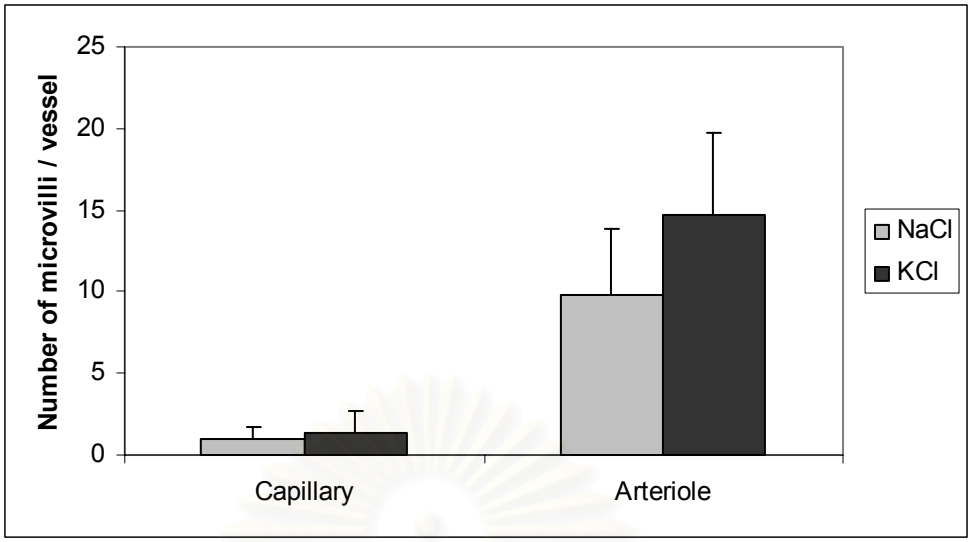


Figure 5-26. Bar graph showing the mean value ± SD of the number of microvilli per vessel in the endothelial cell of cerebral capillaries and arterioles obtained from the NaCl control group and the CSD group.

Table 5-8. The mean value ± SD of the number of pinocytic vesicle/μm2 in the endothelial cell of cerebral capillaries and arterioles obtained from the NaCl control group and the CSD group.

Group	Number of pinocytic vesicles/ μm^2	
	Capillary	Arteriole
NaCl	16 ± 7	18 ± 7
CSD	33 ± 15	43 ± 10

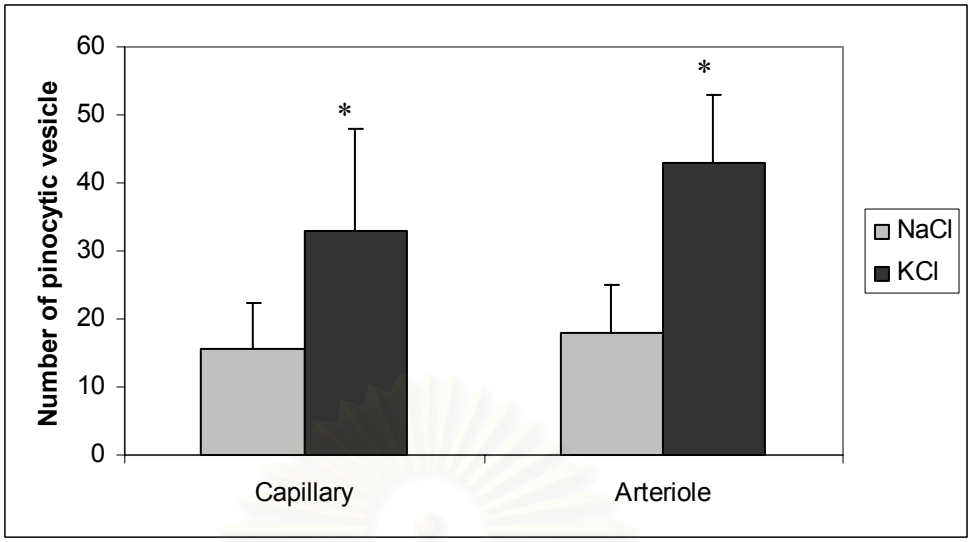


Figure 5-27. Bar graph showing the mean value ± SD of the number of pinocytic vesicle/μm^2 in the endothelial cell of cerebral capillaries and arterioles obtained from the NaCl control group and the CSD group. Significant difference was assessed with ANOVA with the Bonferroni test. *$p < 0.05$ compared with CSD group.

The effects of melatonin on the endothelial cell changes induced by CSD.

Intraperitoneal injection of melatonin 30 minutes before KCl application could attenuate the ultrastructural changes induced by CSD. The number of microvilli was significant decreased from 14.7 ± 5 microvilli/vessel in the CSD group to 8.6 ± 4 microvilli/vessel in the CSD with melatonin pretreated group (40 mg/kg bodyweight). Furthermore the average density of pinocytic vesicles in capillaries was decreased from 33 ± 10 vesicle/μm^2 in the CSD group to 6 ± 3, 19 ± 8 and 8 ± 6 vesicle/μm^2 in 10, 20 and 40 mg/kg bodyweight melatonin pretreated group respectively. The protective effect of melatonin in the pinocytic vesicle formation was also demonstrated in the endothelial cells of arterioles, the average density of these vesicles was 43 ± 10, 16 ± 5, 21 ± 11 and 20 ± 8 vesicle/μm^2 in the CSD group, 10, 20 and 40 mg/kg bodyweight melatonin pretreated group respectively. The data are shown in Table 5-9, Table 5-10 and Figure 5-28, Figure 5-29 and Figure 5-30.

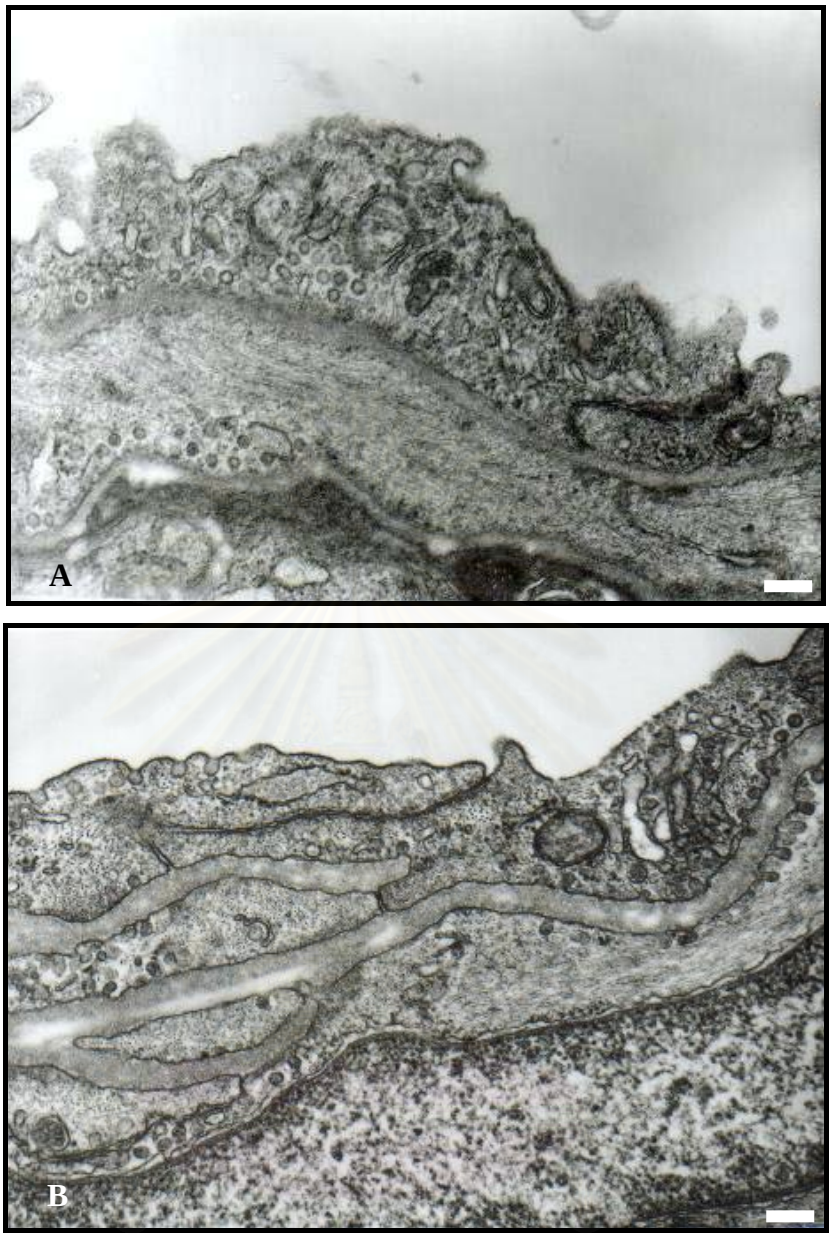


Figure 5-28. Electron micrograph of an endothelial cell obtained from (A) the CSD group and (B) the 40mg/kg bodyweight melatonin pretreated group. (x 60000). Bar = 200 nm.

Table 5-9. The mean value ± SD of the number of microvilli per vessel in the endothelial cell of cerebral capillaries and arterioles obtained from the CSD group and the CSD with melatonin pretreated group.

Group	Number of microvilli / vessel	
	Capillary	Arteriole
KCl	1.4 ± 1.3	14.7 ± 5
Melatonin 10 mg/kg bodyweight + KCl	0.8 ± 1	12 ± 5
Melatonin 20 mg/kg bodyweight + KCl	1.1 ± 1	14.3 ± 1
Melatonin 40 mg/kg bodyweight + KCl	0.9 ± 0.9	8.6 ± 4

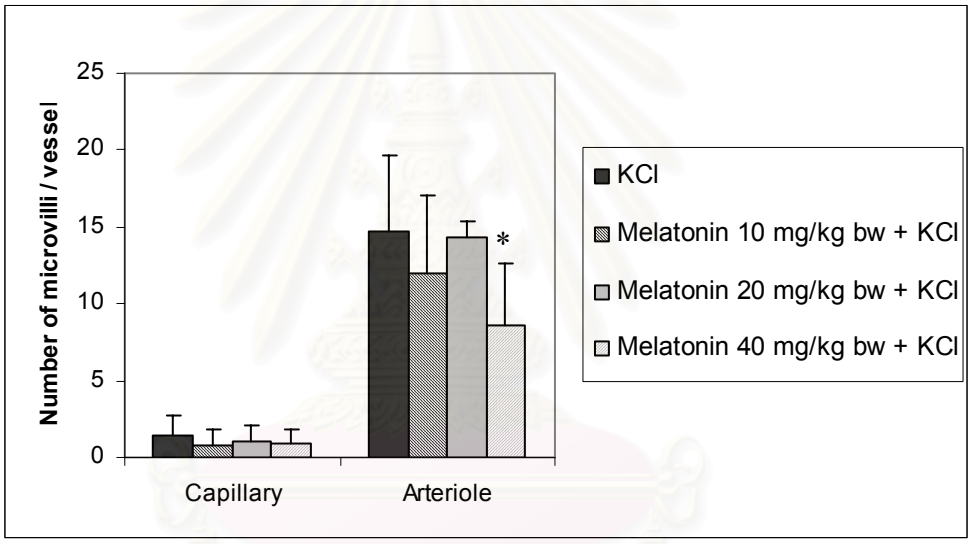


Figure 5-29. Bar graph showing the mean value ± SD of the number of microvilli per vessel in the endothelial cell of cerebral capillaries and arterioles obtained from the CSD group and the CSD with melatonin pretreated group. Significant difference was assessed with ANOVA with the Bonferroni test. $p < 0.05$ compared with CSD group.

Table 5-10. The mean value ± SD of the number of pinocytic vesicle/μm^2 in the endothelial cell of cerebral capillaries and arterioles obtained from the CSD group and the CSD with melatonin pretreated group.

Group	Number of pinocytic vesicle/ μm^2	
	Capillary	Arteriole
KCl	33 ± 15	43 ± 10
Melatonin 10 mg/kg bodyweight + KCl	6 ± 3	16 ± 5
Melatonin 20 mg/kg bodyweight + KCl	19 ± 8	21 ± 11
Melatonin 40 mg/kg bodyweight + KCl	8 ± 6	20 ± 8

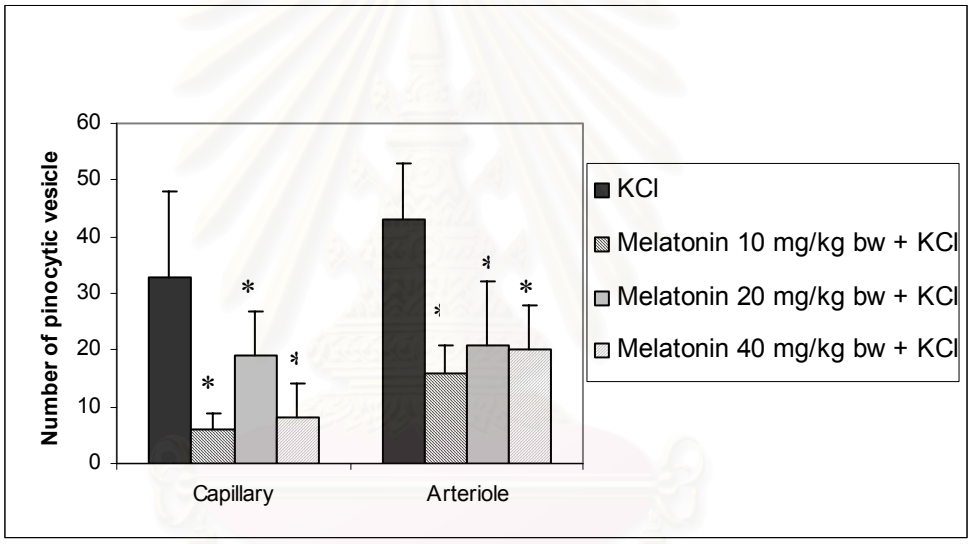


Figure 5-30. Bar graph showing the mean value ± SD of the number of pinocytic vesicle/μm^2 in the endothelial cell of cerebral capillaries and arterioles obtained from the CSD group and the CSD with melatonin pretreated group. Significant difference was assessed with ANOVA with the Bonferroni test. $p < 0.05$ compared with CSD group.

Part III: The effect of melatonin on the trigeminovascular nociceptive system in the NO-donor induced vasodilatation model.

The effects of melatonin on arteriolar dilatation induced by GTN.

The effects of GTN on the pial arteriole

Infusion with 30 % (V/V) GTN in aCSF induced vasodilatation of cerebral arterioles (Figure 5-31). Few minutes after 30 % (V/V) GTN was infused into the cranial window, the dilatation of the cerebral arteriole was observed. The maximal dilation was observed after infusion with 30 % (V/V) GTN for 25 minutes. Thirty minutes after the end of GTN perfusion, the pial arteriolar diameter returned to the baseline value.

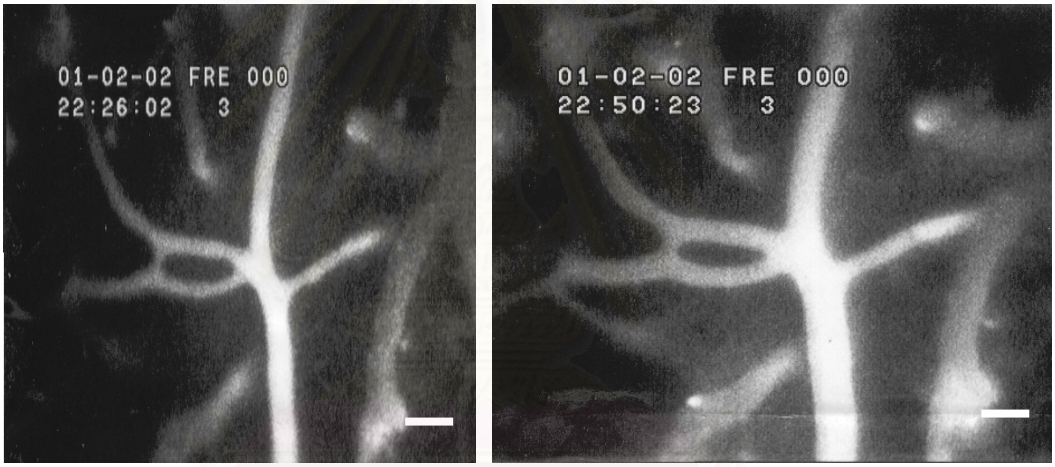


Figure 5-31. The intravital videomicroscope image of cerebral arterioles superfused with 30 % (V/V) GTN. (A) before superfusion, (B) 25 minute after superfusion. Bar = 100 nm.

The effects of melatonin on the pial arteriolar dilatation induced by GTN.

To test the effect of melatonin on the pial arteriolar dilatation induced by GTN, melatonin (10, 20 and 40 mg/kg) was i.p. injected to the rat 30 minutes before suffusion of the pial arterioles with 30 % (V/V) GTN.

Pretreatment with melatonin could minimize the GTN-evoked arteriolar dilatation. The percent change from the baseline of the arteriolar diameter was reduced significantly in the melatonin pretreated group. The data are shown in Table 5-11 and Figure 5-32.

Table 5-11. The percent change from the baseline of the rat pial arteriolar diameter in the 30 % (V/V) GTN superfusion group with and without melatonin pretreatment.

Time (Minute)	Group			
	GTN	melatonin pretreated + GTN		
		10 mg/kg bodyweight	20 mg/kg bodyweight	40 mg/kg bodyweight
-30	0 ± 0	0 ± 0	0 ± 0	0 ± 0
0	2 ± 2	-2 ± 2	0 ± 1	-1 ± 2
5	1 ± 2	0 ± 5	1 ± 2	-1 ± 6
10	17 ± 24	3 ± 2	6 ± 7	7 ± 9
15	40 ± 28	11 ± 11	11 ± 15	19 ± 19
20	52 ± 27	12 ± 13	15 ± 17	25 ± 23
25	53 ± 24	14 ± 14	17 ± 15	28 ± 14
30	58 ± 24	15 ± 14	18 ± 15	27 ± 12
35	55 ± 21	15 ± 10	21 ± 14	26 ± 14
40	43 ± 21	13 ± 9	18 ± 15	19 ± 8
45	29 ± 23	19 ± 10	16 ± 14	11 ± 13
50	24 ± 17	30 ± 12	14 ± 10	7 ± 11
55	21 ± 14	48 ± 31	11 ± 10	7 ± 11
60	18 ± 15	52 ± 39	11 ± 10	10 ± 11

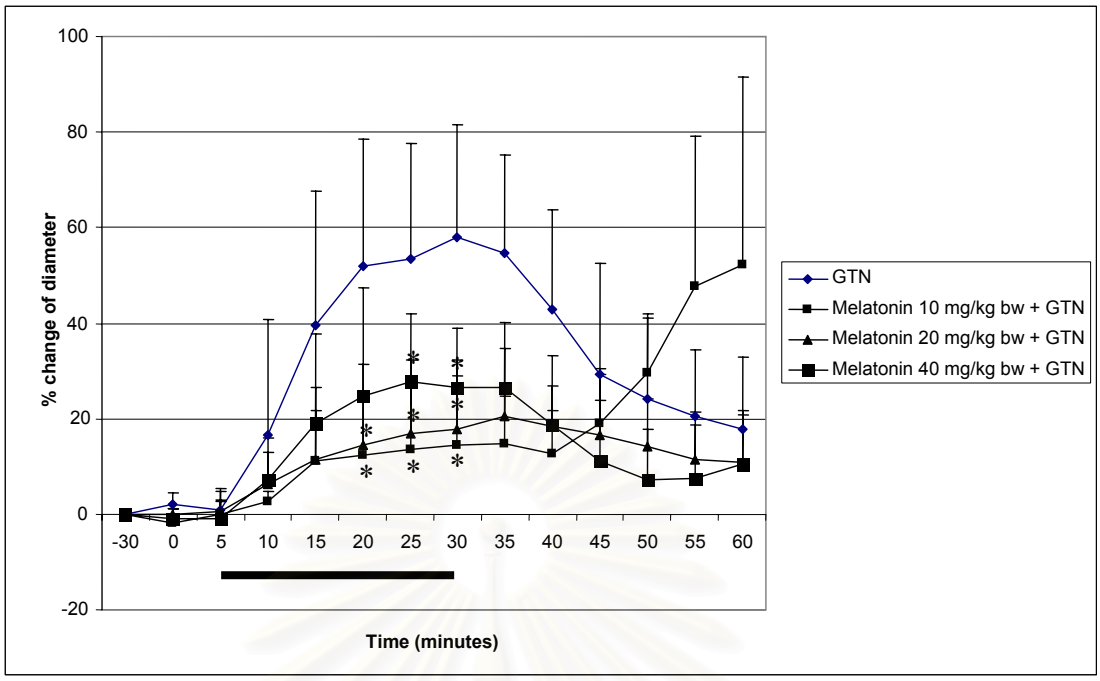


Figure 5-32. The percent change from the baseline of the rat pial arteriolar diameter in 30 % (V/V) GTN superfusion group with and without melatonin pretreatment. Significant difference was assessed with ANOVA with the Bonferroni test. *p < 0.05 compared with GTN group. The solid bar represents the duration of GTN superinfusion.

The effects of melatonin on the nNOS expression in the trigeminovascular nociceptive pathway induced by intravenously infusion of GTN

The effect of GTN infusion on the nNOS expression

In order to determine whether systemic infusion of GTN activates the production of NO from the second order neuron in the trigeminocervical complex, the distribution of the NOS-IR cells in the cervical spinal cord sections following GTN injection was studied. The NOS-IR neurons were counted from both sides of the cervical spinal cord sections. Ten sections were randomly selected from each rat and the NOS-IR cells were counted. The data are reported as the mean value with the

standard deviation. The statistical difference was tested by using ANOVA with a significance level of $p < 0.05$.

The administration of GTN (10 mg/kg) resulted in a substantial increase in the number of NOS-IR cells in the cervical spinal cord as compared with those in the control group; the NOS-IR cells were distributed mainly in the lamina I and II (Figure 5-33). The number of NOS-IR cells raised from 29 ± 10 per section in the control group to 52 ± 8 cells per section in the GTN treated group ($p < 0.05$). The data are shown in Table 5-12, and Figure 5-17.

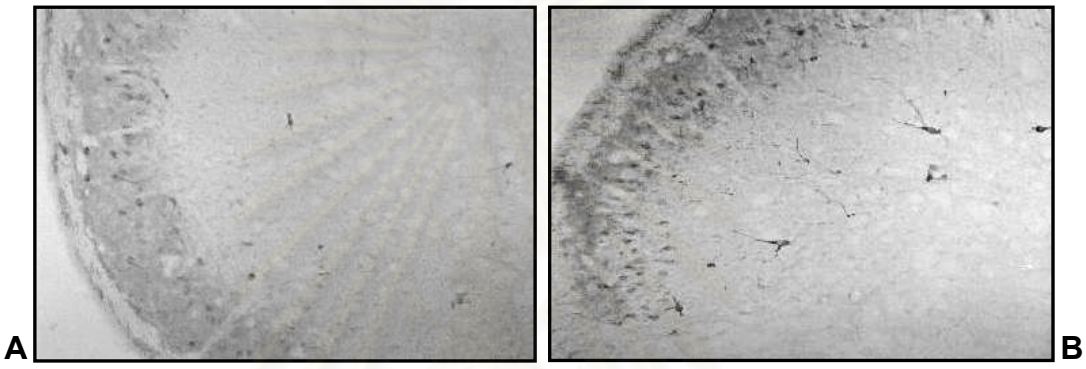


Figure 5-33. The photomicrograph showing the NOS immunoreactivity in the cervical spinal cord sections obtained from A) the control group and B) the GTN treated group.

Table 5-12. The mean value ± SD of the number of NOS-IR cells in the C1 and C2 cervical spinal cord sections obtained from the GTN treated group and the control group.

Group	Number of NOS-IR cells/ section
Control	28 ± 10
GTN	52 ± 10

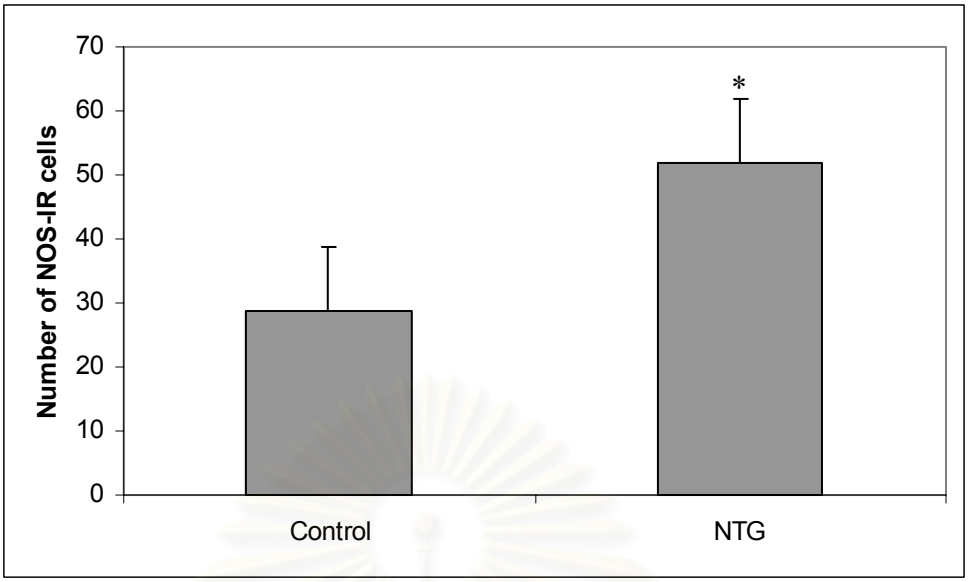


Figure 5-34. Bar graph showing the mean value ± SD of the number of NOS-IR cells in the C1 and C2 cervical spinal cord sections obtained from the GTN treated group and the control group. Significant difference was assessed with ANOVA with the Bonferroni test.* $p < 0.05$ compared with control group.

The effects of melatonin on the nNOS expression induced by GTN infusion

The effect of melatonin on the NOS expression induced by CSD was tested by evaluating the number of NOS-IR cells in the rats given i.v. injection of GTN with and without melatonin pretreatment.

The NOS expression induced by GTN in melatonin pretreated groups was clearly suppressed (Figure 5-35). Quantitative analysis had revealed that the number of NOS-IR cells was significantly reduced from 52 ± 8 cells in the GTN group to 28 ± 18 cells, to 26 ± 8 cells and to 24 ± 8 cells in 10, 20 and 40 mg/kg bodyweight melatonin pretreated group respectively, $p< 0.05$. The data are shown in

Table 5-13, and Figure 5-36.

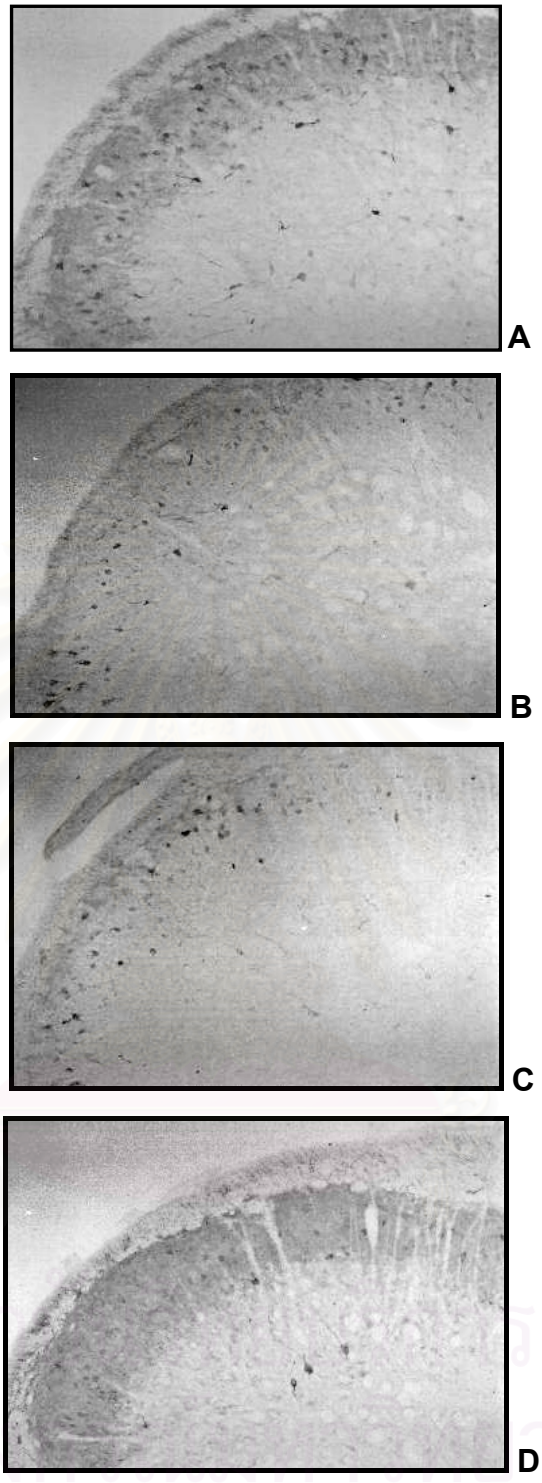


Figure 5-35. The photomicrograph showing the NOS immunoreactivity in the cervical spinal cord sections obtained from the GTN with and without melatonin pretreated groups. A) The GTN group, B) The GTN with 10 mg/kg bodyweight melatonin pretreated group, C) The GTN with 20 mg/kg bodyweight melatonin pretreated group, D) The GTN with 40 mg/kg bodyweight melatonin pretreated group.

Table 5-13. The mean value ± SD of the number of NOS-IR cells in the C1 and C2 cervical spinal cord sections obtained from the GTN with and without melatonin pretreated groups.

Group	Number of NOS cells/section
GTN	52 ± 10
Melatonin 10 mg/kg bodyweight + GTN	29 ± 18
Melatonin 20 mg/kg bodyweight + GTN	26 ± 8
Melatonin 40 mg/kg bodyweight + GTN	24 ± 9

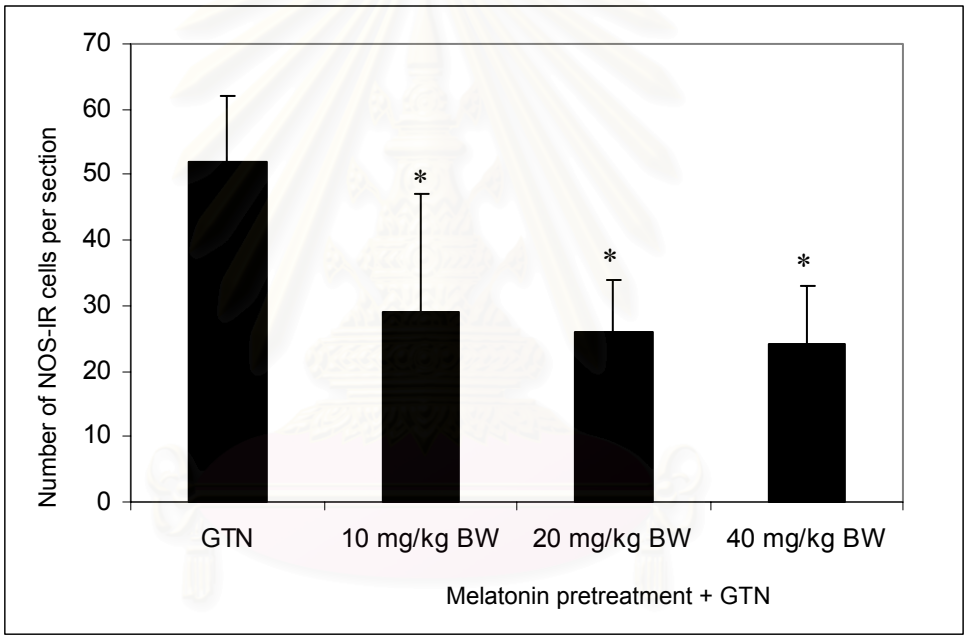


Figure 5-36. Bar graph showing the mean value ± SD of the number of NOS-IR cells in the cervical spinal cord sections obtained from the GTN with and without melatonin pretreated groups. Significant difference was assessed with ANOVA with the Bonferroni test . *$p < 0.05$ compared with GTN group.

5.3 The effects of melatonin on the Fos expression in the trigeminovascular nociceptive pathway induced by intravenously infusion of GTN

The effect of GTN infusion on the Fos expression

The expression of Fos was also examined in this experimental model in order to test whether the second order neuron in the cervical spinal cord are activated by NO-donor infusion. Ten sections were randomly selected from each rat and the Fos-IR cells were counted and reported as the total number of immunoreactive cells in the dorsal horn from both side. The data are reported as the mean value with the standard deviation. The statistical difference was tested using ANOVA with a significant level of $p < 0.05$.

The Fos expression after the GTN infusion for 2 hour was mainly distributed in the lamina I and II of the cervical spinal cord sections in both sides (Figure 5-37). The number of Fos IR cells was significantly increased as compared with that of the control group. Quantitative analysis had revealed that the number of Fos IR cells was significantly increased from 18 ± 5 cells per section in the control group to 30 ± 8 cells per section for the GTN infusion group, $p < 0.05$. The data are shown in Table 5-14 and Figure 5-38.

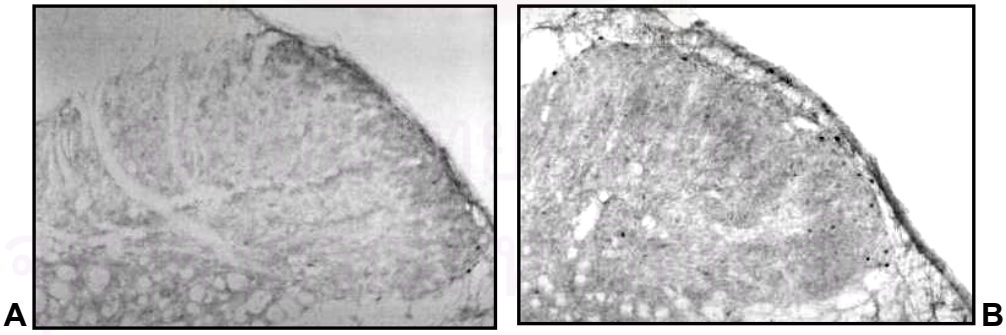


Figure 5-37. The photomicrograph showing the Fos immunoreactivity in the cervical spinal cord sections obtain from A) the control group and B) the GTN treated group.

Table 5-14. The mean value ± SD of the number of Fos-IR cells in the C1 and C2 cervical spinal cord sections obtained from the GTN infusion group and the control group.

Group	Number of Fos-IR cells/section
Control	18 ± 5
GTN	30 ± 8

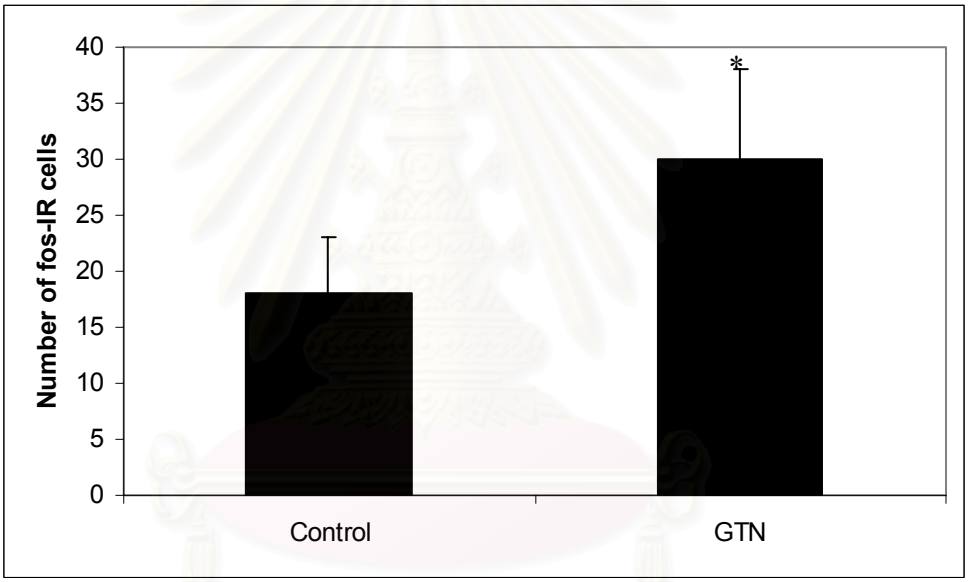


Figure 5-38. Bar graph showing the mean value ± SD of the number of Fos-IR cells in the C1 and C2 cervical spinal cord sections obtained from the GTN infusion group and the control group. Significant difference was assessed with ANOVA with the Bonferroni test (*$p < 0.05$).

The effects of melatonin on the Fos expression induced by GTN infusion

In order to evaluate the effect of melatonin on the Fos expression in the GTN infusion model, three doses of melatonin had been intraperitoneally injected into the rats 30 minutes before GTN infusion. The expression of Fos after 2 hour GTN infusion was then studied.

Similar to the observed decrease in the number of NOS-IR cells, i.p. injection of melatonin before GTN infusion demonstrated the reduction in the number of Fos-IR cells as compared to the GTN infusion group (Figure 5-39). The number of Fos-IR cells in the lamina I and II of the cervical spinal cord was reduced from 29 ± 4 cells in the GTN treated group to 10 ± 4 cells, to 15 ± 3 cells and to 9 ± 2 cells in 10, 20 and 40 mg/kg bodyweight melatonin pretreated group respectively. The data are shown in Table 5-15, and Figure 5-40.

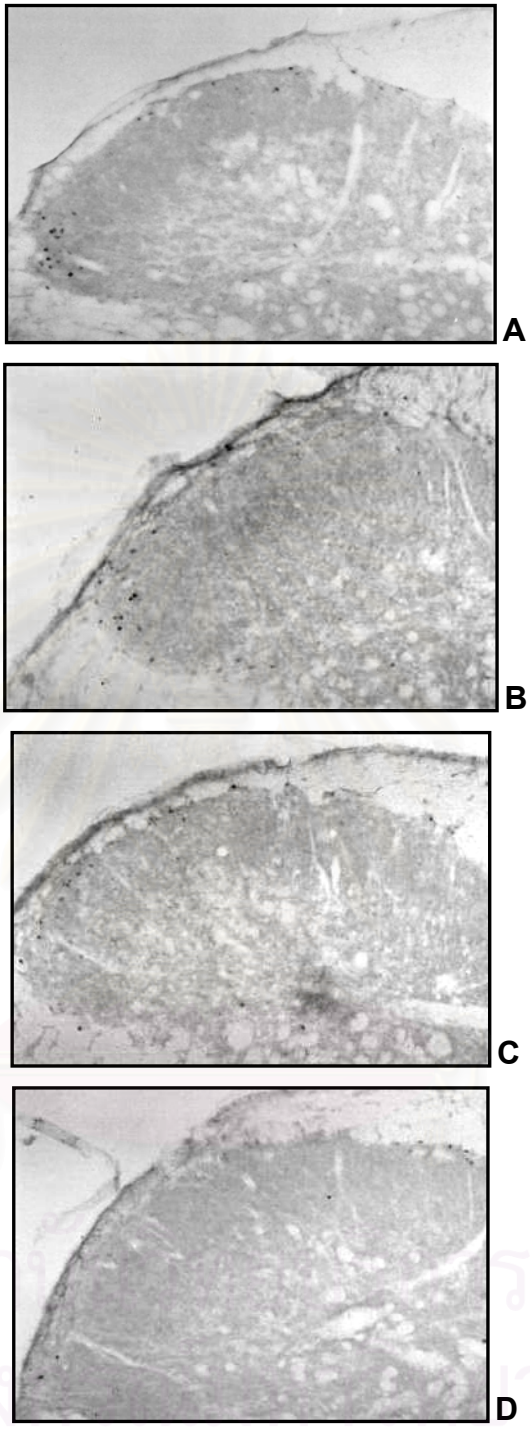


Figure 5-39. The photomicrograph showing the Fos immunoreactivity in the cervical spinal cord sections obtained from the GTN with and without melatonin pretreated groups. A) The GTN group, B) the GTN with 10 mg/kg bodyweight melatonin pretreated group, C) the GTN with 20 mg/kg bodyweight melatonin pretreated group, and D) the GTN with 40 mg/kg bodyweight melatonin pretreated group.

Table 5-15. The mean value ± SD of the number of Fos-IR cells in the C1 and C2 cervical spinal cord sections obtained from the GTN with and without melatonin pretreated groups.

Group	Number of Fos-IR cells/section
GTN	30 ± 8
Melatonin 10 mg/kg bodyweight + GTN	10 ± 4
Melatonin 20 mg/kg bodyweight + GTN	15 ± 4
Melatonin 40 mg/kg bodyweight + GTN	9 ± 7

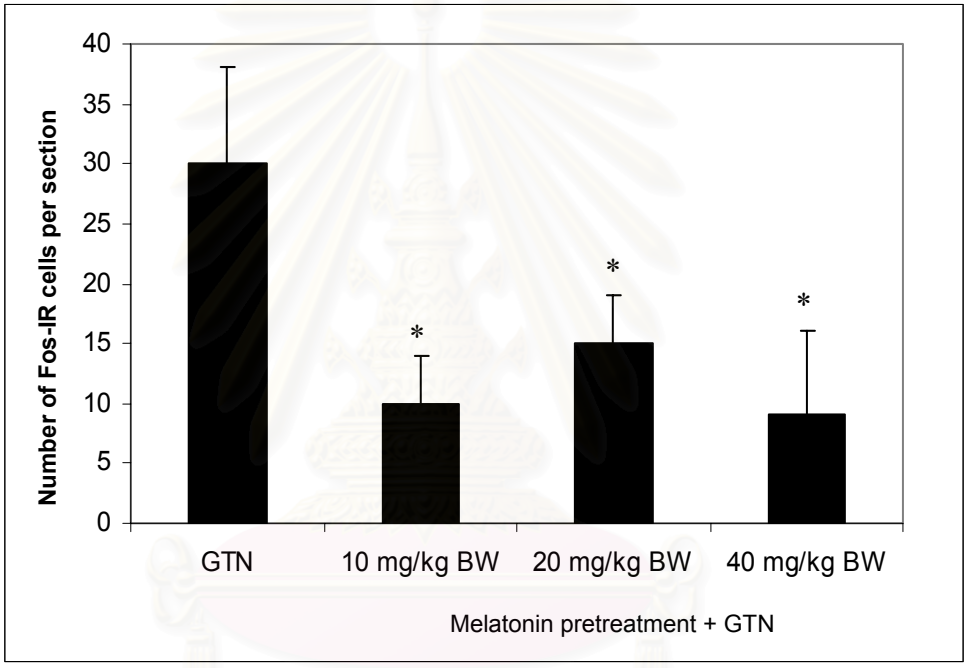


Figure 5-40. Bar graph showing the mean value ± SD of the number of Fos-IR cells in the C1 and C2 cervical spinal cord sections obtained from the GTN with and without melatonin pretreated groups. Significant difference was assessed with ANOVA with the Bonferroni test. *$p < 0.05$ compared with NTG group.

The effects of melatonin on the endothelial cell changes induced by GTN infusion.

The effect of GTN infusion on the ultrastructural changes of cerebral microvessels.

The ultrastructural changes of the endothelial cells were evident in the GTN group. A significant increase in the number of endothelial microvilli was observed in the GTN group as compared with the control group (Figure 5-41). The average number of microvilli in the capillaries was 0.5 ± 0.3 and 3.8 ± 3.9 microvilli per vessel for the control and the GTN group respectively. In arterioles, the average number of microvilli was increased from 1.5 ± 0.5 microvilli per vessel in the control group to 28 ± 14 microvilli per vessel in the GTN group. The data are shown in Table 5-16 and Figure 5-42.

Furthermore, GTN infusion also induced an increased number of pinocytic vesicles in endothelial cells obtained from both capillaries and arterioles (Figure 5-43). The average density of pinocytic vesicles was increased from 9.5 ± 3 vesicle/μm^2 in the control group to 20 ± 10 vesicle/μm^2 in the GTN group for capillaries and from 10 ± 3 vesicle/μm^2 in the control group to 37 ± 15 vesicle/μm^2 in the GTN group for arterioles, respectively (Table 5-17 and Figure 5-44).

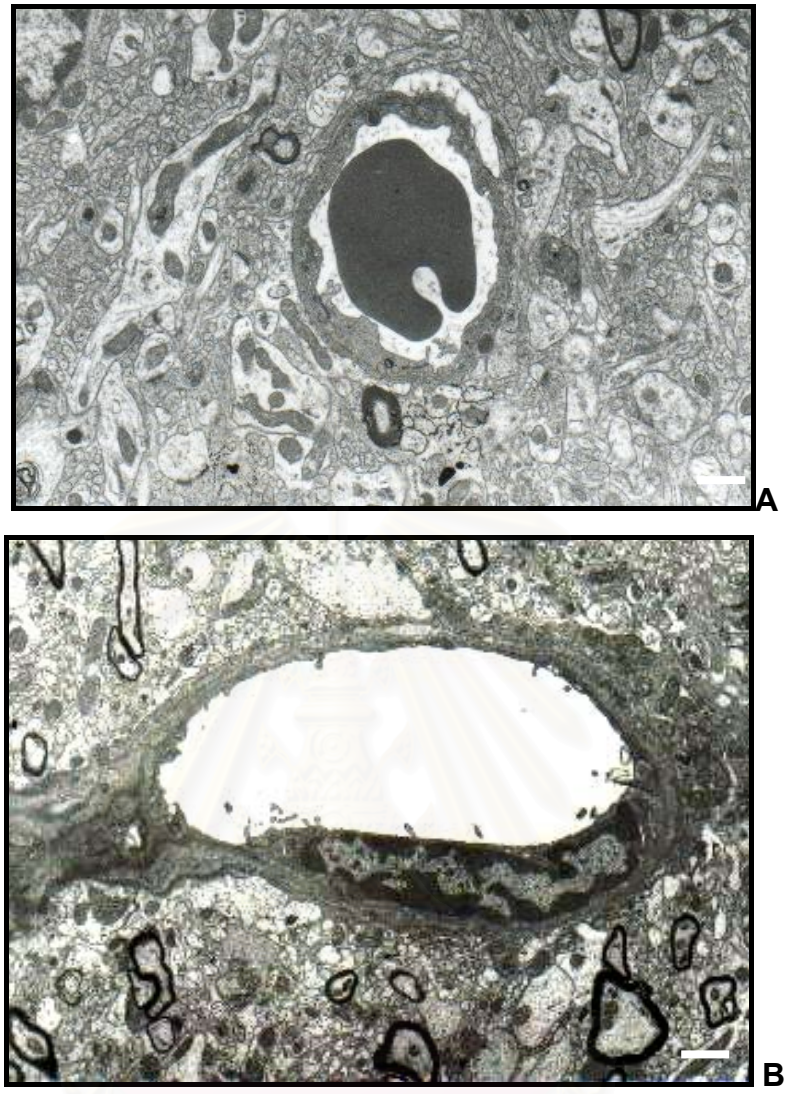


Figure 5-41. Electron micrograph of an endothelial cell obtained from (A) the control group and (B) the GTN group showing an increase in microvillous formation. (x 6800). Bar = 1µm.

Table 5-16. The mean value ± SD of the number of microvilli per vessel in the endothelial cell of cerebral capillaries and arterioles obtained from the control group and the GTN group.

Group	Number of microvilli / vessel	
	Capillary	Arteriole
Control	0.5 ± 0.3	1.5 ± 0.5
GTN	3.75 ± 3.9	28 ± 14

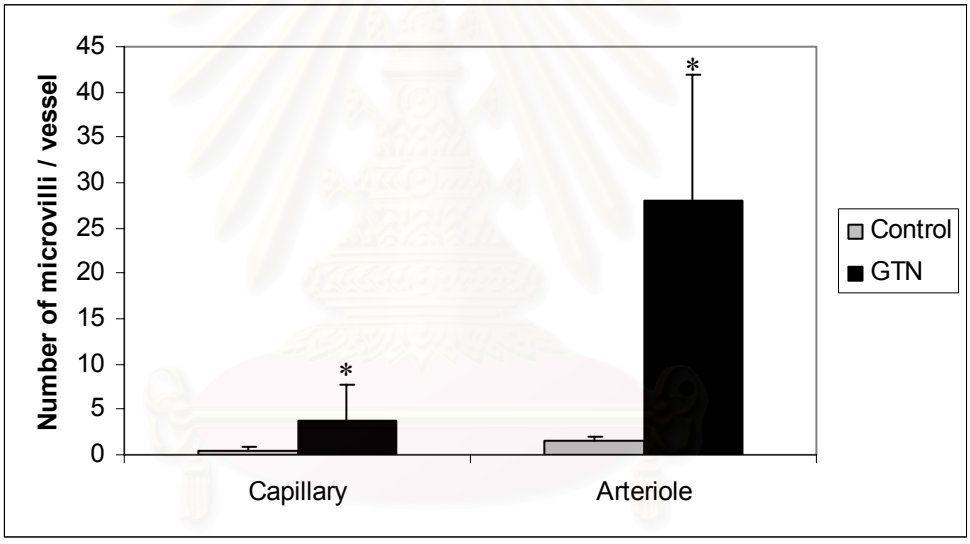


Figure 5-42. Bar graph showing the mean value ± SD of the number of microvilli per vessel in the endothelial cell of cerebral capillaries and arterioles obtained from the control group and the GTN group. *p < 0.05 compared with con

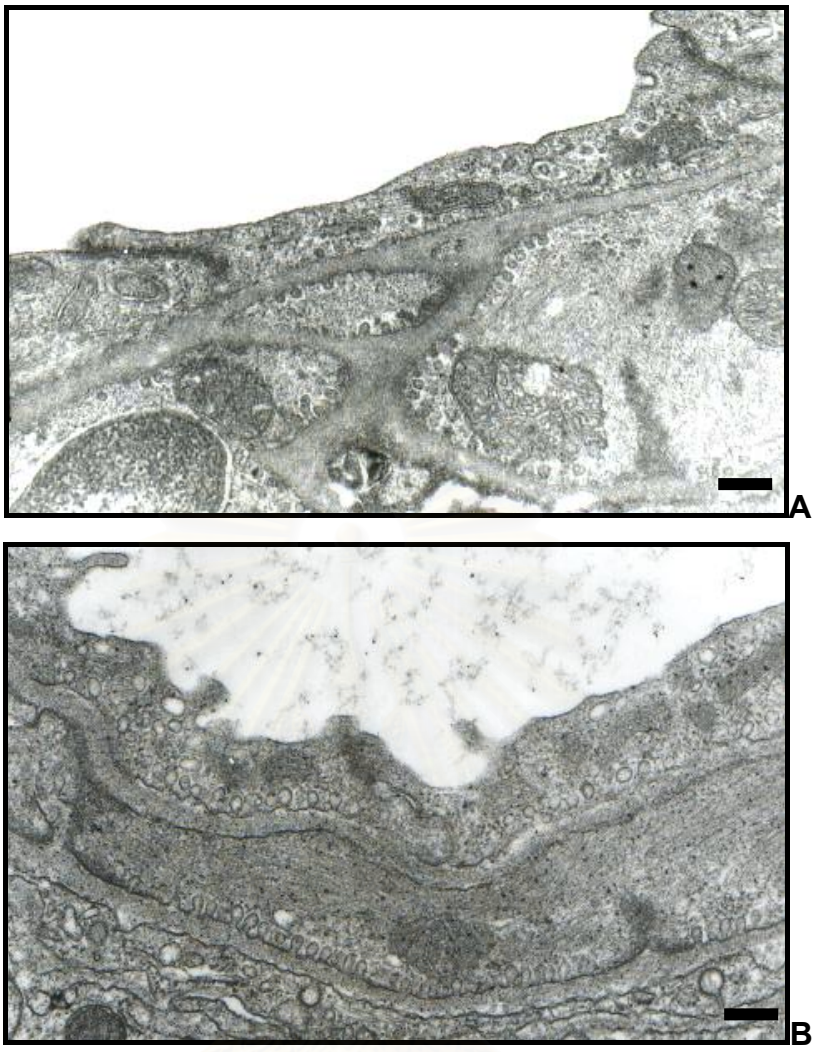


Figure 5-43. Electron micrograph of cerebral microvessels showing a few number of pinocytic vesicles in an endothelial cell obtained from the control group (A) and showing an increase in the number of pinocytic vesicles in an endothelial cell obtained from the GTN group (B). (x 60000). Bar = 200 nm

Table 5-17. The mean value ± SD of the number of pinocytic vesicle/μm^2 in the endothelial cell of cerebral capillaries and arterioles obtained from the control group and the GTN group.

Group	Number of pinocytic vesicle/ μm^2	
	Capillary	Arteriole
Control	9.5 ± 3	10 ± 3
GTN	20 ± 10	37 ± 15

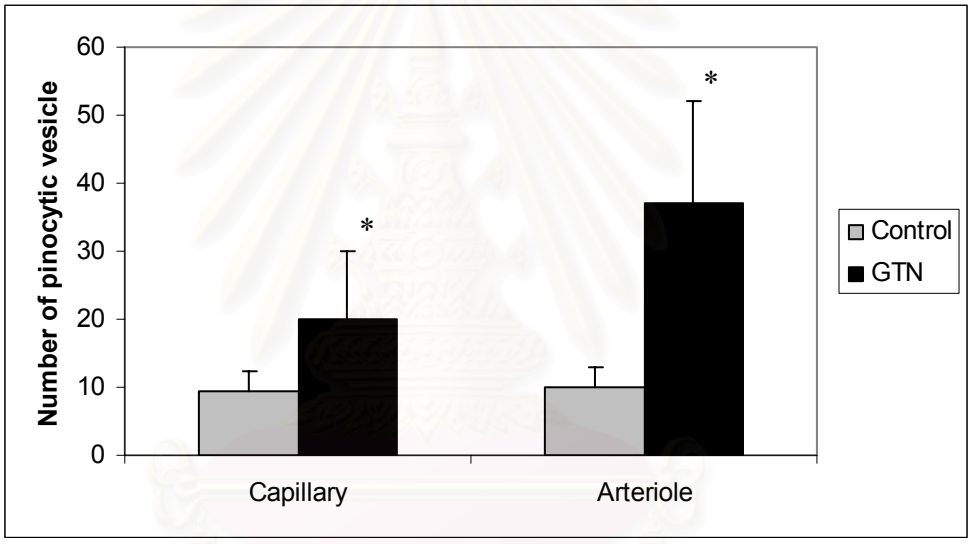


Figure 5-44. Bar graph showing the mean value ± SD of the number of pinocytic vesicle/μm^2 in the endothelial cell of cerebral capillaries and arterioles obtained from the control group and the GTN group. Significant difference was assessed with ANOVA with the Bonferroni test. *p < 0.05 compared with the GTN group.

5.4 The effects of melatonin on the endothelial cell changes induced by GTN infusion

Intraperitoneal injection of melatonin 30 minutes before GTN infusion could attenuate the ultrastructural changes induced by GTN (Figure 5-45). The number of microvilli in arterioles obtained from melatonin pretreated group was significant decreased as compare with those obtained from GTN group. The number of microvilli was decreased from 28 ± 4 microvilli/vessel in GTN group to 18 ± 4, 13 ±6 and 10 ± 5 microvilli/vessel in 10, 20 and 40 mg/kg bodyweight melatonin pretreated group respectively. Furthermore the study demonstrated that The average density of pinocytic vesicles in capillaries was decreased from 20 ± 10 vesicle/μm^2 in the GTN group to 12 ± 6, 8 ± 2 and 12 ± 7 vesicle/μm^2 in 10, 20 and 40 mg/kg bodyweight melatonin pretreated group respectively. The protective effect of melatonin in the pinocytic vesicle formation was also demonstrated in the endothelial cells of arterioles. The average density of these vesicle was 37 ± 15, 23± 15, 12 ± 4 and 21 ± 9 vesicle/μm^2 in the GTN group, 10, 20 and 40 mg/kg bodyweight melatonin pretreated group respectively. The data are shown in Table 5-18, Table 5-19, Figure 5-46 and Figure 5-47.

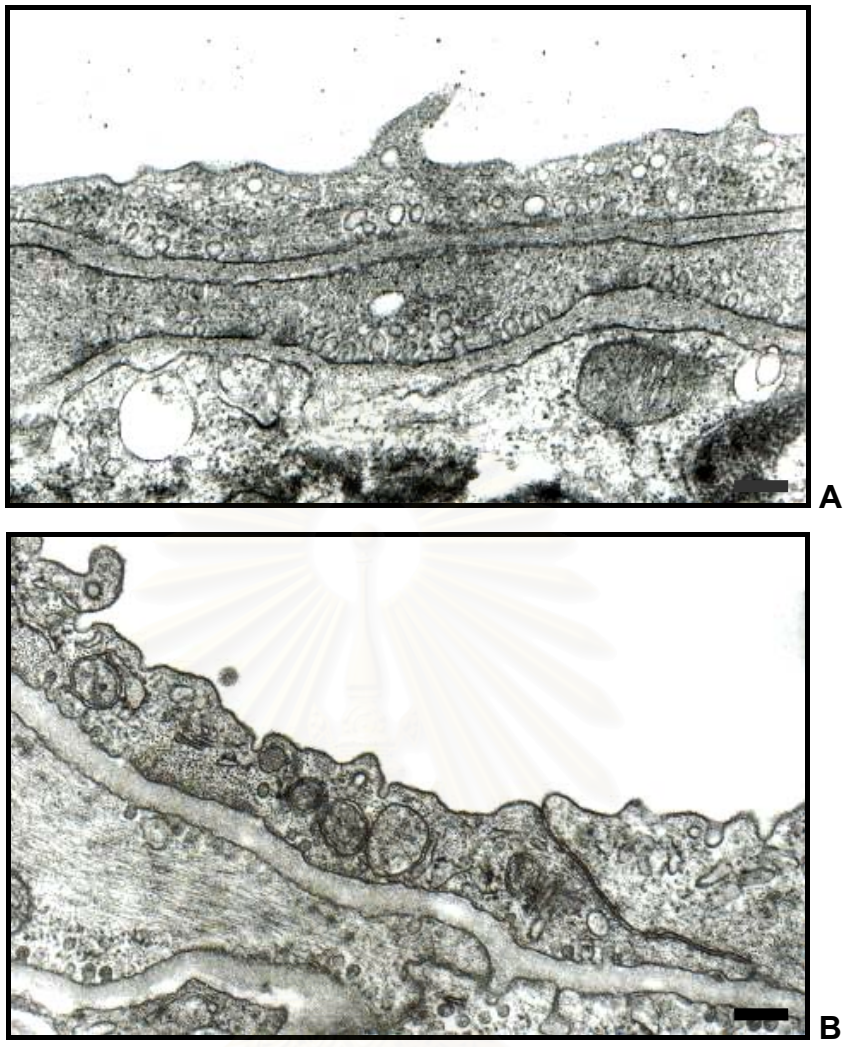


Figure 5-45. Electron micrograph of an endothelial cell obtained from (A) the GTN treated group and (B) the 40mg/kg bodyweight melatonin pretreated group. (x 60000). Bar = 200 nm

Table 5-18. The mean value ± SD of the number of microvilli per vessel in the endothelial cell of cerebral capillaries and arterioles obtained from the GTN and the GTN with melatonin pretreated group.

Group	Number of microvilli / vessel	
	Capillary	Arteriole
GTN	3.75 ± 3.9	28 ± 14
Melatonin 10 mg/kg bodyweight + GTN	1.35 ± 1.6	18 ± 4
Melatonin 20 mg/kg bodyweight + GTN	0.73 ± 0.94	13 ± 6
Melatonin 40 mg/kg bodyweight + GTN	0.55 ± 0.9	10 ± 5

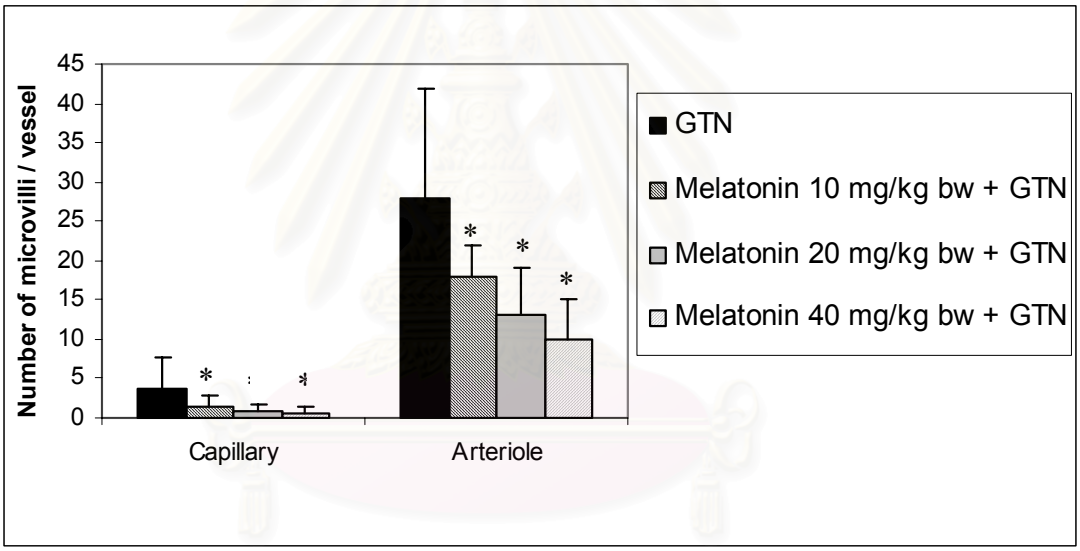


Figure 5-46. Bar graph showing the mean value ± SD of the number of microvilli per vessel in the endothelial cell of cerebral capillaries and arterioles obtained from the GTN and the GTN with melatonin pretreated group. Significant difference was assessed with ANOVA with the Bonferroni test . *$p < 0.05$ compared with the GTN group.

Table 5-19. The mean value ± SD of the number of pinocytic vesicle/μm^2 in the endothelial cell of cerebral capillaries and arterioles obtained from the GTN and the GTN with melatonin pretreated group.

Group	Number of pinocytic vesicle/μm2	
	Capillary	Arteriole
GTN	20 ± 10	37 ± 15
Melatonin 10 mg/kg bodyweight + GTN	12± 6	23 ± 15
Melatonin 20 mg/kg bodyweight + GTN	8 ± 2	12 ± 4
Melatonin 40 mg/kg bodyweight + GTN	12± 7	21 ± 9

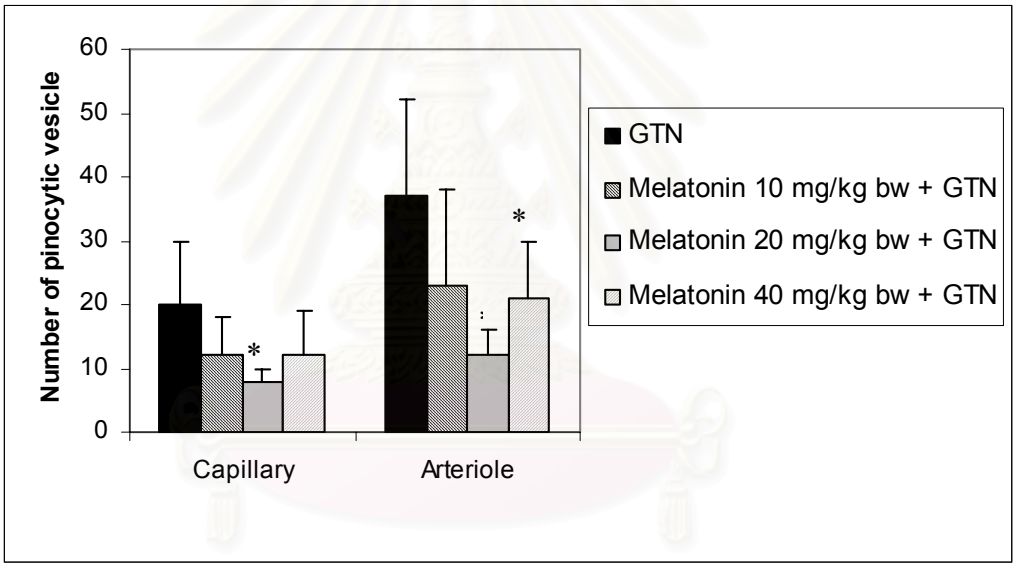


Figure 5-47. Bar graph showing the mean value ± SD of the number of pinocytic vesicle/μm^2 in the endothelial cell of cerebral capillaries and arterioles obtained from the GTN group and the GTN with melatonin pretreated group. Significant difference was assessed with ANOVA with the Bonferroni test. *$p < 0.05$ compared with the GTN group.

CHAPTER 6
Discussion

In this study, a series of experiments was conducted to investigate the effects of melatonin on the trigeminovascular nociceptive system. The first part of this research was conducted to investigate the effects of melatonin on the trigeminovascular nociceptive system in resting condition. The second and the third parts were conducted to investigate the effect of melatonin on the trigeminovascular nociceptive system in pathological conditions (CSD and NO treated).

There are a number of evidences that indicate the relation between trigeminal ganglia and the pineal gland which produces melatonin (Reuss et al., 1992; Reuss 1999 and Shiotani et al., 1986). Retrograde transport of the wheat germ agglutinin upon injection into the superficial pineal of the gerbil labeled neurons in the trigeminal ganglion was demonstrated (Shiotani et al., 1986). Combined tracing and immunohistochemistry demonstrated that some of the trigeminal neurons were SP- and CGRP-positive, suggesting that the immunoreactive fibers, at least in part, stem from the trigeminal ganglia. Similar results were obtained from rats (Reuss et al., 1992). Retrograde tracing with Fluoro-Gold upon application into the pineal gland demonstrated labeled trigeminal ganglion cells. Some of which were also immunoreactive to SP or CGRP (Reuss, 1999). These results reveal that afferent neuropeptidergic entering the superficial pineal gland originates from the trigerminal ganglia.

The effect of melatonin on unprovoked trigeminovascular nociceptive system

The results from the first study show that melatonin, when superfused into the cranial window, induced the vasoconstriction on the cerebral arterioles. The vasoconstrictive effect of melatonin in this study is consistent with the results from several previous studies (Geary et al., 1997; Regringny et al., 1998 and 1999). They concluded that melatonin induced the vasoconstriction effect by activating its vascular MT1 and/or MT2 receptor since pretreatment with Luzindole, a MT1 and/or MT2

receptor antagonist (Krause and Dubocovich, 1991), abolished the vasoconstriction effect of melatonin (Geary et al., 1997; Regringny et al., 1999).

The mechanism underlying the vasoconstriction effect of melatonin is believed to mediate by inhibition of the BK_{Ca} channels (Geary et al., 1997; Regringny et al., 1999). The recent study on receptor distribution demonstrated that the receptor expressed in the cerebral vessel is of the MT1 receptor subtype (Chucharoen et al., 2003). So we can conclude that the vasoconstrictor effect of melatonin is mediated by the inhibition of the BK_{Ca} channels following activation of the MT1 receptor.

The present results from the CBF study using Laser Doppler flowmetry do not show any significant change after i.p. injection (10, 20 and 40 mg/kg bodyweight) of melatonin. The same result was observed in a randomized, placebo-controlled, double-blind, cross-over study. van der Helm-van Mil et al. found that bolus i.v. injection of 10 μg melatonin did not affect CBF and the cerebrovascular resistance (van der Helm-van Mil et al., 2003).

Furthermore in the present study the ultrastructure of endothelial cells obtained from the melatonin treated group does not show any significant difference from the control group. The number of microvilli and the number of pinocytic vesicles were not different from those obtained from the control group. These data can imply that melatonin has no toxic effect to endothelial cells.

Furthermore the role of melatonin in the control of the cerebral arteriolar structure and function has been recently reported by Regringny et al. (Regringny et al., 2001). Their results demonstrated that the arteriolar wall in melatonin deprival rats (pinealectomy) was thinner and stiffer than those obtained from control rats. Because these structural and mechanical alterations were reversed by melatonin treatment, there were probably consecutive to melatonin deprival (Regringny et al., 2001). The changes of the structure of cerebral arterioles were similar to those observed in old rats (Hadju et al., 1990) in which the production of melatonin decreases (Sack et al., 1986).

In this study, there were no differences in the number of NOS-IR and Fos-IR cells between melatonin treated the group and the control group. Intraperitoneal injections of melatonin even in the highest dose (40 mg/kg bodyweight) fail to activate the second order neuron in the trigeminovascular nociceptive pathway. Several experiments in both animal and human demonstrate that the toxicity of melatonin is extremely low. In animals a LD 50 (lethal dose for 50% of the subjects) could not be established. Even 800 mg/kg bodyweight (fantastically high dose) was

not lethal (Barchas et al., 1967). Five human subjects were each given melatonin 1 gram per day for 25-30 days, and were followed with an investigation of physiological and biochemical parameters to detect potential toxicity. All findings were normal at the end of the test period (Nordlund et al., 1977).

The findings in the first part indicated that the i.p. injection of melatonin does not have an effect on CBF, whereas melatonin does constrict pial arterioles when it was directly superfused onto these vessels. It also can be concluded from the results that the i.p. injection of melatonin up to the dose of 40 mg/kg bodyweight do neither harm to endothelial cell nor activate the trigeminovascular nociceptive system.However in some activated conditions (CSD activation or NO-donor activation), the effect of melatonin is clearly demonstrated.

The effect of melatonin on trigeminovascular nociceptive system in animal model of migraine

In this study the effect of melatonin were investigated in two animal models for migraine, namely cortical spreading depression and NO-donor infusion.

In the CSD model, melatonin demonstrated the inhibitory effect on several changes of the trigeminovascular nociceptive system. The alterations in both cortical blood flow and the trigeminal nociceptive system are demonstrated. The CSD induced repetitive hypereamia observed in this study is the same phenomenon previously described by Read et al. (Read et al., 2000). In their study, they also detected for the NO released in the CSD model. Their result showed that the increase in NO current detected from the NO electrode was correlated with the hyperemia observed from Laser Doppler flowmetry. The association between NO and CSD has been previously demonstrated in the anesthetized cat. The potent multiphasic release of NO was associated with CSD. Pretreatment with an NOS inhibitor was found to uncouple the regional cerebral blood flow changes from CSD-induced d.c. depolarization (Read et al., 1997).

The immunohistochemical study in this experiment also reveals that the induction of CSD can increase the c-fos and NOS expression in lamina I and II of the C1/C2 cervical spinal cord sections. Besides these two areas, CSD also induces the expression of NOS in lamina V and lamina X. Lamina V contains the nucleus proprius, which integrates sensory input with information that descends from the brain and the region of the base of the dorsal horn where many of the neurons that project to

the brain stem are located. Furthermore the NOS expression also demonstrates in lamina X which surrounds the central canal and receives afferent input similar to that of laminar I and II.

At the level of the spinal dorsal horn neuron, its excitability depends on the input from the primary afferent as well as the segmental and supra spinal pain-modulating circuits. One important feature of synaptic transmission between primary nociceptive afferents and dorsal horn neurons that underlies the plasticity of dorsal horn neurons is the complexity of its neurotransmitter system. Central terminals of c-fiber neurons contain multiple transmitters including glutamate and the peptides, substance P, and CGRP. Various forms of receptors, both of the ionotropic and metabotropic type , are expressed on the cell membrane of the postsynaptic dorsal horn and trigeminal nucleus caudalis neurons. Activation of the NMDA receptor as well as other metabotropic receptors, eg, neurokinin-1 receptor, results in an increase in the intracellular calcium in the post synaptic neurons. The rising of intracellular calcium will lead to substantial changes in the post synaptic function. The increased calcium will activate NOS and increase the production of NO. This gaseous molecule then diffuses back to the presynaptic terminals and stimulates transmitter release which results in the long-term strengthening of the synaptic function and lead to the development of central sensitization (Srikiatkhachorn, 2001)

It has been suggest by Woolf (2000) that CSD stimulation of trigeminal afferents may induce a central sensitization which is maintained by further afferent input from the sensitized peripheral nerves (Woolf, 2000). Indeed Moskowitz et al. (1993) showed that following induction of the cortical spreading depression there was increased the expression of c-fos like immunoreactivity in the trigeminal nucleus caudalis (TNC). Their results demonstrated a clear association between CSD and nociceptive processing. However, later, some studies have demonstrated the contradictory results (Ingvardsen et al., 1997; Lambert et al., 1999).

Ingvardsen et al. (1997) studied the c-fos expression in the TNC following elicitation of CSD activity with repeated injections of 1 M KCl or NaCl. Lambert et al. (1999) utilized cortical pin prick injury to elicit spreading depression and recorded spontaneous and evoked firing in neurons of the C2 cervical spinal cord. Neither studies documented a robust link between CSD and noxious activation. However, both of these studies may be compromised, in that both evoked a variation of stab

wound to the cortex. This stab wound is not reproducible between or within animals in term of c-fos expression, and therefore the c-fos baseline will be different.

The more recent study by Boley et al (2002) demonstrated that CSD induced changes in CBF and also induced the c-fos expression in second order neurons in the TNC. In the present study, the results support the hypothesis of Moskowitz (1993) and Boley et al. that CSD induced changes in CBF and activation of the trigeminal nociceptive pathway. The c-fos expression is strongly demonstrated in the cervical spinal cord section, particularly in the ipsilateral side to CSD activation.

Boley at al. also assumed that CSD induced perivascular neurogenin inflammation. After activation, these nerve terminals release several substances, i.e. substance P, that promote vasodilatation and increase vascular permeability. The released peptides are also algogenic and can further sensitize the surrounding nociceptors (Boley et al., 2002; Moskowitz, 1993). However the ultrastructural examinations in this study demonstrate the different mechanism underlying CSD activation. Even the endothelial cells obtained from the CSD group demonstrated the increase in the number of pinocytic vesicles and microvilli, but all vessels examined in this study did not show any change in the tight junction. The intact endothelial tight junction was demonstrated in both capillaries and arterioles obtained from the CSD group. These findings suggest that the process of CSD-induced trigeminal nociception can occur despite the absence of perivascular inflammation.

The findings of preserved tight junction in vessels obtained from the CSD group is consistent with the previous study in which albumin was used as a tracer (Ebersberger et al., 2001). The neurogenic inflammation theory is also less likely since KCl at the concentration found during CSD did not alter release of the algogenic peptides, CGRP and prostaglandin E2 (Ebersberger et al., 2001).

The conclusion of no causal relationship between neurogenic inflammation and trigeminal nociception is also supported by pharmacological experiments. In 1999, Goadsby and Hoskin showed that CP122,288, a potent inhibitor of neurogenic inflammation, failed to inhibit the c-fos expression in TNC evoked by activation of the trigemino vascular system (Goadsby and Hoskin, 1999).

Another animal model used in this study is the NO-donor induced vasodilatation model. The result from this experimental headache model demonstrated that the NO-donor when superfused onto the pial arterioles induced vasodilatation on these vessels. The mechanism underlying vasodilatation is that the NO-donor acts

directly on the vascular smooth muscle cell to generate NO, either spontaneously or through interaction with tissue components. NO, then activates soluble guanylate cyclase and thus increases cGMP and cGMP-dependent protein kinase with resultant smooth muscle relaxation. Some studies also demonstrated an indirect mechanism underlying NO-donor mediated vasodilatation. The organic nitrate acts on sensory fibers that innervate pial vessels to release the CGRP. The released CGRP then diffuses to the vascular smooth muscles, where it activates soluble adenylate cyclase to cause vasodilatation. CGRP is the principal regulator of the trigeminovascular system (Tsai et al., 1988), a system formed by trigeminal fibers which innervated intra- and extra- cranial vessels, dura mater and other extracranial structures. Trigeminovascular fibers store neuroactive peptides and release them upon depolarization. A possible role for the trigeminovascular system in the cerebrovascular effect of GTN is also suggested by demonstrating that the chronic trigeminal denervation decreases the responsiveness of cerebral arterioles to GTN (Tassorelli, 1999).

The exact mechanism by which nitrodilators activate the release of polypeptides from sensory nerves is not known. Nitrate might induce the calcium dependent neuropeptide release. Another possibility is that NO generated by nitrodilator interacts with superoxide to form peroxynitrite (Beckman et al., 1990). This agent may then generate hydroxyl radicals, which are important mediators of tissue injuries and may, somehow activate small C fibers that respond to real or threatened tissue injury (Tassorelli, 1999).

Morphologycal changes in the ultrastructure of endothelial cells observed in this study were characterized by an increase in the number of pinocytic vesicles and microvilli in the endothelial cell obtained from the NO-donor treated group. This result is consistent with the previous study (Anuntasethakul et al., 1999). An increase in microvillous formation which expands the luminal surface area may imply increase cellular activity regarding uptake and transcellular transportation. Increased endothelial microvilli have been previously reported in the osmotically impaired blood brain barrier (Lossinsky et al., 1995). The hypothesis of NO-induced derangement of the blood brain barrier can be supported by the observation of a coexisting increase in the density of pinocytic vesicles. Unlike endothelial cells in other organs, brain endothelial cells posses only few pinocytic vesicles during the resting state. An increasing number of these vesicles reflect an enhanced cellular

transport activity. Such an increase may lead to increased permeability of the cerebral microvessel and in the extreme state may cause impairment of blood-brain barrier. An increase in cerebral microvessel pinocytosis was previously observed in other conditions, including concussive brain injury and hypertensive cerebrovascular diseases (Wi et al., 1980; Hazama et al., 1978). In both conditions, increased pinocytosis was related to an increase in cerebral vascular permeability.

The meachanism by which NO can induce endothelial cell injury are not fully understood. NO can interact with oxygen molecules and results in $ONOO^-$ as well as tissue damaging radicals (Beckman et al., 1990). These radicals will lead to endothelial cell injury via the process of lipid peroxidation as well as peroxidation of sulfhydryls (Radi et al., 1991).

The ultrastructural examination in this study demonstrated an increase in the number of pinocytic vesicle and microvilli in the endothelial cell obtained from the NO-donor treated group. This result is consistent with the previous result (Anuntasethakul et al., 1999). Unlike endothelial cells in other organs, brain endothelial cells possess only few pinocytic vesicles during the resting state. An increasing number of these vesicles reflect an enhanced cellular transport activity. Such an increase may lead to increased permeability of the cerebral microvessel and in the extreme state may cause impairment of blood-brain barrier. An increase in cerebral microvessel pinocytosis was previously observed in other conditions, including concussive brain injury and hypertensive cerebrovascular diseases. In both conditions, increased pinocytosis was related to an increase in cerebral vascular permeability.

Furthermore infusion of NO donor can result in various changes in trigeminovascular nociceptive system. Systematic administration of the NO-donor induced a significant increase in NOS-IR and Fos-IR cells in the cervical part of the trigeminal nucleus caudalis. Previous studies by Tassorelli and Joseph (1995) and Pardutz et al. (2000) showed the same results with this study. The localization of NOS and c-fos immunoreactive cells observed in this study also distributed in the same pattern with their study. The majority of NOS-IR and Fos-IR cells were observed in lamina I and II, where nociceptive input are transmitted to the rostal center.

These findings suggest that NO-donor infusion can activate the trigeminovascular nociceptive system.

Both animal models used in this study are proved to be a valid model. Both models demonstrate the activation of the trigeminovascular nociceptive system which is the main mechanism underlying vascular headache.

In this study, the effect of melatonin was studied in these two animal models. The results demonstrate that melatonin pretreatment can attenuate changes in both the vascular and trigemino nociceptive system in both animal models. In the vascular compartment, the hyperemia observed after the induction of CSD and the vasodilatation of arterioles after infusion of NO-donor were substantially attenuated by melatonin pretreatment. This effect is likely to be due to the direct effect of melatonin in constriction of cerebral vessel.

Several studies including this study, in the first part, have confirmed the vasoconstrictive effect of melatonin (Geary et al., 1997; Regringny et al., 1998, 1999).

NO is the key molecule involved in the cerebrovascular system (Read et al., 2000). Melatonin is reported to directly scavenge of NO (Noda et al., 1999; Turjanski et al., 2001) and highly reactive $ONOO^-$ (Gilad et al., 1997; Pappolla et al., 2000; Reiter, 1997, 1999). Besides the direct scavenging NO and $ONOO^-$, melatonin also acts as an indirect antioxidant. It stimulates several important anti-oxidative enzymes such as glutathione peroxidase which is a peroxynitrite reductase (Sies et al., 1997). So the anti-oxidative effect of melatonin may at least partly be responsible for the attenuating effect of melatonin in hyperemia induced by CSD and the NO-donor.

CGRP, as mentioned above, is another key molecule responsible for the vasodilatation after NO-donor infusion. The dilatory action of CGRP in the rat cerebral arterioles is believed to be mediated by the stimulation of the adenylate cyclase, hence increasing the cAMP (Toyada, 2000). Melatonin, coupling with MT1R, is known to inhibit foskolin-stimulate cAMP formation (Capsoni et al., 1991). In recent study, this indole demonstrated the inhibitory action on CGRP mediated vasodilation and stimulation of adenylate cyclase activity in the middle cerebral arteries of the rats (Viswanathan, 2001). Base on these evidences we suggest that melatonin stabilize vascular tone in noxious condition via multiple mechanisms, including direct vasoconstriction, anti oxidative activity and the inhibition of CGRP induced vasodilatation.

Furthermore the protective effect of melatonin also demonstrates in the ultrastructural study of endothelial cell in this study. Several changes in endothelial cells induced by CSD or the NO-donor are attenuated by melatonin pretreatment. The

number of pinocytic vesicles and microvilli was lower in the rat given melatonin pretreatment before the CSD induction or GTN infusion. These results indicate the protective effect of melatonin in vascular compartment.

Besides these effects, melatonin demonstrates the protective effect on the neurons in the trigeminovascular nociceptive system. The number of NOS-IR and Fos-IR cells in the cervical spinal cord obtained from rats given melatonin prior CSD induction or GTN infusion was lower than those obtained from rats without melatonin pretreatment. It is known that NO plays an important role in the process of trigeminovascular nociceptive sensitization (Srikiatkhachorn, 2001). The presence of NOS-IR in the dorsal horn and TNC reflects its involvement in the nociceptive process. In this experiment CSD or GTN infusion enhanced the NOS expression which was diminished with melatonin pretreatment. Several biochemical evidences also indicated that melatonin treatment could significantly reduced the NOS activity in the does-dependent manner in the rat cerebellum as well as in the hypothamus (Bettahi et al., 1996; Pozo et al., 1997). These results were confirmed recently by Chang et al. (2000) and Crespo et al. (1999). Chang et al. found that the nNOS expression in hypoglossal nucleus following a peripheral nerve injury was markedly depressed by melatonin treatment while, Crespo et al. found that melatonin inhibited the expression of the iNOS isoform (Crespo et al., 1999).

Although the detailed mechanisms of melatonin in the prevention of NO mediated neurological deficits remain to be explored, it is likely that the neuroprotective effect of melatonin may be contributed by its antioxidant properties and its free radical scavenging ability (Chang et al., 2000; Reiter et al., 1999 and 2000). It is known that there are no morphophysiological barriers to melatonin (Menendez-Pelaez et al., 1993) and the melatonin –induced suppression of NOS activity is believed to be a consequence of the binding of calmodulin by this indole. It has been suggested that intracellular melatonin can interact with calmodulin and modify the binding of the peptide to the synthetic NOS peptide encompassing the calmodulin-binding domain of constitutive NOS, the natural mechanism by which calmodulin activates this enzyme (Bredt et al., 1992; Pozo et al., 1997; Chang et al., 2000). Besides reducing NO formation in second order neuron in the trigeminovascular nociceptive pathway, in this study melatonin shows that it directly attenuate the changes in the cerebrovascular system induced by the CSD or the NO-donor as well (Reiter et al., 1999, 2000). Thus, the drop in NO synthesis may result

from the less activation of the perivascular nerve which is the peripheral protection effect of melatonin.

In this study, melatonin treatment can decrease the CSD and NO-donor induced c-fos expression. Melatonin may directly protect the neurons with its anti-oxidative effect by decreasing the perivascular activation or indirectly by the decrease of the NOS expression in the trigeminocervical complex (Lamina I and II of C1 and C2 spinal cord). It is known that in the nucleus trigeminalis caudalis, the co distribution of c-fos positive neurons and NOS positive fiber seems of relevance, especially when considering that anatomical evidence has suggested a role for NO in the processing of the sensory and nociceptive information in the medulla and spinal cord (Dun et al., 1994). This hypothesis has been recently confirmed by Srikiatkhachorn. (2001) that NO plays an important role in the process of trigeminovascular nociceptive sensitization. Therefore the decrease of NOS expression by melatonin may lead to the decrease of c-fos expression in the spinal sections obtained from the melatonin treated group in this study.

The results of this study demonstrate that melatonin can attenuate the process of trigeminovascular nociception evoked by cortical spreading depression and NO-donor infusion.

CHAPTER 7
Conclusion

The results of this study confirm the potential anti-migraine effect of melatonin. In the CSD model, melatonin can attenuated the hyperemia, as well as protecting endothelial cells from CSD activation. Furthermore melatonin can attenuate the NO synthesis in the second order neurons of the trigeminovascular nociceptive system which then leads to a decrease of the activation of these second order neurons by CSD.

The similar results are observed in another model using NO-donor infusion. Melatonin attenuates the vasodilatation induced by the NO-donor and also protects the endothelial cell from NO activation. In the central trigeminovascula nociceptive system melatonin protects neurons from NO-activation and also decreases the NO synthesis in these neurons.

The present study demonstrates several biological effects of melatonin which are beneficial in migraine treatment. These effects include (1) Constriction of cranial vessel (2) Inhibition of activation of trigeminovascular system induced by CSD activation. (3) Inhibition of NO activated cascade (4) Attenuating NO biosynthesis (5) Reduction in neuronal activity in central trigeminal neurons. The proposed mechanisms underlying the effect of melatonin on the trigeminovascular nociceptive system in CSD and NO-donor infusion model are shown in Figure 7-1

According to these results, melatonin can be used as an anti-migraine drug. However the suitable pharmacological dose still needs to be explored.

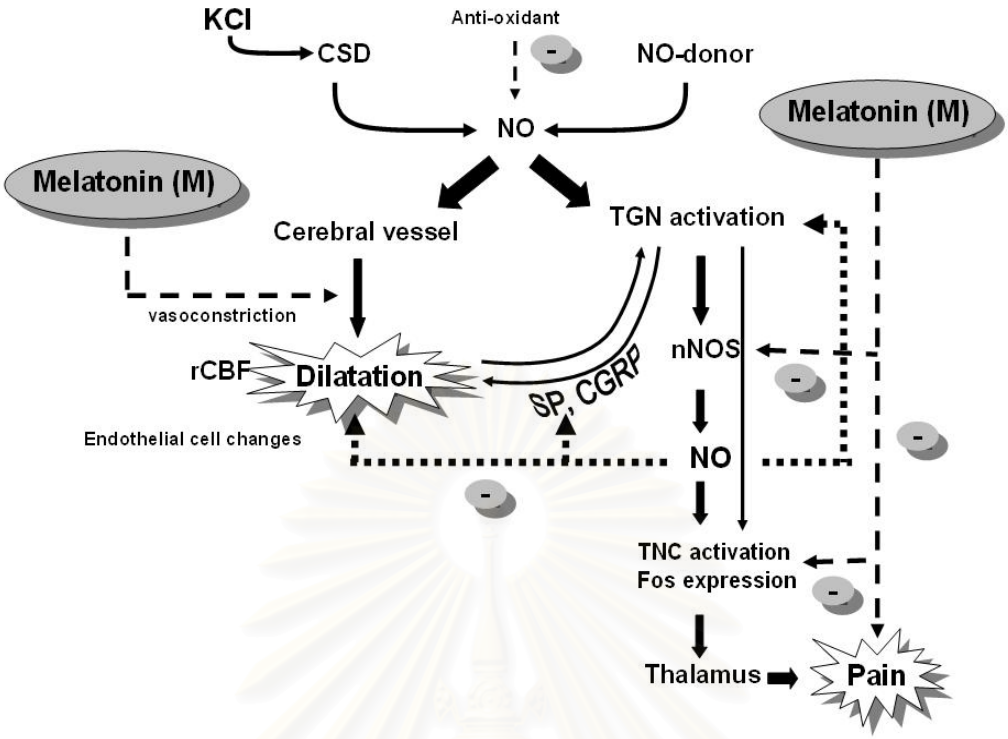


Figure 7-1 The effect of melatonin on trigeminovascular nociceptive system.

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Zanoboni, A., and Zanoboni-Muciaccia, W. Experimental hypertension in pinealectomized rats Life Sci. 6(1967) : 2327-2331.

BIOGRAPHY

NAME	Miss Supang Maneesri
DATE OF BIRTH	9th January 1965
PLACE OF BIRTH	Chiengmai, Thailand
INSTRUCTIONS ATTENDED	Chiengmai University (1986-1987) Bachelor of Science (Medical Technology)
	Chulalongkorn University (1994-1997) Master of Science (Medical Science)
	Chulalongkorn University (1999-2003) Ph.D. candidate (Physiology)
POSITION & OFFICE	1987-1992 : Institude of Dermatology Thailand 1992-present: Department of Pathology Faculty of Medicine Chulalongkorn University