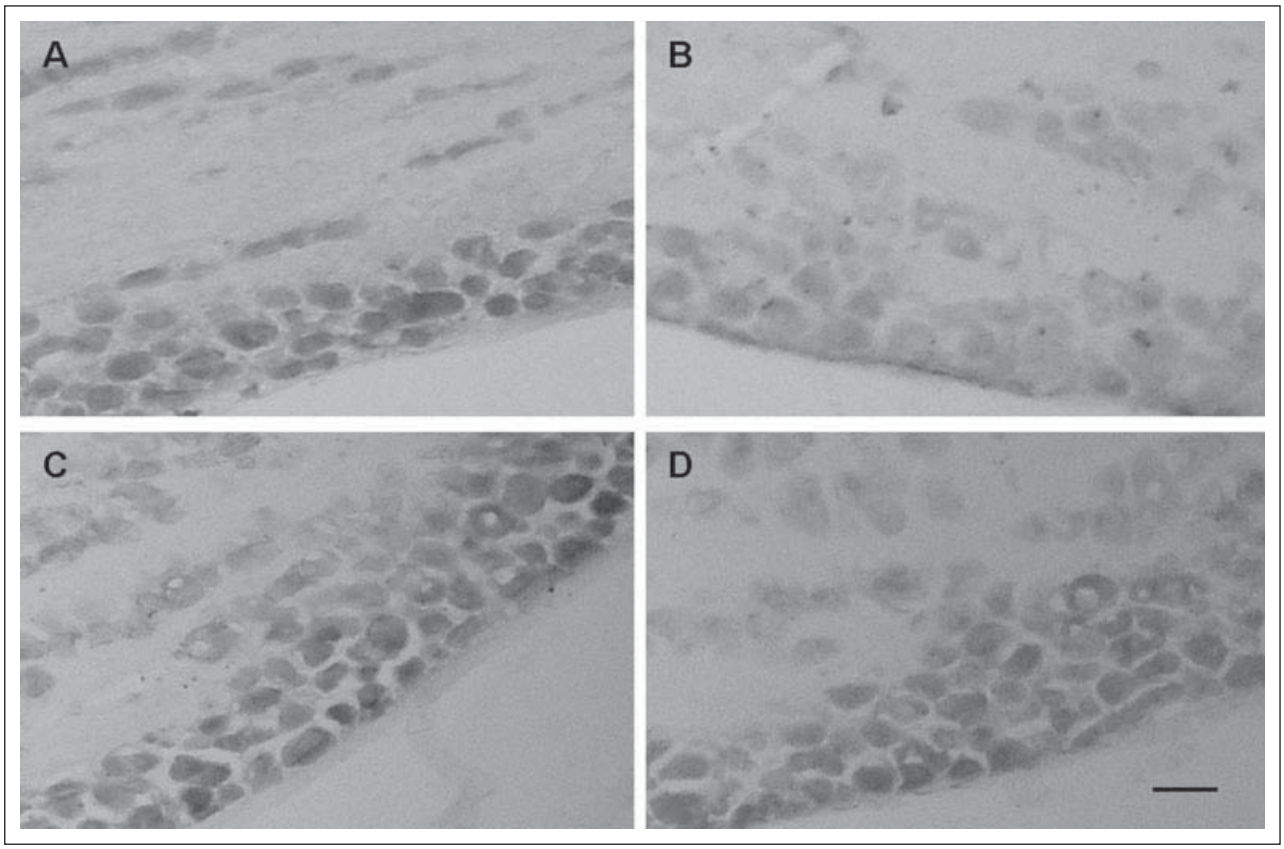

Fig 5.—Effect of chronic paracetamol and ketanserin pretreatment on 5-HT_{2A} expression in TG. The figure demonstrates the 5-HT_{2A} immunohistochemical study in the ophthalmic division of TG. Chronic paracetamol exposure led to an up-regulation of 5-HT_{2A} receptor expression in TG. Pretreatment with ketanserin did not significantly alter this receptor expression (KCl, A; NaCl, B; KCl with chronic paracetamol, C; KCl with chronic paracetamol and ketanserin [10 mg/kg] pretreatment, D; bar = 100 μm). TG = trigeminal ganglion.

STATEMENT OF AUTHORSHIP

Category 1

(a) Conception and Design
Weera Supornsilpchai; Supang M. le Grand; Anan Srikiatkhachorn

(b) Acquisition of Data
Weera Supornsilpchai; Supang M. le Grand; Anan Srikiatkhachorn

(c) Analysis and Interpretation of Data
Weera Supornsilpchai; Supang M. le Grand; Anan Srikiatkhachorn

Table 3.—Effect of Ketanserin on Thermal Nociception and Thermal Hyperalgesia

Test	Control	CFA	NSS + ketanserin	CFA + ketanserin
Inflamed side	8.3 ± 0.6	5.3 ± 1.3*	9.3 ± 1.0	14.3 ± 2.0‡
Noninflamed side	9.2 ± 1.4	9.4 ± 0.9	11.2 ± 2.5	9.2 ± 1.1

Pretreatment with ketanserin did not alter the paw withdrawal latency in the noninflamed paw but prolonged the paw withdrawal latency in the inflamed side (*$P < .001$ compared with the control, ‡$P < .001$ compared with the CFA group without ketanserin treatment).
CFA = complete Freund's adjuvant; NSS = normal saline solution.

Category 2

(a) Drafting the Manuscript
Weera Supornsilpchai; Supang M. le Grand; Anan Srikiatkhachorn

(b) Revising It for Intellectual Content
Weera Supornsilpchai; Supang M. le Grand; Anan Srikiatkhachorn

Category 3

(a) Final Approval of the Completed Manuscript
Weera Supornsilpchai; Supang M. le Grand; Anan Srikiatkhachorn

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Brief Communication (Original)

Enhancing effect of nociceptin in cortical spreading depression: electrophysiological study using an animal model of migraine

Saknan Bongsebandhu-phubhakdi, Thas Phisonkunkasem, Anan Srikiatkhachorn
Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

***Background*:** The alternation in cortical excitability is believed to represent initial manifestations of a migraine attack. Although nociceptin/orphanin FQ (N/OFQ) may be involved in the cortical control, it is still unclear as to what electrophysiological effect of N/OFQ is included in migraine.
***Objective*:** To study the effect of N/OFQ on development of cortical spreading depression (CSD), using an animal model of migraine.
***Methods*:** Wistar rats were divided into three groups: CSD, N/OFQ-CSD, and control. In the N/OFQ rats, N/OFQ was injected intrathecally. CSD was induced by application of crystallized KCl on the cerebral cortex.
***Results*:** KCl application developed a train of depolarizing extracellular potential characterized as CSD. The development of CSD was enhanced by N/OFQ. The peak amplitude and the number of CSD cycles within one hour were greater in N/OFQ-CSD compared with CSD group, while duration and area under curve of each cycle were not changed.
***Conclusion*:** N/OFQ significantly enhanced the development of CSD by increasing cortical excitability. Its receptor, ORL-1 receptor, could be the target of pharmaceutical treatment of migraine.

***Keywords*:** Cortical excitability, cortical spreading depression, migraine, nociceptin, orphan opioid receptor-like 1 (ORL-1) receptor electrophysiological study.

Migraine is currently considered a primary neurobiological headache disorder that relates directly to an excitation of the central nervous system. An increase in the function of cortical excitability leads to paroxysmal head pain with accompanying disturbances [1]. Migraine auras are obviously important neurological disturbances appearing before and during the progress of classical migraine headache [2]. A typical migraine aura is usually visual, apparently starts from the primary visual cortex producing a scintillation included with a scotoma at the central visual field and propagates to the peripheral parts within 10 minutes. The aura indicates a long inhibition after a shorter period of neuronal excitation. Investigations in patients suggest that a visual migraine aura is the clinical manifestation of a Leao's cortical spreading depression (CSD) [3, 4]. CSD phenomenon is a transient failure of brain ionic homeostasis as well as efflux of excitatory amino acids from neurons generating a dramatic neuronal depolarization. CSD is elicited at the occipital pole (area 17), spreading forward to the lateral, medial, and ventral sides of the brain. A link between CSD and migraine auras is hypothesized initially based on increasing evidences of the starting point, the direction and the same propagation speed at 3-5 mm/minute [5, 6]. Similar evaluations can be observed in the somatosensory disturbances propagating along the sensory homunculus.

To search for a novel approach of migraine treatment, the precise molecular and physiological mechanisms of migraine need to be identified. However, it is difficult to perform the experiment in patients during a migraine attack. Therefore, CSD phenomenon in animals has been developed and used

***Correspondence to*:** Dr. Saknan Bongsebandhu-phubhakdi, Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand; E-mail: saknan-tky@umin.ac.jp

as the model of migraine in last decades. In the rats, CSD activates the trigeminovascular afferents, and induced long-lasting blood-flow attenuation within the middle meningeal artery and plasma protein leakage in the dura mater [7, 8]. These findings may represent that hyperexcitability in the cortex during CSD increases the excitability of trigeminal nociceptive pathway connecting with the intracranial nociceptin-sensitive blood vessels with central aspects of the migraine attack in man [9, 10]. The control of cortical excitability is extremely complex, involving several chemical messengers, both small molecule transmitters and peptides. Nociceptin/orphanin FQ (N/OFQ) is a 17-amino acid neuropeptide with close similarity to the opioid peptides [11, 12]. N/OFQ has selective affinity for the orphan opioid receptor-like (ORL-l) G-protein coupled receptor. N/OFQ and ORL-1 receptor are widely distributed in the cortical nociceptive system [13]. Such localization suggests their pathophysiological involvement in the mechanism of cortical excitability, and in neurogenic headaches. It has been demonstrated that plasma nociceptin levels correlates with the frequency of migraine attacks [14]. However, the mechanism that nociceptin triggers migraine attack is not well understood.

In order to study whether nociceptin might be involved mediating the cortical excitability, we evoked CSD by application of crystallized KCl to the cerebral cortex. Furthermore, we determined the effect of nociceptin on electrophysiological waves of CSD.

Materials and methods

Male Wister rats (National Laboratory Animal Center of Mahidol University, Salaya, Nakorn-pathom) weighting 200-300 g were used in all experiments. Experiments were conducted in accordance with the guidelines of experimental animals by the Ethics committee of Faculty of Medicine, Chulalongkorn University.

Rats were divided into three groups: CSD, N/OFQ-CSD and control groups. In the N/OFQ-CSD, N/OFQ (10 μM/100 μL) was administered intrathecally 30 minutes prior to the operation. Saline of the same volume was applied to CSD and control groups. Thirty minutes after pretreatment, rats were prepared for the operation of CSD. In the N/OFQ-CSD and CSD rats, CSD were induced by topical application of 3 mg crystallized KCl on the parietal cortex. Crystallized NaCl of the same weight was placed on the parietal cortex of control group. Electrophysiological study was performed to record extracellular potential in frontal cortex. Cortical excitability was monitored continuously for one hour.

Animal preparation

Rats were anesthetized by intraperitoneal sodium pentobarbital (60 mg/kg). Additional intraperitoneal doses (20 mg/kg) were given to maintain anesthesia on testing of tail pinch reflex. Tracheotomy was performed to assist ventilation. Left femoral vein cannulation was performed for intravenously infusion of anesthetic drug and saline. The head of the animal was fixed in a stereotaxic frame, and the apical and parietal parts of the skull were exposed following a median incision. Over the right hemisphere, the craniotomy procedures were performed by using a saline-cool drill. The anterior and posterior apertures were used for placing electrode and initiating CSD (**Fig. 1**). CSD was induced by topical application of KCl on the cerebral cortical surface.

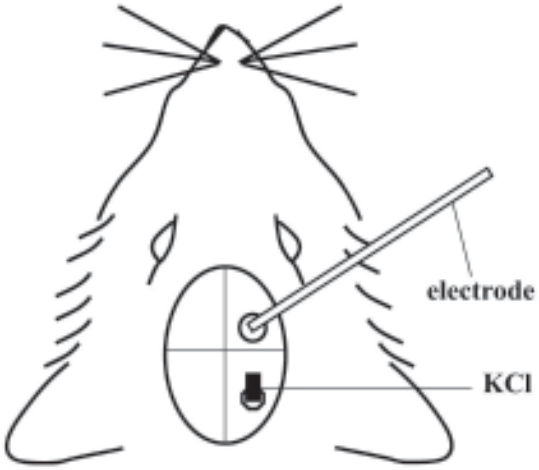

Fig. 1 Schematic representation of the preparation for CSD. The glass microelectrode was arranged in the cerebral cortex via the frontal opening. KCl was placed in the parietal opening.

Recording of extracellular potentials

Intracortical extracellular potentials were recorded using glass micropipettes filled with concentrated NaCl [15]. A silver reference electrode was placed on the unhaired scruff. To record the cortical excitability, another opening was performed at 1 mm anterior and 1 mm lateral to bregma. The electrode assembly was inserted to the cortex to a depth of 500 μm. The signals were recorded using microelectrode amplifier. Analog data were transduced to digital data using the data acquisition system. All traces were analyzed using AcqKnowledge version 3.4 (BioPac), and were stored on personal computer.

Data analysis

All values were expressed as the mean ± SEM. Statistical analysis was performed using Student's *t* test to determine whether there was a statistically significant between the means. Probability values (p) of less than 0.05 were considered to be statistically significant.

Results

KCl induced a repeated cycles of cortical depolarization in CSD group (**Fig. 2A**). The first peak of CSD developed in three minutes after KCl application. The number of cycles was 12.33 ± 0.19; n = 3. The mean of peak amplitude was summarized as 28.07 ± 0.21 mV; n = 3. The interpeak latency (IL), area under curve (AUC) and duration of each cycle were 307.38 ± 7.32 seconds; n = 3, 21.44 ± 0.89 mV-second; n = 3, and 74.48 ± 6.58 seconds; n = 3, respectively. KCl induced a repeated depolarizing trace even in the experiment of N/OFQ-CSD group. Despite no difference in the induction of CSD, the total number of CSD cycles generating within one hour from N/OFQ-CSD group was increased to 14.75 ± 0.24; n = 4; p = 0.012 (**Fig. 2B**). Additionally, the mean of peak amplitude had a statistically significant increase to 36.10 ± 0.37 mV-second; n = 4; p = 0.012 (**Table 1**). However, N/OFQ-CSD group showed no significant difference in the other variables as compared with variables of the CSD group. The AUC of each cycle had a tendency to augment to 30.06 ± 2.49 mV-second; n = 4; p = 0.189. Though, IL and duration tended to decline to 247.98 ± 11.06 seconds; n = 4; p = 0.089, 68.76 ± 5.66 seconds; n = 4; p = 0.540, both were not significant reduction compared with N/OFQ group. Meanwhile, application of NaCl did not cause any change of extracellular potential in the control group (**Fig. 2C**).

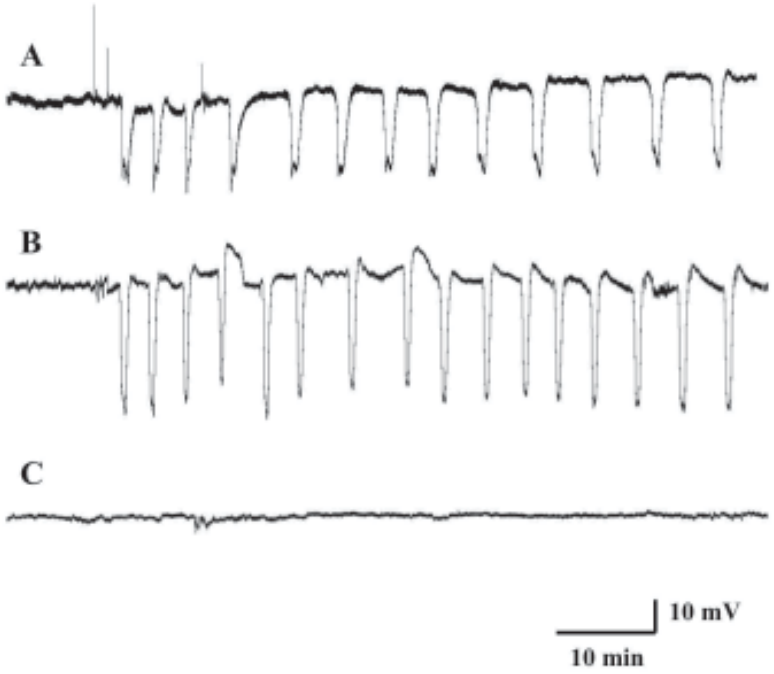

Fig. 2 Tracings of extracellular potential in the experiment. KCl application evoked repetitive depolarization shift in CSD group (**A**), electrophysiological variables of CSD were increased in N/OFQ-CSD group (**B**), and NaCl application did not evoke any change (**C**).

Table 1. Comparing the electrophysiological variables of CSD between CSD and N/OFQ-CSD groups.

	N/OFQ-CSD	CSD	P-value
Number of cycles	14.75 ± 0.24	12.33 ± 0.19	0.012
Amplitude (mV)	36.10 ± 0.37	28.07 ± 0.21	0.001
IL (seconds)	247.98 ± 11.06	307.38 ± 7.32	0.089
AUC (mV-second)	30.06 ± 2.49	21.44 ± 0.89	0.189
Duration (seconds)	68.76 ± 5.66	74.48 ± 6.58	0.540

Discussion

Repetitive CSD in cerebral cortex is a substantial method to study the cortical hyperexcitability during migraine auras [3, 16]. KCl application that was used for evoking CSD is especially developed to provide pathogenetic roles of N/OFQ in migraine attack. The protocol increased the cortical excitability, as it repeatedly surged the extracellular potential in CSD and N/OFQ-CSD groups (**Fig. 2A, 2B**). Typically, potassium existed within the extracellular compartment of the brain. Under certain KCl application, extracellular potassium increased rapidly 20- to 30-fold, similar to the intracellular compartment. Within the central nervous system, neurons are affected by shifts in the extracellular concentration of potassium. Elevated extracellular potassium can equalize the primary chemical force, and lead to augment depolarization by electrical force based on potassium influx. CSD redistributes not only potassium ion, but also other ions (calcium, sodium, chloride, etc.) within the cellular compartment [17].

N/OFQ is signaling molecule that interacts with ORL-1 receptor located both pre- and postsynaptic terminal. The activation of ORL-1 receptor produces the opening of G-protein-activated inward rectifying K^+ (GIRK) channel [18]. It has been recently reported that N/OFQ is involved extensively in central nervous system pathways, including suppression of the neurotransmission [19, 20]. These findings suggest that the effect of N/OFQ is mediated via the decrease of presynaptic neurotransmitter release or postsynaptic receptor activation [21]. However, the results of the present study showed the enhancing effect of N/OFQ in CSD elicited by KCl application (**Fig. 2B**). The electrophysiological variables that indicate the induction and propagation of CSD, represented by number of cycles and peak amplitude, were significantly increased in the N/OFQ-CSD group (**Table 1**). Distinct cellular expressions may explicate the discrepancy of N/OFQ function. In the cerebral cortex, ORL-1 receptor is expressed ubiquitously in presumptive GABAergic interneuron (Golgi type II cells) located layer II [22]. These interneurons terminate axons mainly on a projective glutamatergic neuron (Golgi type I cell) located layer III. Considering that the activation of ORL-1 receptor induces inhibitory modulation, intrathecal administration of N/OFQ may reduce the GABA release from interneurons during CSD development and then the glutamate activation of adjacent projection neurons is enhanced accordingly. On the other hand, AUC and duration were not altered in the N/OFQ-CSD group. These results suggest that N/OFQ may not be included in the sustainment of CSD.

It has been demonstrated that migraine presents as an unstable trigeminovascular reflex with a fragmentary defect in the pain control pathway. The defect permits excessive discharge of the trigeminal nerve and its cortical connections via thalamus in response to excessive afferent input. Diffuse projections from the brain stem to the cerebral cortex initiate cortical hyperexcitability and possibly cortical spreading depression [23]. Therefore, excitability in the trigeminal system can account for the migraine aura. Regarding trigeminovascular system, both N/OFQ and ORL-1 receptor are expressed in high concentration in the central trigeminovascular neurons, which are located in trigeminal nucleus caudalis of the brainstem and in C1 and C2 regions of the spinal cord [13, 22]. The results in this study suggest that N/OFQ might increase the pulsating nature of the headache resulting from the excitation and sensitization of trigeminal system. Further studies for the effect of nociceptin on trigeminal system in CSD model are desirable.

Conclusion

The development of CSD is significantly enhanced by N/OFQ administration. N/OFQ may play an important role in migraine attack. The ORL-1 receptor

could be the target of pharmaceutical treatment of migraine.

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The authors have no conflict of interest to report.

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Cortical hyperexcitability and mechanism of medication-overuse headache

Weera Supornsilpchai, Supang Maneesri le Grand and Anan Srikiatkhachorn

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Cortical hyperexcitability and mechanism of medication-overuse headache

Weera Supornsilpchai[1], Supang Maneesri le Grand[2] and Anan Srikiatkhachorn[1]

Abstract

The present study was conducted to determine the effect of acute (1 h) and chronic (daily dose for 30 days) paracetamol administration on the development of cortical spreading depression (CSD), CSD-evoked cortical hyperaemia and CSD-induced Fos expression in cerebral cortex and trigeminal nucleus caudalis (TNC). Paracetamol (200 mg/kg body weight, intraperitonealy) was administered to Wistar rats. CSD was elicited by topical application of solid KCl. Electrocorticogram and cortical blood flow were recorded. Results revealed that acute paracetamol administration substantially decreased the number of Fos-immunoreactive cells in the parietal cortex and TNC without causing change in CSD frequency. On the other hand, chronic paracetamol administration led to an increase in CSD frequency as well as CSD-evoked Fos expression in parietal cortex and TNC, indicating an increase in cortical excitability and facilitation of trigeminal nociception. Alteration of cortical excitability which leads to an increased susceptibility of CSD development can be a possible mechanism underlying medication-overuse headache.

Keywords

paracetamol, cortical spreading depression, medication-overuse headache, trigeminal nociception, cortical excitability

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Introduction

Cortical spreading depression (CSD) is an electrical phenomenon of cerebral cortex which underlies the development of migraine aura. Accumulating evidence indicates the relationship between CSD development and vulnerability to headache attacks. For instance, Ayata and colleagues (1) showed that various migraine preventive agents, regardless of their formulae and therapeutic mechanisms, share the similar effect in reducing the frequency of CSD in experimental animals. They also demonstrated the relationship between degree of CSD suppression and dose and duration of treatment. On the other hand, the increase in cortical susceptibility to develop CSD was reported in serotonin (5-HT)-depleted animals (2). This finding may explain an increase in headache frequency in chronic daily headache, the condition in which 5-HT level is low (3). The reduction in threshold for the induction of CSD was reported in female mice (4). The gender difference in cortical excitability may contribute to an increased prevalence of migraine in women.

Analgesic over-consumption is known to worsen headache in patients with primary headaches – either migraine or tension-type headache. A survey in a tertiary headache centre in the US showed that overused substances included butalbital containing combination products, paracetamol, opioids, aspirin, ergotamine tartrate, and various triptans (5). The mechanism underlying this phenomenon is not well defined. Possible explanations include the sensitisation of central trigeminal nociceptive neurones, derangement of endogenous pain control pathway, etc. Based on the relationship between CSD development and clinical headache described above, it is possible that chronic analgesic exposure may cause headache deterioration by increasing the cortical excitability which leads to an increased susceptibility of CSD development.

The objectives of this study were to determine the effect of acute and chronic analgesic exposure on the development of CSD and CSD-evoked

[1]Department of Physiology, Faculty of Medicine, Chulalongkorn University, Patumwan, Bangkok, Thailand.
[2]Department of Pathology, Faculty of Medicine, Chulalongkorn University, Patumwan, Bangkok, Thailand.

Corresponding author:
Anan Srikiatkhachorn MD, Department of Physiology, Faculty of Medicine, Chulalongkorn University, Rama IV Rd, Patumwan, 10330 Bangkok, Thailand. Email: anan.s@chula.ac.th

trigeminal nociception. Paracetamol was chosen for the study because this drug is widely used among patients with chronic headaches. In addition, since the change in vascular compartment is also important in the process of headache pathogenesis, the effect of analgesic administration on the CSD-evoked cortical hyperaemia was also investigated.

Materials and methods

Animals and drug treatment

Adult, male, Wistar rats weighing 200–250 g (National Animal Centre, Mahidol University, Thailand) were housed and maintained on normal rat food and tap water *ad libitum* under controlled environmental conditions. The study protocol was approved by the Ethical Committee of Faculty of Medicine, Chulalongkorn University (003/2551).

The study had two components. The first part was to determine the acute effect of paracetamol on CSD development. In this experiment, rats were divided into paracetamol-treated and control groups (10 rats each). A single dose of paracetamol (200 mg/kg body weight, intraperitoneally) was given to the treatment group whereas vehicle (12.5% of 1,2-propanediol in 0.9% sterile saline) of the same volume was given to the control. CSD was elicited at 1 h after paracetamol injection. The second experiment aimed at investigating the effect of prolonged paracetamol exposure on CSD development and CSD-evoked trigeminal nociception. Rats were divided into paracetamol-treated and control groups (10 rats each). Paracetamol (200 mg/kg body weight, intraperitoneally) or vehicle was administered once daily for the period of 30 days. Twenty-four hours after the last injection, animals were prepared for CSD induction.

In both experiments, CSD was induced in the parietal cortex by the topical KCl application method. Electrocorticograms were recorded continuously for 1 h using a glass micro-electrode. Cortical blood flow was monitored in frontal area using laser Doppler flowmetry. Two hours after CSD induction, rats were humanely killed. Brain and upper cervical cord were removed for Fos immunohistochemical study. The liver was also removed for histopathological examination using the standard haematoxylin and eosin method to exclude the hepatotoxicity induced by paracetamol. The presence of centrilobular or panacinar necrosis and sinusoidal congestion were used to indicate the paracetamol-induced hepatotoxicity.

Induction of CSD

Rats were anaesthetised with sodium pentobarbital (60 mg/kg body weight, intraperitoneally). Additional doses were given as required to maintain surgical anaesthesia based on testing of corneal reflex and response to tail pinch. Ventilation was assisted by a positive-pressure ventilator (rodent ventilator model 683, Harvard Apparatus, Holloston, Massachusetts, USA) via a tracheotomy opening. Arterial blood pressure was continuously recorded using a pressure transducer (Nihon model TP-300T, Nihon Khoden, Tokyo, Japan) inserted into the femoral artery. Rats were firmly positioned in a stereotaxic frame. A midline surgical incision was done and skin and soft tissue overlying the skull were removed. A 2-mm craniotomy was performed with the saline-cooled drill on the parietal bone (7 mm posterior and 1 mm lateral to bregma). Dura mater was carefully opened by microneedle to expose the cortical surface. Artificial cerebrospinal fluid (NaCl 118 mM, KCl 4 mM, $Na_2HPO_4.H_2O$ 1 mM, $NaHCO_3$ 25 mM, $CaCl_2.2H_2O$ 1.5 mM, $MgSO_4.7H_2O$ 1.2 mM, dextrose 5 mM in distilled water, pH 7.4, 37°C) was superfused to prevent the cortical surface from dryness. After all operation procedures had been completed, a single 3-mg KCl crystal was placed directly on the surface of parietal cortex to generate the CSD and was left on the cortical surface for the entire experimental period. This technique can generate a series of unifocal CSD. To avoid the variation in the CSD induction method, the amount of KCl, size of craniotomy well and amount of artificial cerebrospinal fluid were strictly controlled.

Electrocorticographic recording

To record cortical activity, another craniotomy (diameter 5 mm) was done at 1 mm anterior and 1 mm lateral to bregma. Microelectrode (internal diameter 5 μm) was prepared from bromosilicate glass, pulled with micropipette puller (Industrial Science Associates, Inc., Forest, Ridgewood, New York, USA). The micro-electrode was filled with NaCl (4 M) and then an Ag/AgCl wire was inserted. The completely filled glass micro-electrode was inserted perpendicular to the cortex to the depth of 500 μm from the cortical surface using an hydraulic micromanipulator (Narishige, Scientific Instrument Lab., Tokyo, Japan). An Ag/AgCl reference electrode was placed on the skin at the back. The obtained electrical signal was amplified using a micro-electrode amplifier (P16B; Grass Instrument, Quincy, Massachusetts, USA). The frequency band of electrophysiological acquisition was between 1–10 kHz. Due to the very high signal-to-noise ratio, the data filtration process was not necessary. Analogue data were converted to digital form using data acquisition system (PowerLab 4SP; Bella Vista, NSW, Australia). All tracings were analysed using PowerLab computer software (PowerLab 4SP). Measured variables included amplitude and area under the curve (AUC) of each CSD

wave as well as the number of CSD waves occurring within a 1-h period.

Cortical blood flow recording

In order to monitor the CSD-evoked change in the cortical blood flow, the fibre-optic needle probe of a laser Doppler flowmeter (ALF 21; Advance Co. Ltd, Tokyo, Japan) was placed adjacent to the glass micro-electrode. The probe was inserted perpendicularly at a distance of 2 mm above the cortical surface. The wavelength of the laser beam was 780 nm. Data were recorded on a polygraph (Nihon RM 6000; Nihon Khoden, Tokyo, Japan). Peak amplitude of each hyperaemic cycle was calculated as percentage change from baseline value.

Fos immunohistochemical study

After completion of cortical activity and blood flow recording, rats were humanely killed by excessive dose of sodium pentobarbital and perfused transcardially with 250 ml of phosphate buffer, followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Brain and cervical spinal cord were removed and immediately immersed in 4% paraformaldehyde in 0.1 M phosphate buffer. After overnight fixation, tissue was placed in a cryoprotectant solution (30% sucrose in 0.1 M phosphate buffer, pH 7.4) before being cut with a cryomicrotome at –20°C. Somatosensory cortex was identified according to the co-ordinates described by Paxinos and Watson. [6] and was coronally sectioned (30 μm thick). The caudal medulla (3 mm. caudal to the obex) to the first cervical cord was transversely cut and one in every five sections was collected. Sections were kept in cold 0.01 M PBS. All sections were incubated with 3% hydrogen peroxide in 50% ethanol for 30 min to minimise endogenous peroxidation. After repeated rinses in PBS, the sections were pre-incubated in PBS containing 3% normal horse serum, 1% bovine serum albumin for 60 min at room temperature and incubated overnight with rabbit anti-Fos (Santa Cruz Biotechology; (1:500 dilutions)). They were then incubated for 30 min with biotinylated goat anti-rabbit antiserum (Dako LSAB 2 system, Glostrup, Denmark) and rinsed again in PBS before incubating for 30 min in a streptavidin-horseradish peroxidase solution (Dako LSAB 2 system, Glostrup, Denmark). Bound peroxidase was revealed by incubating all sections in a solution containing 0.05% 3,3-diaminobenzidine, 0.005% hydrogen peroxide for 10 min. The reaction was stopped by repeated rinses in PBS. Sections were mounted on gelatinised slides, dehydrated in a graded series of ethanol, mounted, cover-slipped with Permount, and examined with a light microscope.

The number of Fos-immunoreactive (Fos-ir) cells was determined using image analysis software (ImagePro® Plus; Media Cybernetics Inc., Bethesda, Maryland, USA). To determine the number of Fos-ir cells in somatosensory cortex, a 250×250 μm square was drawn in layer V of the parasaggital part of this cortex and Fos-ir cells confined in the square were counted. Data from 10 areas sampled from each section (10 sections per rat) were averaged and expressed as number per 6.25×10^4 μm^2. Expression of Fos in TNC was quantitated by counting Fos-ir neurones in lamina I and II of TNC from 10 sections of the cervical spinal cord and 10 sections from caudal medulla. The data were averaged and expressed as number per section. The identity of each rat was concealed throughout the counting process.

Statistical analysis

All cortical activity variables as well as numbers of Fos-ir neurones are expressed as mean ± SD. Possible statistically significant differences between paracetamol-treated and control groups was determined by Mann–Whitney U-test. Probability values of less than 0.05 were considered to be statistically significant.

Results

Exposure to paracetamol, acute and chronic, did not alter animal behaviour, including feeding as shown by the comparable body weights between the two groups. The averaged body weight of the chronic paracetamol-treated and control groups were 410 ± 20 g and 392 ± 29 g, respectively. The histological examination of liver was normal. There was no change in hepatocyte morphology or evidence of inflammation was observed.

Effect of acute paracetamol exposure

Effect on cerebral cortex. Cortical KCl application elicited a series of cortical depolarisations characterizing the CSD. Comparison between the two groups, showed the number of CSD waves generated in the first hour was similar (13.1 ± 1.1 and 13.7 ± 1.5 peak for control and paracetamol treated groups, respectively; $P = 0.332$). Despite the similarity in the frequency of CSD occurrence, the feature of individual DC shift observed in the control and paracetamol groups was different. The amplitude of DC shift was significantly greater in the paracetamol-treated group (31.5 ± 2.3 mV) as compared to the control (28.1 ± 3.3 mV; $P = 0.001$). Minimal change in CSD amplitude over time was observed with larger amplitude in the early waves. However, the difference was

not statistically significant. The AUC of the DC shift was also greater in the paracetamol-treated group. The AUCs of the DC shift were 93.8 ± 24.7 and 73.4 ± 18.5 mV-s, for the paracetamol-treated and control groups, respectively; $P = 0.006$). The magnitude of increase was 12.8% from the baseline value (Figure 1 and Table 1). No significant difference was observed when the durations of DC shift between acute paracetamol and control were compared.

The cortical blood flow study revealed the cyclical hyperaemia which perfectly corresponded to the cycle of CSD. The rise in cortical blood flow usually occurred prior to the DC shift. Pretreatment with paracetamol lowered the degree of hyperaemia induced by CSD. The peak amplitude of CSD-evoked cortical hyperaemia observed in the paracetamol-treated and control groups were $185 \pm 37\%$ and $222 \pm 62\%$ from the baseline value, respectively. The difference between paracetamol-treated and control groups was statistically significant ($P = 0.001$).

The anti-Fos immunohistochemical study of cerebral cortex showed high density of Fos-ir neurones in the hemisphere ipsilateral to side of CSD induction. Only a few Fos-ir neurones were observed in the non-stimulated contralateral hemisphere. Pretreatment with paracetamol can reduce the density of CSD-evoked Fos expression in cerebral cortex. This reduction was more prominent in layer V. The density of Fos-ir cells in layer V in the paracetamol and control groups were 54.2 ± 16.1 and 113.9 ± 15.2 cells per $6.25 \times 10^4\,\mu m^2$, respectively ($P = 0.026$; Figure 2 and Table 1). The Fos-ir cells were also visualised in amygdala, dorsal raphe, thalamus, and hypothalamus (especially paraventricular nucleus). There was no significant difference between control and paracetamol groups.

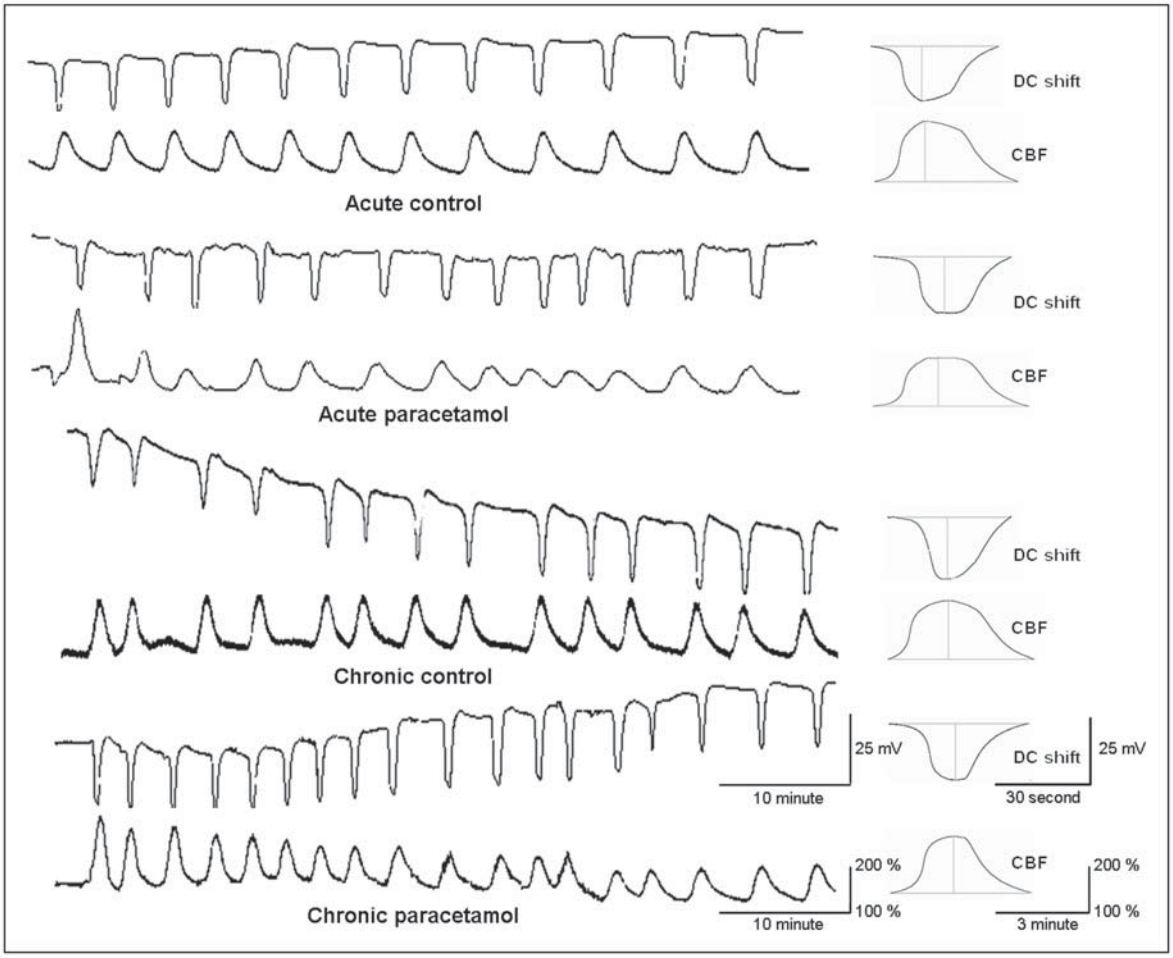

Figure 1. Effect of acute and chronic paracetamol administration on development of CSD (upper trace) and CSD-evoked cortical hyperaemia (lower trace). Acute paracetamol exposure leads to an increase in CSD amplitude and area-under-the curve without change in frequency (upper two panels). On the other hand, chronic exposure results in an increase in CSD frequency (lower two panels). A decrease in CSD-evoked cortical hyperaemia is observed in both acute and chronic paracetamol groups compared with the respective controls.

Effect on CSD-evoked trigeminal nociception. Application of KCl increased Fos expression in laminar I and II of the TNC especially in the ipsilateral side. The average numbers of Fos-ir cells in ipsilateral and contralateral TNC were 10.5 ± 2.2 and 4.9 ± 2.1 cells per slide, respectively. Pretreatment with paracetamol reduced the number of CSD-evoked Fos-ir neurones in both sides of TNC. The numbers of Fos-ir cells in the ipsilateral and contralateral TNC in the acute paracetamol treated group were 5.0 ± 2.3 and 2.4 ± 1.1 cells per slide, respectively. The difference between the paracetamol and control groups was statistically significant in both sides ($P = 0.001$; Figure 3).

Table 1. The effect of acute and paracetamol administration on the development of CSD and CSD-evoked Fos expression in the cerebral cortex and TNC

Measure variables	Acute			Chronic		
	Control ($n = 10$)	Paracetamol ($n = 10$)	*P*-value	Control ($n = 10$)	Paracetamol ($n = 10$)	*P*-value
Electrocorticographic variables						
Number of peak in first hour (range)	13.1 ± 1.1 (11–14)	13.7 ± 1.5 (12–15)	0.332	12.5 ± 1.3 (12–14)	15.7 ± 1.5 (14–18)	0.032
Amplitude (mV) (range)	28.1 ± 3.3 (26.0–30.5)	31.5 ± 2.3 (28.2–34.1)	0.001	27.3 ± 2.9 (25.1–31.7)	29.7 ± 3.4 (26.8–35.5)	0.100
Duration (s)	41.1 ± 8.5	44.0 ± 8.5	1.362	36.8 ± 6.2	33.5 ± 7.4	0.060
Area under the curve (mV-s)	73.4 ± 18.5	93.8 ± 24.7	0.016	70.0 ± 19.8	65.4 ± 16.8	0.355
CSD-evoked cortical hyperaemia						
Amplitude (% change from baseline)	222 ± 62	185 ± 37	0.001	244 ± 43	199 ± 49	0.001
CSD-evoked Fos expression						
Ipsilateral cortex (cells per $6.25 \times 10^4\ \mu m^2$)	75.1 ± 18.7	54.2 ± 16.1	0.026	79.0 ± 17.7	113.9 ± 15.2	0.001
Ipsilateral TNC (cells per slide)	10.5 ± 2.2	5.0 ± 2.4	>0.001	11.5 ± 2.8	16.4 ± 2.9	0.004
Contralateral TNC (cells per slide)	4.9 ± 2.1	2.4 ± 1.0	>0.001	7.4 ± 3.1	10.2 ± 3.2	0.015

All variables were expressed as mean $\pm$ SD.

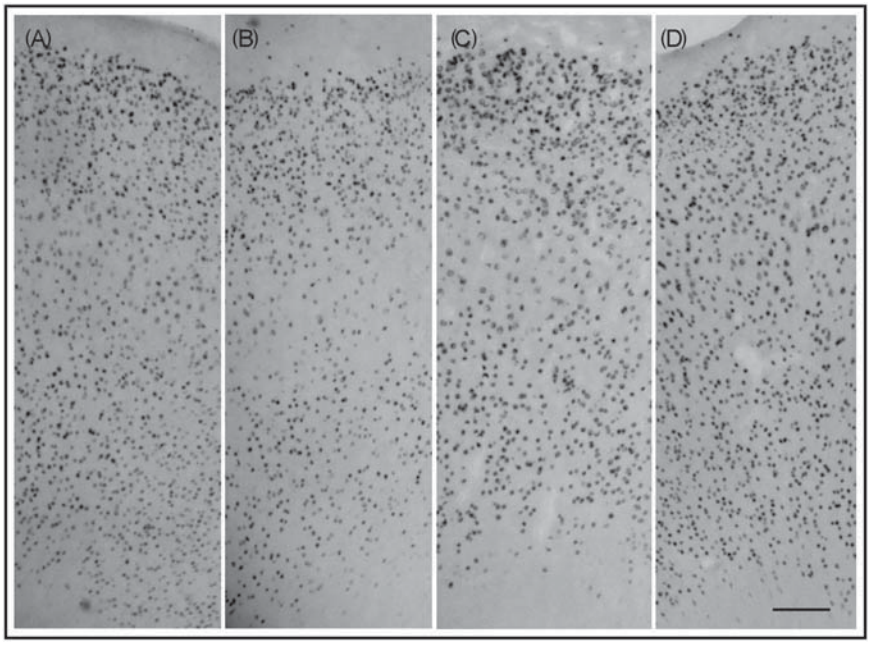

Figure 2. Effect of acute and chronic paracetamol administration on CSD-evoked Fos expression in cerebral cortex. Acute treatment with paracetamol decreases the number of Fos-immunoreactive cells in layer 3 while chronic exposure increases CSD-evoked Fos expression. (A) Acute control; (B) acute paracetamol; (C) chronic control; (D) chronic paracetamol. Bar = 250 μm.

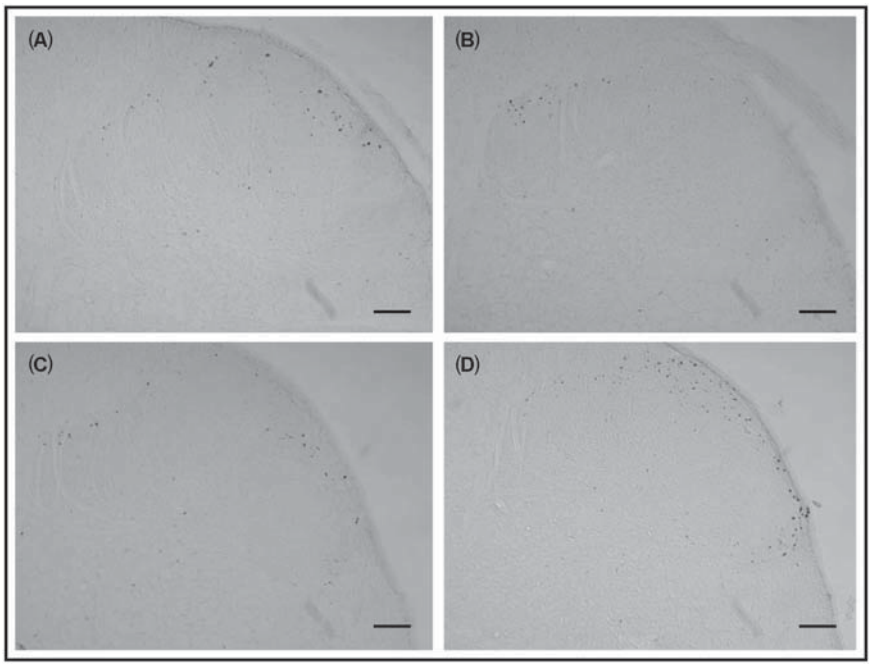

Figure 3. Effect of acute and chronic paracetamol administration on CSD-evoked Fos expression in trigeminal nucleus caudalis. Acute treatment with paracetamol minimises the number of Fos-immunoreactive cells in the TNC, reflecting the anti-nociceptive effect. Conversely, chronic treatment enhances the CSD-evoked nociception as shown by an increase in the number of Fos-immunoreactive cells in the TNC. (A) Acute control; (B) acute paracetamol; (C) chronic control; (D) chronic paracetamol. Bar = 250 μm.

Effect of chronic paracetamol exposure

Effect on CSD and Fos-immunoreactivity in cerebral cortex. Chronic exposure to paracetamol increased the frequency of CSD waves. The number of CSD waves observed in 1 h from chronic paracetamol treated group and the control group were 15.8 ± 1.5 and $12.5 \pm +1.3$ peak per hour, respectively ($P = 0.032$). Unlike those observed in the acute treatment group, neither change in peak amplitude nor AUC was evident between chronic paracetamol-treated and control groups. The average amplitude and AUC of CSD waves from the chronic paracetamol group were 29.76 ± 3.38 mV and 65.38 ± 16.77 mV-s, respectively, while those of the control group were 27.30 ± 2.98 mV and 70.01 ± 19.84 mV-s, respectively ($P = 0.100$ and $P = 0.355$ for peak amplitude and AUC, respectively). The increase in CSD frequency was parallel to the increase in the density of Fos-ir cells in cerebral cortex. The number of Fos-ir cells in the chronic paracetamol and control groups were 113.9 ± 15.2 and 79.0 ± 17.7 per $6.25 \times 10^4\ \mu m^2$, respectively ($P = 0.001$; Figure 1 and Table 1).

Similar to results observed in the acute paracetamol experiment, the cortical blood flow study showed significant lower degree of CSD-induced hyperaemia in the animals receiving chronic paracetamol administration. The peak amplitude of CSD-evoked hyperaemia in the chronic paracetamol and control groups were $199 \pm 49\%$ and $244 \pm 43\%$ from baseline value, respectively ($P = 0.010$).

Effect on CSD-evoked trigeminal nociception. In the chronic paracetamol treated group, the average number of Fos-ir cells in the ipsilateral and contralateral sides were 16.4 ± 2.9 and 10.2 ± 3.2 cells per slide, respectively. The numbers of Fos-ir cells in the ipsilateral and contralateral TNC in the control group were 11.5 ± 2.8 and 7.4 ± 3.1 cells per slide, respectively. The number of Fos-ir cells in the chronic paracetamol treated group was significantly higher than control group (*P*-values 0.04 and 0.015 for ipsilateral and contralateral sides, respectively; Figure 3).

Discussion

The present study shows that the trigeminal system responds differently to acute and chronic analgesic exposure. Short-term exposure to paracetamol reduces the number of CSD-evoked Fos-ir neurons in the TNC without alteration in CSD frequency. On the other hand, chronic exposure leads to an increase in CSD frequency and CSD-evoked Fos expression in the cerebral cortex and the TNC. Concerning cortical blood flow, paracetamol reduces the amplitude of CSD-evoked cortical hyperaemia regardless of the duration of exposure.

CSD is believed to be the physiological mechanism underlying the aura phase of migraine. Changes in CSD frequency may reflect the deterioration in the excitability of cerebral cortex and susceptibility to develop migraine attacks. Several factors have been reported to affect the frequency of CSD waves. In general, factors or conditions that reduce neuronal excitability will reduce the frequency of CSD while those which increase the excitability will increase the CSD frequency. For instance, CSD frequency was reduced in rats treated with anaesthetics such as halothane, isofurane and sevoflurane (7). N_2O can reduce CSD frequency when combined with isofurane or urethane, but not α-chloralose(8). Reduction in CSD frequency has also been observed in animals chronically treated with various classes of anti-migraine preventive medications (1). On the other hand, the high frequency of CSD as well as the increase in the trigeminal nociception has been reported in animal with low serotonin (2). Several lines of evidence indicate that the development of CSD can affect the synaptic transmission in cerebral cortex. Using an *ex vivo* brain slice technique, Wernsmann et al. (9) demonstrated that tetanus-induced long-term potentiation was significantly enhanced in hippocampal slices obtained from the ipsilateral site to the hemisphere in which CSD was evoked. A more recent study with a human brain slice model also showed that the induction of CSD significantly increased the amplitude of field excitatory post-synaptic potential and increased the induction of LTP in the third layer of neocortical tissues (10). These findings are compatible with our observation of an increase in CSD-evoked Fos expression in cerebral cortex especially in those with greater numbers of CSD waves. All this evidence indicates the facilitating effect of CSD on synaptic excitability which may contribute to neocortical hyperexcitability. Extending this hypothesis, an increase in the number of CSD waves occurring in the prolonged paracetamol-treated group may further enhance the excitability of the cortex, resulting in the cortical hyperexcitability state.

Besides frequency, changes in other CSD variables have also been reported. The reduction in threshold and increases in propagation velocity of CSD have been demonstrated in R192Q knock-in mice, an animal model for familial hemiplegic migraine (11). The recent study by Tong and Chesler (12) has revealed that acidosis can increase the threshold for CSD induction but decrease the duration and velocity of CSD. Oestrogen and progesterone have been reported to enhance the repetition rate as well as the amplitude of spreading depression in neocortical slices treated with hypotonic artificial cerebrospinal fluid or KCl micro-injection (13). In this study, we observed an increase in the amplitude of CSD without frequency change in rats treated with a single dose of paracetamol. The increase in CSD amplitude disappeared in rats with chronic paracetamol administration. Although the mechanism by which acute paracetamol exposure increases the CSD amplitude remains unclear, alteration in astrocyte functions is a possible explanation. It is known that astrocytes play an important role in the regulation of extracellular calcium and potassium as well as transmitters, especially glutamate, during CSD. Therefore, derangement in astrocyte function can alter the cortical excitability. The mutation in the Na^+/K^+-ATPase pump gene ATP1A2 expressed in astrocytes has been reported to associate with familial hemiplegic migraine (14). Effects of paracetamol on some astrocyte functions have been reported. For instance, Mancini et al. (15) demonstrated that paracetamol can down-regulate the nuclear translocation of nuclear factor-kappa B and inhibits prostaglandin E_2 production. However, information concerning the effect of paracetamol on extracellular ion regulation of astrocytes is still unavailable.

In this study, we observed the dissociation between amplitude of CSD and degree of cortical hyperaemia. In the acute group, the increase in CSD amplitude co-existed with the decreased degree of CSD-induced cortical hyperaemia. The decrease in CSD-evoked cortical hyperaemia was also evident in chronically treated animals without changes in CSD amplitude. The alteration in CSD-evoked cortical hyperaemia may be the result of a direct effect of paracetamol on cerebral circulation. The mechanism underlying the CSD-evoked cortical hyperaemia is complex and involves several chemical messengers (16). Some of these messengers can be affected by paracetamol. For instance, it has been shown that paracetamol can affect cerebral endothelial cells causing the inhibition of prostaglandin production (17). Anti-oxidant and anti-inflammatory effects of paracetamol on the cerebral vasculature have also been demonstrated [18). These effects may alter cerebrovascular motor tone and diminish the degree of cortical hyperaemia evoked by CSD.

The present findings lead to two further questions, what is the mechanism underlying these changes and whether these observations can be generalised to other analgesics. Recently, Mallet et al. (19) have suggested roles for the endocannabinoid and serotonin systems in the anti-nociceptive mechanism of paracetamol. They proposed that the analgesic effect of paracetamol is mediated via the action of its metabolite AM404 upon the CB(1) receptors. Activation of the endocannabinoid system will reinforce the serotonergic bulbospinal pathways and attenuate the nociceptive process in the spinal cord. Serotonin also plays a role in paracetamol-induced analgesia by increasing transcript and protein levels of low-affinity neurotrophin

receptor, insulin-like growth factor-1 (IGF-1) receptor alpha subunit, and growth hormone receptor. This neurotransmitter can reduce the amount of somatostatin receptor (sst3R) mRNA via the action of spinal 5-HT_{1A} receptors (20). The involvement of serotonin in paracetamol-induced analgesia is supported by the observation that pretreatment with serotonin receptor antagonists can attenuate the analgesia induced by paracetamol (21). This serotonin-dependent nociceptive inhibition is also evident in humans. In 2006, Pickering et al. (22) showed that co-administration of paracetamol with tropisetron or granisetron, 5-HT_3 antagonists, completely blocked the analgesic effect of paracetamol in humans. It has been proposed that paracetamol may act centrally by reinforcing the descending inhibitory pain pathway (23). Our previous experiments also support the role of serotonin in the analgesic mechanism of this agent. Acute exposure to paracetamol increases 5-HT levels and down-regulates the pro-nociceptive 5-HT_{2A} receptor whilst chronic exposure decreases the 5-HT level and up-regulates this receptor (24). The reverse relationship between 5-HT level and its receptors reflects the fact that receptor up-regulation may be secondary to the reduction in the transmitter level. Since activation of 5-HT_{2A} receptor leads to an increase in neuronal excitability, an up-regulation of this receptor in cerebral cortex may increase the cortical neuronal response. This mechanism may explain the increased CSD response and cortical Fos expression observed in rats following chronic treatment with paracetamol in this study. Of note, the alteration in 5-HT_{2A} receptor expression cannot explain the effect of paracetamol in attenuating the CSD-evoked cerebral hyperaemia. In addition to neural tissue, 5-HT_{2A} receptors are also expressed in cerebral vessels (25). This vascular receptor is likely to mediate the vasodilator effect (26). Therefore, up-regulation of this receptor would increase cerebral blood flow rather than decrease it, as shown in this study. To our knowledge, there is no study investigating the effect of paracetamol on vascular 5-HT_{2A} receptor expression.

Concerning the issue of generalisability, clinical observations show that medication-overuse headache can result from the prolonged use of analgesics, ergots or triptans. The medication-overuse headaches share common clinical features regardless of the variety of its causative agents. This observation suggests that all drugs may share a common mechanism. This hypothesis is supported by the observation that chronic exposure to triptans also alters the central serotonin-dependent pain controlling system (26). Therefore, it is possible that chronic use of other acute anti-migraine medications may increase cortical excitability as observed in rats with prolonged paracetamol exposure. It should be noted that, although paracetamol has been reported as a common drug used by chronic headache sufferers, it is mostly used in combination with other compounds such as opioid analgesics. Therefore, more studies with other classes of medication are needed to confirm this hypothesis.

Conclusions

The present study demonstrates the plasticity of the cerebral cortex and trigeminal nociceptive system in response to acute and chronic analgesic exposure. While acute exposure elicits the anti-nociceptive effect, chronic administration of analgesics increases the excitability of cerebral cortex as well as facilitating the trigeminal nociceptive mechanism. The temporal profile resembles the clinical features of chronic headache observed in patients with analgesic-induced headache. Therefore, neuronal hyperexcitability may explain the development of chronic headache in these patients.

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Original article

Effect of serotonin depletion on cortical spreading depression evoked cerebrovascular changes

Supang Maneesri[a], Weera Supornsilpchai[b], Chonlawan Saengjaroentham[a], Juntima Pleumsamran[c], Anan Srikiatkhachorn[d]

[a]Department of Pathology , Faculty of Medicine, Chulalongkorn University, Bangkok 10330, [b]Department of Physiology, Faculty of Dentistry, Chulalongkorn University, Bangkok 10330, [c]Department of Physiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50000, Thailand. [d]Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330

Background: The cortical spreading depression (CSD) is a phenomenon associated with several pathological conditions including migraine. It can induce alterations in both neural and vascular compartments. Serotonin (5-HT) depletion is known as a condition involved in migraine pathophysiology. The hyper-excitability of the cortical neurons to the CSD activation in the low 5-HT state has been previously reported. However, the cerebrovascular responses to CSD activation in this condition have never been studied yet.

Objectives: Determine the effect of 5-HT depletion on the cerebrovascular responses to CSD activation.

Methods: Wistar rats (weighing 250-300 grams) were divided into three groups: control, CSD, and low 5-HT with CSD group (five rats per group). To induce the low 5-HT state, the para-chlorophenylalanine was injected intraperitoneally into the rats three days before the experiment. CSD was induced by the application of solid KCl (3 mg) on the parietal cortex. NaCl instead of KCl was applied to the control group. Cerebral cortical blood flow was monitored using Laser Doppler flowmetry. The ultrastructure of cerebral microvessels was examined using electron microscopy to determine the cerebral microcirculatory responses to CSD.

Results: Depletion of serotonin induced a significant increase in the peak amplitude of CSD-evoked cerebral hyperaemia. This condition also enhanced the development of CSD-induced endothelial pinocytosis and microvillus formation in cerebrocortical microvessels.

Conclusion: 5-HT was an important neurotransmitter involved in the control of cerebrovascular responses to CSD activation. The hypersensitivity of the cerebrovascular responses observed in the 5-HT depleted state may explain the relationship between headache and 5-HT depletion.

Keywords: Cerebral blood flow, cortical spreading depression, endothelial cell, low serotonin, ultrastructural changes

The cortical spreading depression (CSD) is a depolarization wave moving across the surface of the cerebral cortex at the speed of 2-5mm/minute [1]. CSD is accompanied by series changes of the neurovascular responses. This includes the marked alteration of the ions homeostasis, the release of several neurotransmitters and the short-lasting increase in regional cerebral blood flow [2-5].

Many studies have indicated that CSD can occur following several pathological conditions such as head trauma, stroke, exacerbates brain injuries and migraine. In migraine, several lines of evidence have indicated the tight association between CSD and the aura phase. This is the phenomenon happening prior to the headache phase in migraine with aura [6, 7]. Based on the CSD theory, CSD activation can induce the release of proinflammatory neuropeptides, which later provokes a neurogenic inflammation. This inflammation could activate the sensory C-fiber of the trigeminal system and finally cause pain [8]. Several neurotransmitters are involved in the pathway of this pain process. Among them, serotonin (5-HT) seems to be the important neurotransmitter involved in both mediating and modulating this process. The

Correspondences to: Dr. Supang Maneesri le Grand, Department of Pathology, Faculty of Medicine, Chulalongkorn University, Bangkok 1330, Thailand. E-mail: le.grand.maneesri.s@gmail.com

involvement of 5-HT in the migraine pathophysiology has been evident by the results of several studies that indicated the abnormality of the serotonin system in migraine patients. The decrease in the level of 5-HT in the brain, plasma, and platelets has been observed in these patients [9-11].

In the brain, 5-HT is a neurotransmitter involved in the control of both the neuronal function and the cerebral circulation. Therefore, the abnormality of neuronal and cerebrovascular responses could be expected in the condition with low 5-HT.

Previously, we have demonstrated that the low level of 5-HT could induce the hyper-excitability of the cortical neurons after CSD activation [12]. With the application of KCl (3 mg), the rats with low 5-HT could develop the CSD wave in the higher frequency than the rats of the control group. However, the effect of low 5-HT on the cerebrovascular responses to the CSD has never been studied yet. To clarify this point, we studied the effect of CSD on the alteration of cerebral cortical blood flow (CBF) and the ultrastructure of cerebral microvessels in the rats with and without 5-HT depletion. The CBF was measured using Laser Doppler flowmetry, while the ultrastructural changes of the cerebral endothelial cells were examined using electron microscopy.

Materials and methods

Study design

All the protocols in this experiment were approved by the Chulalongkorn University Animal Care and Use Committee. Male Wistar rats weighing 250-300 grams were divided into three groups (five animals each) as follows: control, CSD and low 5-HT with CSD. They were housed in a light/dark cycle with light on from 6.00 to 18.00. Food and water was provided ad libitum.

To induce the low 5-HT condition, the tryptophan hydroxylase inhibitor, para-chlorophenyl alanine (PCPA), at the dosage of 100 mg /kg of bodyweight, was intra-peritoneally injected into rats three days prior to the experiment. This PCPA treatment can deplete the 5-HT in the brain to < 20% of the normal amount [13]. In the control group, instead of PCPA, the physiological saline of the same volume was given to the rats three days prior to the experiment.

To induce the CSD, 3 mg of solid potassium chloride was placed on the cerebrocortical surface of the rat to elicit CSD. Solid sodium chloride at the same weight was applied on the cortex of the control animals. The CBF was continuously monitored for 60 minutes using Laser Doppler flowmetry. After completion of the CBF recording, all rats were sacrificed using an excessive dose of pentobarbital. The brains were immediately removed. The ultrastructure of the cerebral microvessels was examined using electron microscopy.

Surgical operation and induction of CSD

The rats were anaesthetized with pentobarbital sodium (50 mg/kg body weight, intraperitoneally) and were mechanically ventilated by a positive pressure ventilator (Rodent ventilator model 683, Harvard Apparatus, South Natick, USA) via the tracheostomy opening. The blood pressure was monitored continuously with an intra-arterial pressure transducer (Nikhon model TP-300T, Nihon Khoden, Tokyo, Japan). It was placed in a femoral artery and recorded on a polygraph (Nikhon RM 6000, Nihon Khoden, Tokyo, Japan). Arterial blood was collected periodically for the determination of pH, PaO_2 and $PaCO_2$ by a pH/blood gas analyser (238 pH/blood gas analyzer, Ciba Corning Diagnostics, Essex, UK). The blood gas and pH were controlled in the physiologic range throughout the experiment.

After tracheostomy and cannulation, the rat was placed on a surgical frame with the head fixed to the head holder. Craniotomy (2 mm in diameter) was performed in the parietal bone at 7 mm posterior and 1 mm lateral to the bregma. The dura was opened to expose the cortical surface. Three milligram of solid KCl was placed directly on the surface of the parietal cortex. Sodium chloride was given to the rats of the control group.

CBF monitoring

To measure the cortical blood flow, an anterior craniotomy (7 mm in diameter) was performed in the frontal bone at 1 mm anterior and 1 mm lateral to the bregma. A glassless window was placed over the craniotomy opening. The cortical surface was prevented from drying and hypothermia by superfusion with artificial cerebrospinal fluid (NaCl 118 mM, KCl 4 mM, $Na_2HPO_4.H_2O$ 1 mM, $NaHCO_3$ 25 mM, $CaCl_2.2H_2O$ 1.5 mM, $MgSO_4.7H_2O$ 1.2 mM, dextrose 5 mM in distilled water, pH 7.4, 37^oC). The fiber optic needle probe of the Laser Doppler flowmeter (ALF 21, Advance, Tokyo, Japan) was placed perpendicularly with a distance of 2 mm above the cortical surface. The wavelength of the laser beam was 780 nm. The data were recorded and the

amplitude of each hyperemic peak was calculated as a percent change from the baseline value.

Ultrastructure examination

Portions of the frontal cortex were removed and cut into multiple cubes (1x1 mm). All specimens were immediately immersed in 2.5% glutaraldehyde for four hours and post-fixed with 1% osmium tetroxide. The tissues were dehydrated through graded series of ethanol. They were passed through two changes of propylene oxide, before being embedded in plastic media (Epon 812; Electron Microscopy Sciences, Ft. Washington, USA). After polymerization, semi-thin and ultrathin sections were cut using ultramicrotome with a glass knife. The semi-thin sections (0.5 μm thick) were stained with toluidine blue in order to select suitable sections for electron microscopy. The ultrathin sections (70-90 nm thick) were stained with uranyl acetate and lead citrate and were examined under the transmission electron microscope (JEM 1210; JEOL, Tokyo, Japan). The measured variables included the number of endothelial pinocytic vesicles and the number of microvilli.

Ten capillaries (diameter: 8-10 μm) and five small arterioles (diameter: 15-20 μm) per sample were selected. The number of microvilli was counted and reported as an average number of microvilli per vessel. To evaluate the alteration in pinocytic vesicle formation, two electron micrographs that covered the area of endothelial cells were taken from every capillary at the direct magnification of 20,000 times while four electron micrographs were taken at the same magnification from every selected arteriole.

A 200x200 nm square grid was fixed to the electron micrograph. Then, the number of endothelial pinocytic vesicles was counted and reported as an average number of pinocytic vesicles per μm^2.

Statistical analysis

All data were expressed as mean±standard deviation (SD), and were analyzed for possible statistical significance using ANOVA for repeated measurements with the post hoc Tukey test. The result of CBF was presented in percent change from the baseline value. The average of the peak amplitude of the hyperemic cycle was compared for possible statistically significant difference between groups using the Kruskal-Wallis method. Probability values of less than 0.05 were considered statistically significant.

Results

Effect of 5-HT depletion on CSD-evoked changes in CBF

The induction of CSD by application of KCl caused repeated cycles of cerebral hyperemia while the application of NaCl had no effect on the CBF. The average duration of these cycles was 4.0±0.5 minutes. The amplitude of each peak was calculated as a percent change from the baseline flow. The median number of the hyperemic cycles within one hour was 11 peaks (ranging from 10 to 13 peaks). Compared with the control group, the average amplitude of CSD-induced hyperemic waves in the low 5-HT group were higher, which was clearly observable starting from the first peak. In the low 5-HT group the average percent change from the baseline of CBF after CSD activation was 298±50%, which was significantly higher than those obtained from the group of normal 5-HT rats (140±70%). The latency between two subsequent peaks was not different (**Fig. 1** and **Table 1**).

Effect of 5-HT depletion on the CSD-evoked changes in the ultrastructure of the cerebral microvessels

The ultrastructural examination in both capillary and small arteriole revealed that CSD induced the ultrastructural changes of cerebral endothelial cells compared with the rats with NaCl application. The changes were characterized by an increased number of pinocytic vesicles and the formation of endothelial microvilli. An increase in pinocytic vesicles was observed both in luminal and abluminal surfaces. Furthermore, the ultrastructural changes were enhanced in the rats with 5-HT depletion. In capillaries, the average number of pinocytic vesicles was significantly increased from 27±10 vesicles per μm^2 in the CSD group to 45±12 vesicles per μm^2 in the 5-HT depleted rats with CSD activation. The average number of microvilli was increased from 1.4±1.3 microvilli/vessel in the CSD group to 3.7±1.3 microvilli/vessel in the low 5-HT with CSD group. The study in arteriole had revealed a similar result as observed in capillary. The average number of pinocytic vesicles and microvilli obtained from the arteriole in 5-HT depleted rats were significantly higher than those measured in the CSD group (**Fig. 2, 3**, and **Table 2**).

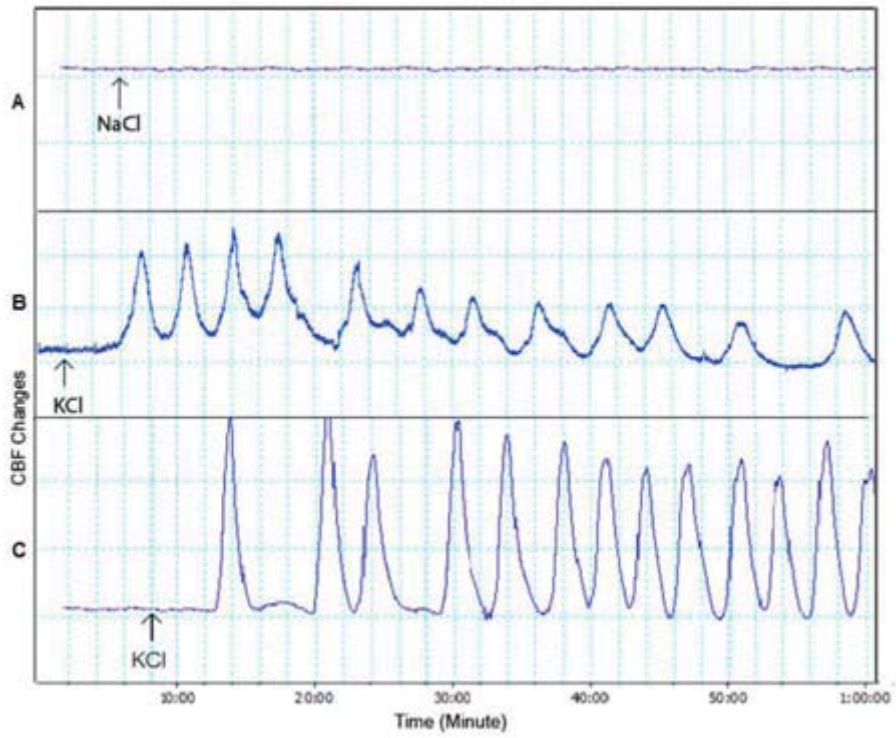

Fig. 1 The recording example of CBF changes after the application of 3 mg NaCl in the control group (**A**), the application of 3 mg KCl in CSD group (**B**) and the application of 3 mg KCl in low 5-HT group.

Table 1. The effect of 5-HT depletion on CSD-evoked hyperaemic changes of the CBF. The data were expressed as mean ± S.D.

Group	Average of % change from baseline
Control	0
CSD	140 ± 70
Low 5-HT with CSD	298 ± 50 *

*$p<0.01$ compared with CSD group.

Besides the alteration in the endothelial cell, the swelling of the perivascular astrocytic footplates was frequently demonstrated around the capillary obtained from the low 5-HT with the CSD group (**Fig. 2**). This abnormality was not observed in the control and CSD group.

Discussion

The present results demonstrate that the cerebrovascular responses to the CSD activation increase in the rats with low 5-HT compared with the control group. In the control group, the induction of CSD can provoke the cyclical changes of CBF and the alteration of the ultrastructure of cerebral endothelial cells. However, in low 5-HT rats, those responses are increased. An increase in the CSD induced cerebral hyperemia as well as an increase in the number of the pinocytic vesicles and microvilli are observed in the serotonin-depleted rats. These findings indicate the important role of 5-HT in the control of the cerebrovascular responses to the CSD activation.

It has been known that the CSD can induce activation in both the neural and vascular compartment [4]. This results in the alteration of the cortical neuron activities and cerebrovascular responses. In this study, the ultrastructural changes of cerebral endothelial cells and the cyclical hyperemic changes of the CBF were observed after CSD activation. Several studies have confirmed the cerebral hyperemia after the CSD activation, but the mechanism underlying this phenomenon and the function of this hyper-perfusion is still unclear. Since the repeated CSD does not induce

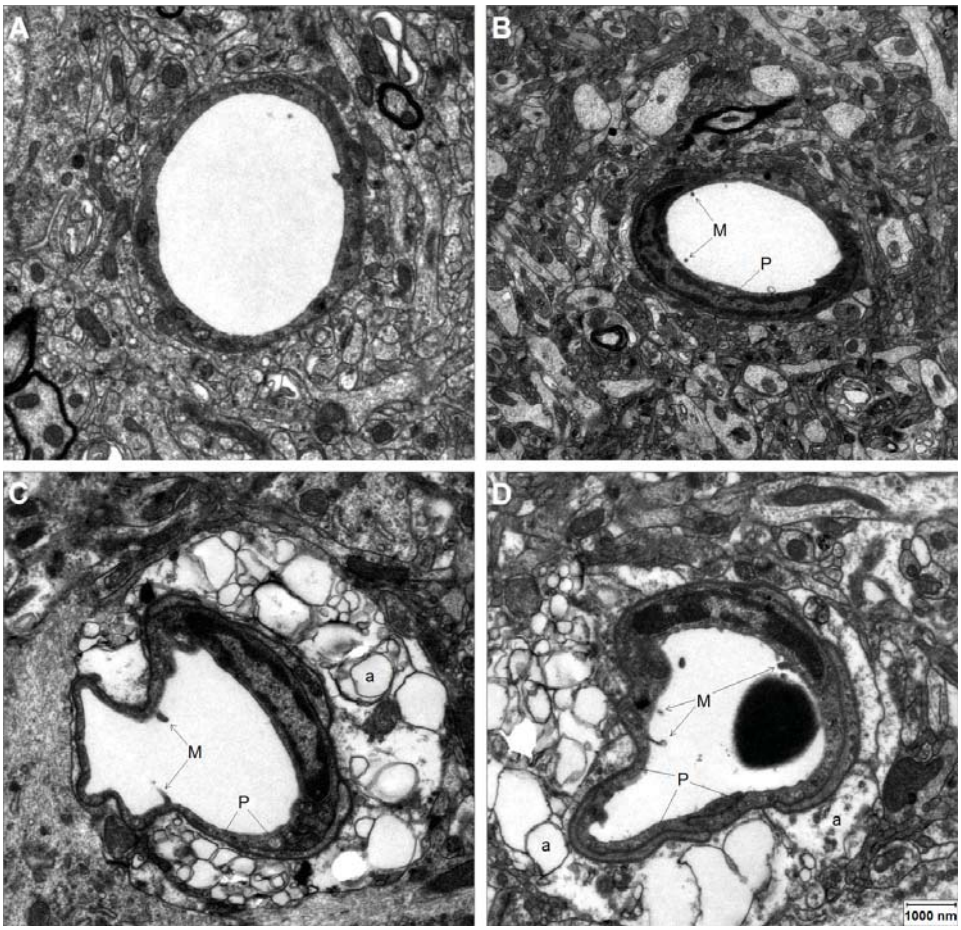

Fig. 2 Electronmicrographs showing the capillaries obtained from the control (**A**), CSD (**B**) and low 5-HT with CSD (**C**, **D**) groups. The microvilli and pinocytic vesicle are hardly observed in the endothelial cell in the control group (**A**). Few microvilli, M, and pinocytic vesicles, P, are observed in the capillary obtained from the CSD group (**B**). In the low HT group, the induction of CSD induces an increase in the pinocytic vesicle and microvillus formation in the capillary endothelial cell. In this group, the swelling of astrocytic foot plate (**a**) is clearly demonstrated as well (**C**, **D**). Bar = 1000 nm.

the damage to the neural tissue under the physiological condition, the protection of neural tissue from injury by increasing the CBF is one hypothesis that could explain the function of CSD in healthy brain [14]. However, the induction of CSD in a physiological impaired condition can result in the damage of neural tissue [15].

In this study, the activation by CSD in the low 5-HT group could induce higher cerebrovascular responses than those observed from the normal 5-HT group. The CSD induced changes of CBF and ultrastructure of cerebral endothelial cells were significantly increased in this group.

All cerebral vessels are surrounded by neurons and the astrocytic footplate. With this anatomical arrangement, both neurons and astrocytes play an important role in the adjustment of the CBF to local metabolic activity [16-18]. In this study, besides the increase of pinocytic vesicles and microvillus formation, the swelling of the astrocytic footplate around the cerebral vessels was clearly demonstrated in the low 5-HT group. These abnormalities were not detected in rats with a normal level of 5-HT. The increase in the number of pinocytic vesicles and microvillus formation have been demonstrated in several conditions of an impaired blood brain barrier (BBB) such as in the concussive and traumatic brain injury, and hypertensive encephalopathy [19-22]. The swelling of the astrocytic footplate is known as another characteristic indicating the abnormality of the BBB. This abnormality was reported in parallel with the impairment of the BBB and the BBB breakdown [19, 23]. For these reasons, the higher degree of the ultrastructural alteration of the cerebral endothelial cells

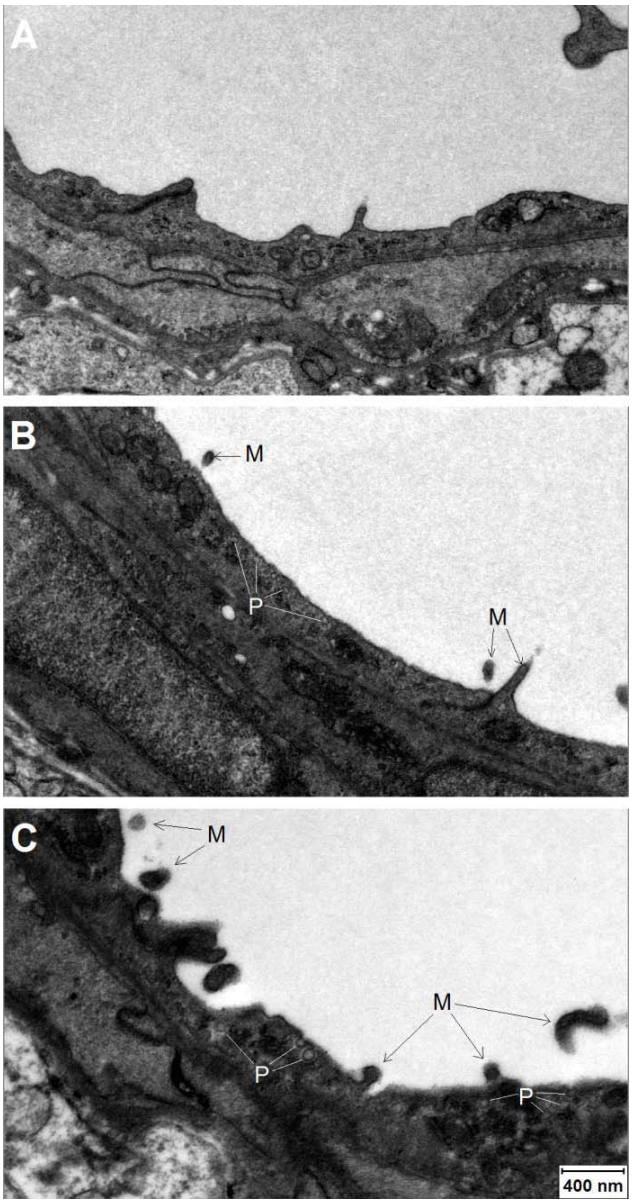

Fig. 3 Electron micrographs showing the ultrastructure of an arteriolar endothelial cell obtained from the control group (A) which demonstrates a few number of pinocytic vesicle and microvilli. In the CSD group, the induction of CSD can induce an increase in the pinocytic vesicles, P, and microvilli, M, in the endothelial cell. These CSD-evoked ultrastructural changes are facilitated in the condition with low 5-HT (C). Bar = 400 nm.

Table 2. The effect of low 5-HT on CSD-evoked ultrastructural changes of the cerebral endothelial cells

Variables	Control group	CSD group	Low 5-HT with CSD group
Capillaries			
Pinocytic vesicles *(number per m^2)*	16 ± 7	27 ± 10	45 ± 12*#
Microvilli (number per vessel)	1 ± 0.7	1.4 ± 1.3	3.7 ± 1.3*#
Arterioles			
Pinocytic vesicles *(number per m^2)*	18 ± 7	34 ± 10*	49 ± 11*#
Microvilli (number per vessel)	8 ± 4	15 ± 5*	26.5 ± 5*#

*$p < 0.05$ compared with the control group. #$p < 0.05$ compared with the CSD group.

and the swelling of astrocytic footplate observed in low 5-HT rats may imply that the activation by CSD in the 5-HT depleted state can induce higher cerebrovascular responses than in the normal 5-HT condition. If the activation is persistent, the cerebral endothelial cells in low 5-HT condition will damage quicker than those in the condition with normal 5-HT.

According to the current knowledge on the regulation of CBF, there are a number of neurotransmitters and substances involved in the hyperemic changes during CSD. Among them, nitric oxide (NO) is one of the important neurotransmitters involved in both vascular and neuronal alteration during CSD [24]. NO itself has a vasodilatation effect via the cGMP dependent mechanism. After release, NO can directly react with superoxide (O_2^-) resulting in the formation of the peroxynitrite ($ONOO^-$) which is the strong reactive oxygen free radical. $ONOO^-$ has been reported to be the crucial molecule in the alteration of the blood flow as well as the integrity of BBB in several pathological conditions [25]. The role of NO in the control of the integrity of BBB has been confirmed by the number of findings that suggest close relation between the increase in the synthesis/ release of NO and an increase in the permeability of cerebral circulation in response to a variety of mediators [26-28].

Interestingly, there are several lines of evidence demonstrating the abnormality of NO production in the low 5-HT state. Ramos et al. revealed that the depletion of serotonin by the PCPA injection could induce an immediate increase in the nNOS activity in several parts of the brain (striatum, hippocampus, and parietal cortex) [29]. This hypothesis has been confirmed by the study of the postnatal development of the nitrergic system after depletion of 5-HT. The results showed that after depletion of serotonin by the para-chloroamphetamine treatment, the nNOS immunoreactivity in striatum, frontal cortex, and hippocampus was higher than those observed in the control rats. The results also demonstrated that the more serotonin was depleted, the higher the expression of NO marker was. This suggested the close relation between serotonin and the NO system [30]. Altogether, it can be suggested that the low 5-HT state can induce a larger increase in the NO production in the brain than in the control group. Furthermore, the increase of the level of the NO released after CSD activation can be at least one explanation for the increase in the CBF and the ultrastructure of cerebral endothelial cells observed in the low 5-HT group.

In conclusion, 5-HT is the neurotransmitter involved in the control of cerebrovascular responses to CSD activation. The depletion of 5-HT can induce an increase in the CBF hyperperfusion as well as the damage of the cerebral endothelial cells from CSD activation. The increase in the production of NO might explain these abnormalities of the cerebrovascular responses observed in this study. However, since the hyper-excitability of the cortical neurons have been demonstrated in the 5-HT depleted state, the increase of several vasoactive neurotransmitters released after CSD activation cannot be excluded for these cerebrovascular responses in 5-HT depleted condition.

Abbreviations

CBF: cortical blood flow,
CSD: cortical spreading depression,
5-HT: serotonin,
PCPA: para-chlorophenylalanine,
NO: nitric oxide.

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Research Submission

Nociceptin/Orphanin FQ Modulates Cortical Activity and Trigeminal Nociception

Saknan Bongsebandhu-phubhakdi, PhD; Thas Phisonkulkasem, MSc; Anan Srikiatkhachorn, MD

Background.—Alterations in the levels of nociceptin/orphanin FQ (N/OFQ) have been reported in patients with primary headaches, including migraines and cluster headaches. These clinical observations suggest that N/OFQ is involved in the pathogenesis of primary headaches.

Objectives.—The present study was conducted to determine the role of N/OFQ in the control of trigeminal nociception and cortical excitation.

Methods.—Cortical spreading depression (CSD) was elicited in Wistar rats by cortical application of potassium chloride, and electrocorticograms were recorded. N/OFQ was administered via an intracisternal injection. The presence of CSD-evoked trigeminal nociception was determined with Fos and transient receptor potential vanilloid 1 (TRPV1) immunoreactivity.

Results.—Nociceptin/orphanin FQ produced a biphasic effect on CSD generation, characterized by an initial attenuation followed by delayed potentiation. The amplitude of CSD waves were lower in the initial period but increased in the later period. The total number of CSD waves recorded in 1 hour was greater in the N/OFQ-treated group. Exposure to N/OFQ significantly increased the number of Fos-immunoreactive cells in the trigeminal nucleus caudalis and the number of TRPV1-immunoreactive cells in the trigeminal ganglia, indicating the enhancement of trigeminal nociception.

Conclusion.—These results indicate that N/OFQ can lead to biphasic effect characterized by an initial inhibition, and delay potentiation that eventually intensify CSD-evoked trigeminal nociception.

Key words: migraine, nociceptin, nociceptin/orphanin FQ, cortical spreading depression, trigeminal nociception

Abbreviations: CSD cortical spreading depression, Fos protein encoded by the c-Fos immediate early gene, N/OFQ nociceptin/orphanin FQ, ORL1 orphan opioid receptor-like 1, TG trigeminal ganglia, TNC trigeminal nucleus caudalis, TRPV1 transient receptor potential vanilloid 1

(*Headache* 2011;51:1245-1253)

INTRODUCTION

Nociceptin/orphanin FQ (N/OFQ) is one of several neuropeptides that are expressed in the nociceptive system, including the trigeminal pathway. In the rat central nervous system (CNS), this peptide is similar to dynorphin A in both structure and distribution.[1] N/OFQ selectively binds to the N/OFQ receptor, a G protein-coupled receptor previously known as the opioid receptor-like receptor 1 (ORL1). This receptor belongs to the opioid receptor family and is expressed widely in both the CNS and peripheral nervous tissue.[2] In the CNS, the N/OFQ receptor is particularly abundant in the cerebral cortex, limbic system, and several other areas involved in pain perception.[3]

Nociceptin/orphanin FQ immunoreactivity and ORL1 mRNA have been detected in human and cat

From Department of Physiology, Faculty of Medicine, Chulalongkorn University, Patumwan, Bangkok, Thailand.

Address all correspondence to A. Srikiatkhachorn, Department of Physiology, Faculty of Medicine, Chulalongkorn University, Rama IV Road, Patumwan 10330 Bangkok, Thailand, email: anan.s@chula.ac.th

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Conflict of Interest: None

trigeminal ganglia and have been shown to co-localize with the calcitonin gene-related peptide (CGRP), substance P, nitric oxide synthase, and pituitary adenylate cyclise-activating peptide.[4] This anatomical co-localization suggests that N/OFQ plays a role in trigeminal sensory transmission and vascular regulation. Because these 2 processes are crucial in the control of trigeminovascular nociception, it is likely that N/OFQ is involved in the pathogenesis of vascular headaches. This hypothesis is supported by clinical observations; plasma levels of N/OFQ are decreased in patients with migraine without aura compared to non-headache controls, and these levels are further reduced during the initial period of migraine onset.[5] Reductions in the plasma concentration of this peptide are also observed during cluster headaches.[6] These suggest that N/OFQ levels during the migraine and cluster period may contribute to a defective regulation of trigeminal activity. The role of N/OFQ in the modulation of cortical activity and trigeminal nociception, however, remains unclear.

The objective of the present work was to determine the role of N/OFQ in the pathogenesis of migraines by investigating its effects on the development of cortical spreading depression (CSD) and the expression of c-Fos and transient receptor potential vanilloid 1 (TRPV1). Immunohistochemistry was used to identify the presence of c-Fos protein, an indicator of trigeminal nociceptive neuron activation, in the trigeminal nucleus caudalis (TNC). TRPV1 is a non-selective cation channel that is activated by a wide variety of exogenous and endogenous physical and chemical stimuli. TRPV1 may play an important role in the pathogenesis of migraines because it integrates painful stimuli ranging from noxious heat to endovanilloids involved in inflammation.[7] We hypothesized that N/OFQ regulates cortical activity and trigeminal nociception. Using electrophysiological recordings of CSD development and immunohistochemical analysis of the trigeminal system, we investigated whether intracisternal administration of N/OFQ can modulate trigeminal nociception.

MATERIALS AND METHODS

Adult male Wistar rats weighing 200 to 300 g were obtained from the National Laboratory Animal Centre (Mahidol University, Bangkok, Thailand). The animals were housed 5 per cage in stainless steel-bottom cages kept in a well-ventilated room. The room was equipped with an automated lighting timer, and the temperature was held at 25°C. The animals were provided *ad libitum* access to food and tap water. Protocols for this study were approved by the Faculty of Medicine Ethics Committee at Chulalongkorn University.

Chemicals.—Pentobarbital sodium (Nembutal®) was purchased from Sanofi (Bangkok, Thailand). Normal saline was purchased from King Chulalongkorn Memorial Hospital Product Public (Bangkok, Thailand). Purified N/OFQ was purchased from Tocris (Bristol, UK). Potassium chloride (KCl), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), sodium carbonate ($NaHCO_3$), hydrogen peroxide (H_2O_2), and ethanol were purchased from Merck (Whitehouse Station, NJ, USA). Liquid DAB+ and Envision+ System–horseradish peroxidase (DAB) for use with rabbit primary antibodies were purchased from Dako (Glostrup, Denmark). Paraformaldehyde (95%) was purchased from Sigma (St. Louis, MO, USA). Rabbit anti-TRPV1 was purchased from Genetex (Irvine, CA, USA). Rabbit anti-Fos was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Normal goat serum was purchased from Dako (Glostrup, Denmark).

Experimental Design.—The rats were divided into control (n = 8) and N/OFQ-treated groups (n = 8). In both groups, CSD waves were induced by topical application of solid KCl (3 mg) to the parietal cortex. In the N/OFQ-treated groups, N/OFQ (10 μM/100 μL), which is a concentration that achieves maximum effect in the rat dorsal horn,[8] was administered intracisternally after completion of the third depolarization shift wave. The same volume of saline was administered to controls. Electrocorticograms were recorded continuously for 1 hour, and then the brain was removed for immunohistochemical analysis.

Animal Preparation.—The rats were anesthetized with an i.p. injection of pentobarbital sodium (60 mg/kg). Additional doses (20 mg/kg) were given as needed to maintain surgical anesthesia based on the tail pinch reflex. A tracheotomy was performed to assist with ventilation. The left femoral vein was

cannulated to allow for i.v. administration of anesthetic drugs and saline.

After the tracheotomy and cannulation were performed, the animals were placed on a surgical frame, and their heads were fixed on a stereotaxic frame. The right parietal bone was exposed by mobilizing the skin along either side of the midline incision. Two craniotomies were performed using a saline-cooled drill. The recording electrode was placed in the anterior opening that was located in the frontal bone 1 mm anterior and 1 mm lateral to the bregma. The other opening was placed in the parietal bone 7 mm posterior and 1 mm lateral to the bregma and used for the application of solid KCl for CSD initiation.

Electrocorticographic Recording.—Cortical depolarization was measured with a glass microelectrode (internal diameter 5 μm) prepared from a borosilicate glass capillary tube pulled with a microelectrode puller (P-30 Vertical Micropipette Puller; Sutter Instrument, Novato, CA, USA). The microelectrode was filled with 4 M NaCl solution, and then an Ag/AgCl wire was inserted. A hydraulic micromanipulator (Narishige; Scientific Instrument Lab, Tokyo, Japan) was used to insert the filled glass microelectrode perpendicular to the cortex at a depth of 500 μm from the cortical surface. Another Ag/AgCl wire was placed on the back of the animal and served as a reference point. The electrical signal was amplified with a microelectrode amplifier (MEZ-8301; Nihon Koden, Tokyo, Japan). Analog data were digitized with a data acquisition system (Biopac Physiograph MP100A; Biopac, Santa Barbara, CA, USA).

The amplitude, duration, number of cycles, and area under the curve (AUC) of each CSD wave occurring within the 1-hour recording period were analyzed with AcqKnowlege version 3.7.3 (Biopac). The amplitude was measured as the vertical length from the baseline to the peak of each depolarization shift. The duration was measured as the horizontal length (temporal difference) between the start and end point of each depolarization shift. The number of cycles was defined as the total number of depolarization shifts that occurred within 1 hour of N/OFQ or saline administration. The AUC was the total area under the curve for each depolarization shift. All of the measured variables were converted to absolute values for analysis.

Immunohistochemical Study.—After the electrocorticographic recordings were completed, the rats were euthanized with an overdose of sodium pentobarbital, and thoracotomies and laparotomies were performed. A cannula was inserted into the apex of the heart and advanced distally into the aortic arch. The animals were perfused transcardially with 300 mL of 0.1 M phosphate-buffered saline (PBS), followed by 300 mL of 4% paraformaldehyde in 0.1 M PBS (pH = 7.4). The brain, cervical spinal cord, and trigeminal ganglia were dissected and fixed overnight at 4°C in 0.01 M PBS.

The caudal medulla and cervical spinal cord (C1 and C2 region; approximately –1 to –6 mm from the obex) were cut into 5-mm blocks and immersed in a cryoprotectant solution (30% sucrose in 0.01 M PBS, pH = 7.4) for 24 hours at 4°C. The trigeminal ganglia were immersed in the cryoprotectant solution whole. Tissues were then placed on a stage and completely covered with an optimal cutting temperature embedding medium. After freezing the optimal cutting temperature at –20°C, the samples were coronally sectioned (20-μm thick) with a cryostat. The sections were then washed 3 times and stored in cold 0.01 M PBS.

Immunohistochemistry was performed using the free floating technique. All of the sections were incubated at room temperature. The sections were initially rinsed 3 times with washing buffer (0.01 PBS). The sections were then incubated with 50% ethanol for 30 minutes followed by 3% hydrogen peroxide in 50% ethanol for 30 minutes to minimize endogenous peroxidation. After another 3 rinse cycles with PBS, the brainstem and cervical spinal cord sections were incubated for 1 hour in PBS containing 3% normal goat serum and 1% bovine serum albumin. Sections from these regions were also incubated with anti-Fos polyclonal antibody (1:1000 dilution in PBS-containing normal goat serum).

The trigeminal ganglia sections were incubated with rabbit anti-TRPV1 polyclonal antibody (1:500 dilution) for 20 hours at 4°C. These sections were then rinsed 3 times with PBS and incubated for 45 minutes with envision and rabbit anti-horseradish peroxidase.

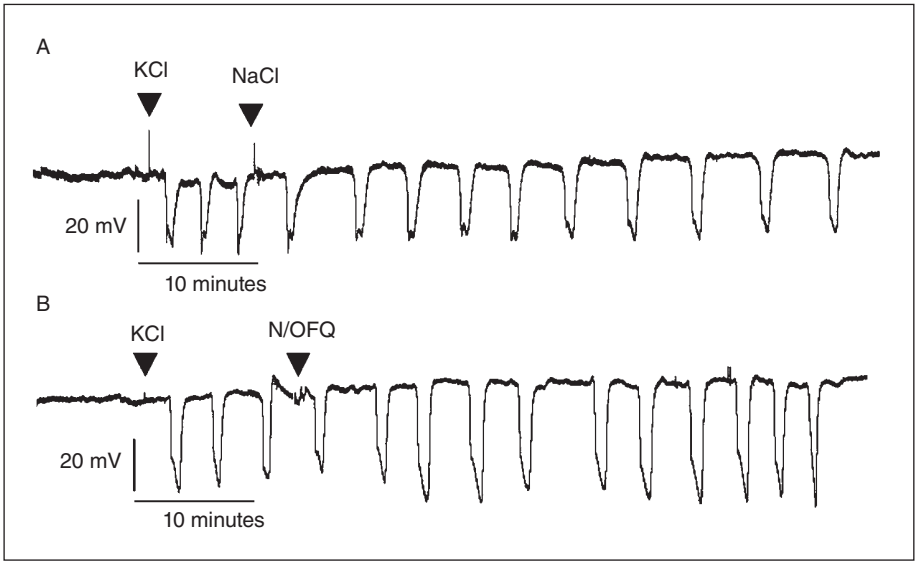

Fig 1.—Representative traces showing the pattern of cortical spreading depression (CSD) in (A) control and (B) nociceptin/orphanin FQ (N/OFQ)-treated rats. The second triangle indicates administration of normal saline or N/OFQ after the third CSD cycle. The effect of N/OFQ on CSD is characterized by a short initial period (approximately 10 minutes) of CSD attenuation followed by an extended period of CSD potentiation.

The levels of bound peroxidase were assessed by incubating the sections with liquid DAB for 10 minutes. The reaction was terminated with 2 successive rinses with distilled water. The sections were then mounted onto gelatin-coated slides, air dried overnight, and coverslipped with Permount.

c-Fos-immunoreactive (Fos-IR) cells were identified by a dark brown stain in the nucleus. Only cells located in the laminae I and II of the TNC with nuclei visible on the focal plane were included for analysis. Expression of Fos in the TNC was quantified by counting the number of Fos-IR neurons in laminae I and II of the TNC from 10 sections of the cervical spinal cord and 10 sections of the caudal medulla. The data are expressed as the mean number of Fos-IR cells per section.

Transient receptor potential vanilloid 1-immunoreactive (TRPV1-IR) cells were distinguished by their darkly stained cell bodies and processes. Expression of TRPV1 in the trigeminal ganglia (TG) was determined by counting the number of TRPV1-IR cells from 500 randomly selected small- to medium-sized (diameter < 50 μm) ganglionic cells. The data are expressed as the percentage of TRPV1-IR cells per rat. Researchers were blinded to the treatment group during counting.

Data Analysis.—Data are expressed as the mean ± SD. The amplitude and onset time of the CSD waves were plotted. The differences between means were analyzed with a 1-way analysis of variance (1-way ANOVA) followed by a Student's *t*-test. Statistical significance was defined as $P < .05$.

RESULTS

Administration of KCl induced repeated CSD cycles (Fig. 1), with the first CSD peak developing 3 minutes after KCl application. There were 12.2 ± 0.3 cycles during the 1-hour electrocorticographic recording period. In the control group, the CSD wave amplitude was stable throughout the recording period, with a mean value of 27.0 ± 1.5 mV. The average AUC duration was 63.3 ± 8.4 seconds, and each cycle lasted 17.3 ± 2.9 mV-seconds. Figure 1A shows a representative control electrocorticographic recording, and the electrophysiological data are presented in Table 1.

Effect of N/OFQ on CSD.—Nociceptin/orphanin FQ exerted dual effects on CSD; there was an initial attenuation period lasting 10 to 15 minutes (Fig. 1B) followed by a long-lasting potentiation (Fig. 2). Prior to N/OFQ treatment, the CSD amplitude was 29.7 ± 1.7 mV, but this value was reduced to

Table 1.—Electrophysiological CSD Data for the Control and N/OFQ-Treated Groups

Electrophysiological Variables	Control (N = 8)	N/OFQ-Treated (N = 8)	*P* Value
Number of cycles	12.2 ± 0.3	14.9 ± 0.3	.012
Amplitude (mV)			
Pre-treatment	27.0 ± 1.5	29.7 ± 1.7	.100
Initial 2 waves	27.0 ± 1.9	21.3 ± 1.1	.029
Delayed waves	26.8 ± 1.1	33.3 ± 1.8	<.001
AUC (mV-seconds)			
Pre-treatment	17.3 ± 2.9	20.0 ± 1.7	.050
Initial 2 waves	19.0 ± 1.6	17.8 ± 3.3	.442
Delayed waves	21.1 ± 3.2	26.0 ± 2.9	.007
Duration (seconds)			
Pre-treatment	63.3 ± 8.4	57.0 ± 5.3	.130
Initial 2 waves	65.3 ± 9.9	54.3 ± 5.3	.038
Delayed waves	65.4 ± 8.9	63.5 ± 8.9	.959

Data are expressed as the mean ± SD.
AUC = area under the curve; CSD = cortical spreading depression; N/OFQ = nociceptin/orphanin FQ.

21.3 ± 1.1 mV during the attenuation period. There was a statistically significant difference ($P = .029$) between the initial waves of the N/OFQ-treated and control groups. The average amplitude of the delayed CSD waves in the N/OFQ-treated group was 33.3 ± 1.8 mV compared to 26.8 ± 1.1 mV for the controls ($P < .001$). The AUC of the delayed CSD wave was also significantly greater in the N/OFQ-treated group (N/OFQ-treated animals = 26.0 ± 2.9 mV-seconds; control group = 21.1 ± 3.2 mV-seconds; $P = .007$). Although there was no change in the duration of the CSD waves, the total number of CSD waves was greater in the N/OFQ-treated group (N/OFQ-treated group = 14.88 ± 0.29 waves; control group = 12.25 ± 0.31 waves; $P = .012$).

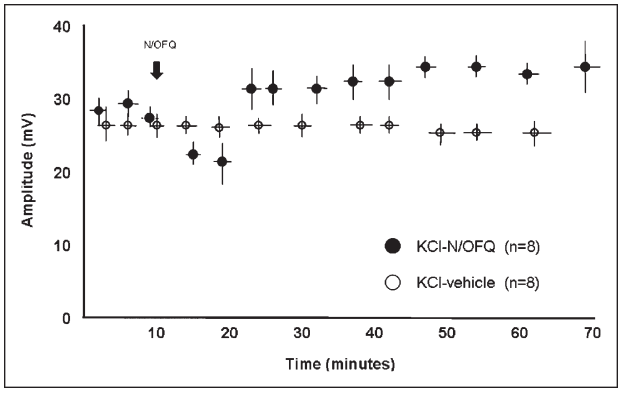

Fig 2.—The effect of nociceptin/orphanin FQ (N/OFQ) on cortical spreading depression (CSD) amplitude. N/OFQ administration transiently reduced the CSD amplitude. This attenuation was followed by a long-lasting period of increased CSD wave amplitude and frequency.

Effect of N/OFQ on CSD-Evoked Fos Expression in TNC.—Nociceptin/orphanin FQ treatment significantly increased the number of Fos-IR cells in the TNC (Fig. 3). The number of Fos-IR cells in the ipsilateral TNC was 34.7 ± 3.3 cells/slide for the N/OFQ-treated group and 24.6 ± 2.0 cells/slide for the control group ($P < .001$). A significant difference was also observed when we compared the number of Fos-IR cells in the contralateral TNC (Table 2), with 17.6 ± 2.1 cells/slide in the N/OFQ-treated group and 12.7 ± 2.5 cells/slide in the control group ($P < .001$). The observed increase in the number of Fos-IR cells in the N/OFQ-treated group suggests that this peptide facilitates the process of trigeminal nociception.

Effect of N/OFQ on TRPV1 Receptor Expression in the TG.—In addition to increasing the number Fos-IR cells in the TNC, N/OFQ administration also increased the expression of the TRPV1 receptor in the TG (Fig. 4 and Table 2). The percentage of TRPV1-IR cells in the ipsilateral TG was 47.3 ± 5.5% for the N/OFQ-treated group and 20.2 ± 2.1% for the control group ($P < .001$). A lower number of TRPV1 cells were observed in the con-

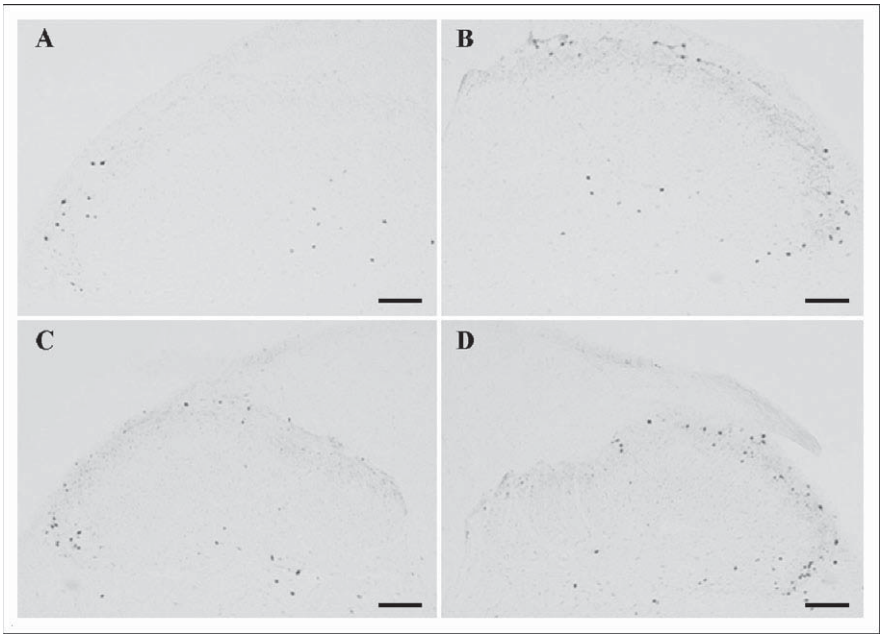

Fig 3.—Representative micrographs showing c-Fos-immunoreactive (Fos-IR) cells in the trigeminal nucleus caudalis (TNC). An increased number of Fos-IR cells were observed in the nociceptin/orphanin FQ (N/OFQ)-treated group. (A) Contralateral control; (B) ipsilateral control; (C) contralateral N/OFQ-treated; (D) ipsilateral N/OFQ-treated. Scale bar = 250 μm.

tralateral TG, with 40.2 ± 6.3% in the N/OFQ-treated group and 17.0 ± 3.0% in the control group ($P < .001$).

DISCUSSION

In the present study, we demonstrated that N/OFQ can alter CSD development and CSD-evoked trigeminal nociception. Electrophysiological variables indicative of CSD induction, namely amplitude, number of cycles, and AUC, were enhanced by N/OFQ administration. We also observed that exposure to N/OFQ increased the expression of nociception-related proteins in the trigeminal system. Since several reports have indicated that intracistenal administration of N/OFQ inhibits nociceptive effect in spinal dorsal horn,[9-11] it is unlikely that N/OFQ can produce its direct effect in trigeminal nociception.

The present results show that N/OFQ has dual effects on cortical activity, characterized by an initial attenuation and followed by delayed potentiation of

Table 2.—CSD-Induced Fos-IR Cells and TRPV1-IR Cells in the Trigeminal System

Measured Variables	Control	N/OFQ-Treated	*P* Value
The number of Fos-IR (cells/slide)			
Ipsilateral TNC	24.6 ± 2.0	34.7 ± 3.3	<.001
Contralateral TNC	12.7 ± 2.5	17.6 ± 2.1	<.001
The percentage of TRPV1-IR cells (%)			
Ipsilateral TG	20.2 ± 2.1	47.3 ± 5.5	<.001
Contralateral TG	17.0 ± 3.0	40.2 ± 6.3	<.001

Data are expressed as the mean ± SD.
CSD = cortical spreading depression; Fos-IR = c-Fos-immunoreactive; N/OFQ = nociceptin/orphanin FQ; TG = trigeminal ganglia; TNC = trigeminal nucleus caudalis; TRPV1-IR = transient receptor potential vanilloid 1-immunoreactive.

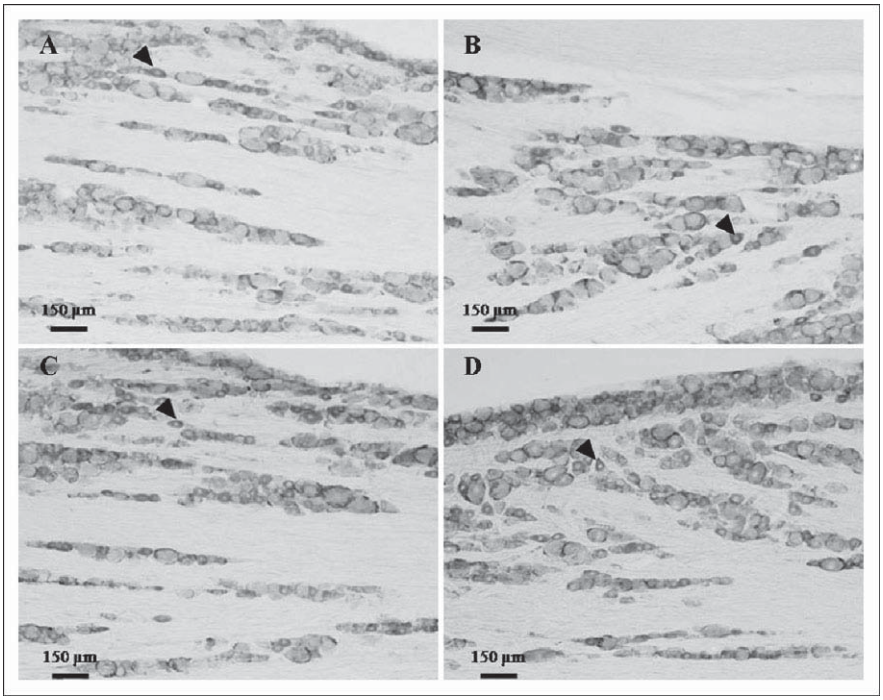

Fig 4.—Representative micrographs showing transient receptor potential vanilloid 1-immunoreactive (TRPV1-IR) cells in the trigeminal ganglia. TRPV1 was expressed in small- to medium-sized ganglionic neurons (arrowhead). Nociceptin/orphanin FQ (N/OFQ) treatment increased the number of TRPV1-IR cells. (A) Contralateral control; (B) ipsilateral control; (C) contralateral N/OFQ-treated; (D) ipsilateral N/OFQ-treated. Scale bar = 150 µm.

CSD development. This dual effect indicates that the role of N/OFQ in controlling cortical activity is complex. Several potential mechanisms could underlie these observations. It is known that N/OFQ can exert several cellular effects, including inhibition of adenylyl cyclase and Ca^{2+} channel currents.[12] N/OFQ can also increase conductance of the G-protein inwardly rectifying potassium (GIRK) channel.[13] Studies have also indicated that N/OFQ-induced currents prolong hyperpolarization by 10 to 15 minutes.[14,15] These effects result in membrane hyperpolarization and cellular inhibition and may explain the initial attenuation that was seen in this experiment.

The delayed facilitation effect of N/OFQ is interesting. The most likely explanation is that N/OFQ causes cellular hyperexcitability by modulating the function of inhibitory interneurons. N/OFQ is known to modulate the release of several neurotransmitters, such as serotonin and norepinephrine, in the rostral ventromedial medulla and spinal cord.[16] In the cerebral cortex, the ORL1 receptor is expressed ubiquitously in presumptive GABAergic interneurons (Golgi type II cells) located in layer II.[1,17] The axons of these interneurons primarily terminate on projective glutamatergic neurons (Golgi type I cell) located in layer III. Because activation of ORL1 receptors induces inhibitory modulation, intracisternal administration of N/OFQ may reduce GABA release from interneurons during CSD development, resulting in enhanced glutamate activation in adjacent projection neurons.

The effects of N/OFQ appear to result from the summation of 2 opposing actions. It has been suggested that N/OFQ produces analgesia that is readily antagonized by opioid antagonists,[18] thus functionally reversing the analgesic effects of a number of opioids.[19] At low concentrations, N/OFQ also shows bidirectional effects on monosynaptic transmission with GIRK activation.[20] Higher concentrations, however, reverse inhibition of the polysynaptic local circuit in the spinal cord.[21] Thus, our results suggest that N/OFQ modulates cortical activity and produces antinociception by initial attenuation. This initial anti-

nociception is later reversed to nociception by delayed potentiation of CSD development.

In the present study, N/OFQ administration increased the number of TRPV1-IR cells in the TG. Of the many stimuli that activate cation currents in primary sensory neurons, CSD is believed to be one of the physiological activators of TRPV1.[22] During CSD development, extracellular K^+ is elevated to 40 to 60 mM in the grey matter of the cortex.[23] Elevated K^+ may cause depolarization of primary sensory neurons in the cortex, which can contribute to elevated TRPV1 expression and prolonged migraine headaches that are associated with TRPV1 sensitization in the TG.[24] This hypothesis is strengthened by our finding that N/OFQ increases TRPV1 expression in the TG. The N/OFQ receptor antagonist has also been shown to have anti-allodynic and anti-hyperalgesic effects in rats after spinal nerve injury and inflammation.[25] This study also demonstrated increases in TRPV1 receptor expression in the TG. Up-regulation of this receptor may increase nociceptive sensitivity, resulting in increased pain perception.

The present study showed that N/OFQ administration led to an increase in the number of CSD-evoked Fos-IR cells in the TNC, indicating enhancement of CSD-evoked trigeminal nociception. In addition, N/OFQ receptor blocker reduced Fos-IR that was induced by L5/L6 spinal nerve ligation, suggesting that N/OFQ system might be involved in the enhancement of allodynic and hyperalgesic effects in rats.[25] Therefore, the increase in CSD-evoked trigeminal nociception reported here is more likely to be explained by an increase in CSD leading to increased nociceptive activation and up-regulation of the TRPV1 receptor.

In conclusion, the present study demonstrates that N/OFQ has a biphasic effect on cortical activity and CSD-induced trigeminal nociception. N/OFQ attenuates CSD in the early phase but enhances CSD protein expression in the trigeminal system in the late phase. Additionally, our results indicate that TRPV1 channel in trigeminal pathway is sensitized to the activation by CSD in the presence of N/OFQ. This finding provides another example of an ion channel in which N/OFQ acts to modify channel expression. This may have important clinical implications in view of the role of N/OFQ in trigeminal nociception.

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STATEMENT OF AUTHORSHIP

Category 1

(a) Conception and Design
Saknan Bongsebandhu-phubhakdi; Anan Srikiatkhachorn

(b) Acquisition of Data
Thas Phisonkulkasem

(c) Analysis and Interpretation of Data
Saknan Bongsebandhu-phubhakdi; Thas Phisonkulkasem; Anan Srikiatkhachorn

Category 2

(a) Drafting the Article
Saknan Bongsebandhu-phubhakdi; Anan Srikiatkhachorn; Thas Phisonkulkasem

(b) Revising It for Intellectual Content
Saknan Bongsebandhu-phubhakdi; Anan Srikiatkhachorn; Thas Phisonkulkasem

Category 3

(a) Final Approval of the Completed Article
Saknan Bongsebandhu-phubhakdi; Anan Srikiatkhachorn; Thas Phisonkulkasem

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Brief Communications

Serotonin Depletion Leads to Cortical Hyperexcitability and Trigeminal Nociceptive Facilitation via the Nitric Oxide Pathway

Supang Maneesri le Grand, PhD; Weera Supornsilpchai, PhD; Chonlawan Saengjaroentham, BSc; Anan Srikiatkhachorn, MD

Objective.—To investigate the role of nitric oxide (NO) in the development of cortical hyperexcitability and trigeminal nociceptive facilitation induced by serotonin (5-HT) depletion.

Background.—Nitric oxide and 5-HT are important in the pathogenesis of primary headaches. An increase in cortical excitability and trigeminal nociception has been demonstrated in animals with low 5-HT levels. Although the mechanism underlying this increase is unclear, an alteration of the NO system is one possible explanation.

Methods.—Male Wistar rats were divided into control and 5-HT-depleted groups. 5-HT was depleted by i.p. injection of parachlorophenylalanine (100 mg/kg). Three days after injection, a microelectrode was inserted into the cerebral cortex for electrocorticograph recording and waves of cortical spreading depression (CSD) were triggered with KCl application. N-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg by i.v. injection) or saline was given after the second CSD wave. Following the experiment, the cerebral cortex and brain stem were removed for anti-neuronal nitric oxide synthase (nNOS) and anti-Fos immunohistochemistry.

Results.—Relative to the control group, the 5-HT-depleted group exhibited a higher frequency of CSD waves, more nNOS-immunoreactive cells in both the cerebral cortex and brainstem and more Fos-immunoreactive cells in the trigeminal nucleus caudalis (TNC). In the control group, L-NAME application led to fewer nNOS-immunoreactive cells in the cerebral cortex and TNC, and fewer Fos-immunoreactive cells in the TNC; however, L-NAME was without effect on the CSD pattern. By contrast, in addition to decreased nNOS and Fos expression, L-NAME significantly reduced the frequency of CSD events in the 5-HT-depleted group.

Conclusions.—Inhibition of NO production can counter both the cortical hyperexcitability and facilitation of trigeminal nociception that develop in the depleted 5-HT state. Therefore, NO is likely involved in the increase in both CSD events and CSD-evoked trigeminal nociception under decreased 5-HT conditions.

From Department of Pathology, Faculty of Medicine, Chulalongkorn University, Patumwan, Bangkok, Thailand (S.M. le Grand and C. Saengjaroentham); Departments of Physiology, Faculty of Dentistry, Chulalongkorn University, Patumwan, Bangkok, Thailand (W. Supornsilpchai); Department of Physiology, Faculty of Medicine, Chulalongkorn University, Patumwan, Bangkok, Thailand (A. Srikiatkhachorn).

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Address all correspondence to A. Srikiatkhachorn, Department of Physiology, Faculty of Medicine, Chulalongkorn University, Rama IV Road, Patumwan, Bangkok 10330, Thailand.

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Key words: nitric oxide, serotonin, cortical spreading depression, trigeminal nociception

Abbreviations: 5-HT serotonin, CSD cortical spreading depression, Fos-IR Fos-immunoreactive, L-NAME nitro-L-arginine methyl ester, nNOS neuronal nitric oxide synthase, nNOS-IR nNOS-immunoreactive, NO nitric oxide, PCPA parachlorophenylalanine

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INTRODUCTION

Serotonin (5-HT) is widely accepted as a key neurotransmitter in the pathophysiology of migraine. The first evidence supporting this role of 5-HT was an observed increase in the urinary excretion of the 5-HT metabolite 5-hydroxyindole acetic acid (5-HIAA) during an episode of migraine.[1] Since then, numerous studies have reported abnormal serotonin systems in migraine patients. For instance, during a migraine attack, central 5-HT concentration is decreased and 5-HT release is increased.[2-4] Similarly, a neuroimaging study reported decreased 5-HT synthesis in migraine patients during headache-free periods and increased 5-HT synthesis during attacks.[5] Moreover, the depletion of 5-HT in migraine patients by administration of parachlorophenylalanine (PCPA) decreased their pain threshold, and 20% of patients complained of generalized body pain.[6] A decrease in 5-HT levels was also observed in patients with chronic daily headache, including those with medication-overuse headache.[7]

We previously reported that rats with low 5-HT levels exhibited cortical hyperexcitability; this was based on an increased generation of cortical spreading depression (CSD) events. This increase was accompanied by enhanced trigeminal nociception in the trigeminal nucleus caudalis (TNC).[8] It is well recognized that CSD can trigger substantial changes in neurovascular responses, including the release of nitric oxide (NO).[9] In the trigeminovascular system, NO has a strong influence on both the vascular and neural compartments, including the modulation of trigeminal nociceptive processing.[10] It is therefore possible that modulating NO might influence the enhanced trigeminovascular nociception observed in the low 5-HT state. In this study, we investigated the effect of N-nitro-L-arginine methyl ester (L-NAME), a non-specific nitric oxide synthase (NOS) inhibitor, on CSD-evoked trigeminal nociception in serotonin-depleted rats. In addition, we examined neuronal NOS (nNOS) expression within the cerebral cortex and TNC of 5-HT-depleted rats, as the activity of nNOS is involved in modulating trigeminal nociception.

MATERIALS AND METHODS

Study Design.—Male Wistar rats weighing 250-300 g were divided into control and 5-HT-depleted groups (24 rats per group). Within each group, half (12 rats) received an injection of L-NAME. The 5-HT-depleted condition was generated as previously described.[8] Briefly, PCPA (100 mg/kg) was i.p. injected 3 days prior to the start of the experiment. On the day of the experiment, a craniotomy was performed to expose the cerebral cortex and CSD was triggered by applying solid KCl (3 mg) to the surface of the parietal cortex as previously described.[8,11] During the experiment, the amplitude, duration, and frequency of CSD waves occurring within the first hour were recorded and measured.

To investigate the effect of decreasing NO, the non-specific NOS inhibitor L-NAME (or saline) was injected i.v. (10 mg/kg) after complete cessation of the second CSD wave.

All protocols used in this experiment were approved by the Chulalongkorn University Animal Care and Use Committee.

Neuronal Nitric Oxide Synthase and Fos Immunohistochemistry.—After completion of electrocorticograph recording, rats were deeply anesthetized with a high dose of sodium pentobarbital and perfused transcardially, first with 250 mL of phosphate buffer followed by 250 mL of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The spinal cord and brain were removed and immediately immersed in 4% paraformaldehyde in 0.1 M phos-

phate buffer for overnight storage. After fixation, the C_1-C_2 cervical spinal cord and the brain at 7 mm anterior to Bregma were selected and cryoprotected by overnight storage in 30% sucrose in 0.1 M phosphate buffer, pH 7.4. Serial 30-μm thick transverse sections were cut with a cryostat (Microm HM 505N; Walldorf, Germany) at –25°C. One in 4 sections was collected. The brain sections were processed for nNOS immunohistochemistry while the spinal cord sections were processed for both nNOS and Fos immunohistochemistry, as previously described.[7,10]

All slides were scanned (Aperio ScanScope; Aperio, Vista, CA, USA) and analyzed using the computer program Aperio ImageScope version 10.0.36.1805 (Aperio). To determine the density of nNOS-immunoreactive (nNOS-IR) cells in the cerebral cortex, 5 sections from each rat were selected. A 1 mm × 1 mm square region of interest (ROI) was drawn and nNOS-IR neurons within the ROI were counted. Data from 5 selected ROIs in the parasagittal cortex from each slide were summed and expressed as the number of positive cells per 5 mm^2. To determine the expression of nNOS and Fos in the TNC, 10 cervical cord sections were selected from each animal. The neurons with Fos and/or nNOS immunoreactivity (indicated by a dark brown nucleus or cytoplasm, respectively) within the area of laminae I and II were counted, averaged, and reported as the number of cells per slide. The experimenter was blinded to the treatment groups during the counting process.

Statistical Analysis.—All data are expressed as mean ± standard deviation (SD). Electrophysiological variables were analyzed for possible statistical significance using the Kruskal–Wallis test. The numbers of Fos-immunoreactive (Fos-IR) and nNOS-IR cells were compared using the Mann–Whitney method. All statistical analyses were performed using the computer program SPSS version 10 for Windows (SPSS, Chicago, IL, USA), and differences with a probability value of less than 0.05 were considered to be statistically significant.

RESULTS

The injection of PCPA did not noticeably alter the behavior of the animals, including their feeding, and mean body weight was similar among all groups.

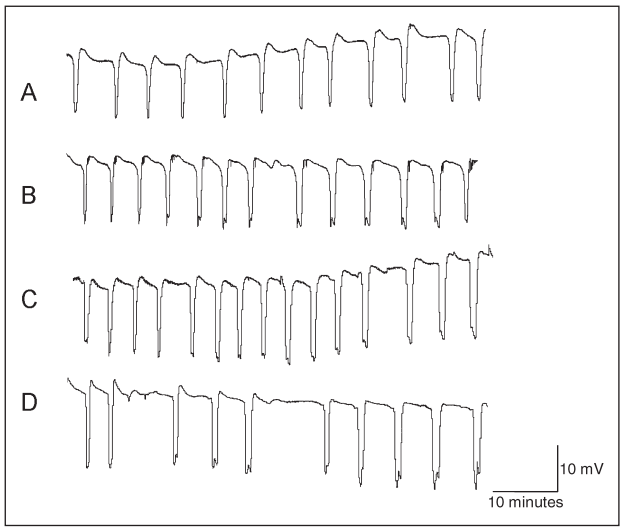

Fig 1.—Effect of L-NAME on cortical spreading depression (CSD). Cortical application of KCl triggered a series of DC shifts consistent with waves of CSD (A). The NOS inhibitor L-NAME was without effect on CSD events in control rats (B). Depleting 5-HT with parachlorophenylalanine treatment increased the frequency of CSD events (C), which can be attenuated by L-NAME (D).

In addition, physiological parameters including blood pressure, heart rate, and respiration were stable throughout the experiment.

Effect of L-NAME on CSD.—Cortical application of KCl generated a series of depolarization shifts characteristic of CSD, with a mean of 12.4 ± 1.2 waves in the 1st hour. The frequency of CSD waves was significantly higher in the 5-HT-depleted group (14.8 ± 1.6 waves; $P < .05$ vs control), and this effect was normalized by pretreatment with L-NAME; in the control group L-NAME did not affect the pattern of CSD waves. Apart from the frequency of CSD waves, other measured electrocorticographic values were unchanged (Fig. 1 and Table 1).

Effect of L-NAME on nNOS Expression.—Depletion of 5-HT by PCPA injection increased nNOS expression in both the brain and upper spinal cord. In the cerebral cortex, the mean densities of nNOS-IR neurons in the control and 5-HT-depleted groups were 18 ± 3 and 28 ± 5 cells per 5 mm^2, respectively ($P < .01$). In the ipsilateral TNC the mean numbers of nNOS-IR neurons in the control and 5-HT-depleted groups were 25 ± 5 and 32 ± 5 cells per slide, respectively ($P < .01$). Pretreatment

Table 1.—Effect of L-NAME on Cortical Spreading Depression in Control and Parachlorophenylalanine (PCPA)-Treated Groups

	Control Group			PCPA-Treated Group		
Variable	Saline	L-NAME	% Change	Saline	L-NAME	% Change
Number of peaks	12.4 ± 1.2	11.2 ± 1.4	–9.6	14.8 ± 1.6*	11.7 ± 1.4[#]	–20.9
Amplitude (mV)	27.1 ± 2.4	30.2 ± 3.4	+11.4	29.3 ± 3.8	28.6 ± 2.8	–2.3
Duration (s)	40.2 ± 6.5	43.0 ± 9.4	+6.9	39.8 ± 7.3	42.5 ± 8.4	+6.7

*$P < .05$ compared with the control group.
[#]$P < .05$ compared with the saline subgroups within the PCPA-treated group.

with L-NAME significantly reduced the number of nNOS-IR cells in both the cerebral cortex and TNC. This effect was much more pronounced in the 5-HT-depleted group, particularly in the cortical regions; in the ipsilateral cerebral cortex, nNOS-IR cells were reduced by 46.4% in the 5-HT-depleted group compared with a 27.8% reduction in the control group (Figs. 2 and 3, and Table 2).

Effect of L-NAME on CSD-Evoked Fos Expression.—We found that the trigeminal nociceptive system was activated by CSD, as evidenced by the presence of Fos-IR neurons within laminae I and II of the TNC (with 21 ± 3 and 12 ± 4 cells per slide for ipsilateral and contralateral TNC, respectively). Depletion of 5-HT by PCPA injection further increased the density of Fos-IR neurons in the TNC, reflecting nociceptive facilitation. In the ipsilateral TNC, we measured 25 ± 4 and 21 ± 3 Fos-IR cells per slide for the PCPA-treated and control groups, respectively ($P < .05$). In contrast, pretreatment with L-NAME decreased the number of Fos-IR neurons in the 5-HT-depleted and control groups by 28% and 14.3%, respectively (Fig. 4 and Table 2).

DISCUSSION

In this study, we found that depleting 5-HT with PCPA treatment increased both cortical excitability and trigeminal nociception. These enhancements coincided with increased nNOS expression in both the cerebral cortex and TNC. In addition, these effects were attenuated by injection of the nitric oxide synthase inhibitor L-NAME. Taken together, these results provide compelling evidence for the involvement of NO in facilitating trigeminovascular nociception in the serotonin-depleted state.

A relationship between 5-HT depletion and increased NOS expression was previously reported. For instance, Ramos and colleagues reported that depleting serotonin by PCPA injection rapidly increased nNOS activity in several brain regions including striatum, hippocampus, and parietal cortex.[12] Moreover, a study of postnatal CNS development in rats demonstrated a direct, positive correlation between 5-HT depletion and expression of an NO marker.[13] In the present study, the increase in nNOS expression in the cerebral cortex coincided with cortical hyperexcitability, both of which were blocked by an NOS inhibitor. This suggests that increased NO production is an important factor contributing to increased cortical excitability in the low 5-HT state. The mechanism by which NO enhances neocortical activity may resemble that which generates long-term potentiation in the hippocampus.[14] A recent study of the auditory brainstem demonstrated that NO, a highly diffusible gas molecule, is important in modulating synaptic transmission at glutaminergic synapses by acting on both target and adjacent inactive neurons.[15]

In contrast to the present study, the effect of NO has been reported to decrease CSD susceptibility. In 2008, Petzold and colleagues found that CSD events were triggered with a significantly lower threshold and propagated with a wave of ischemia when NO production was inhibited; this threshold effect was

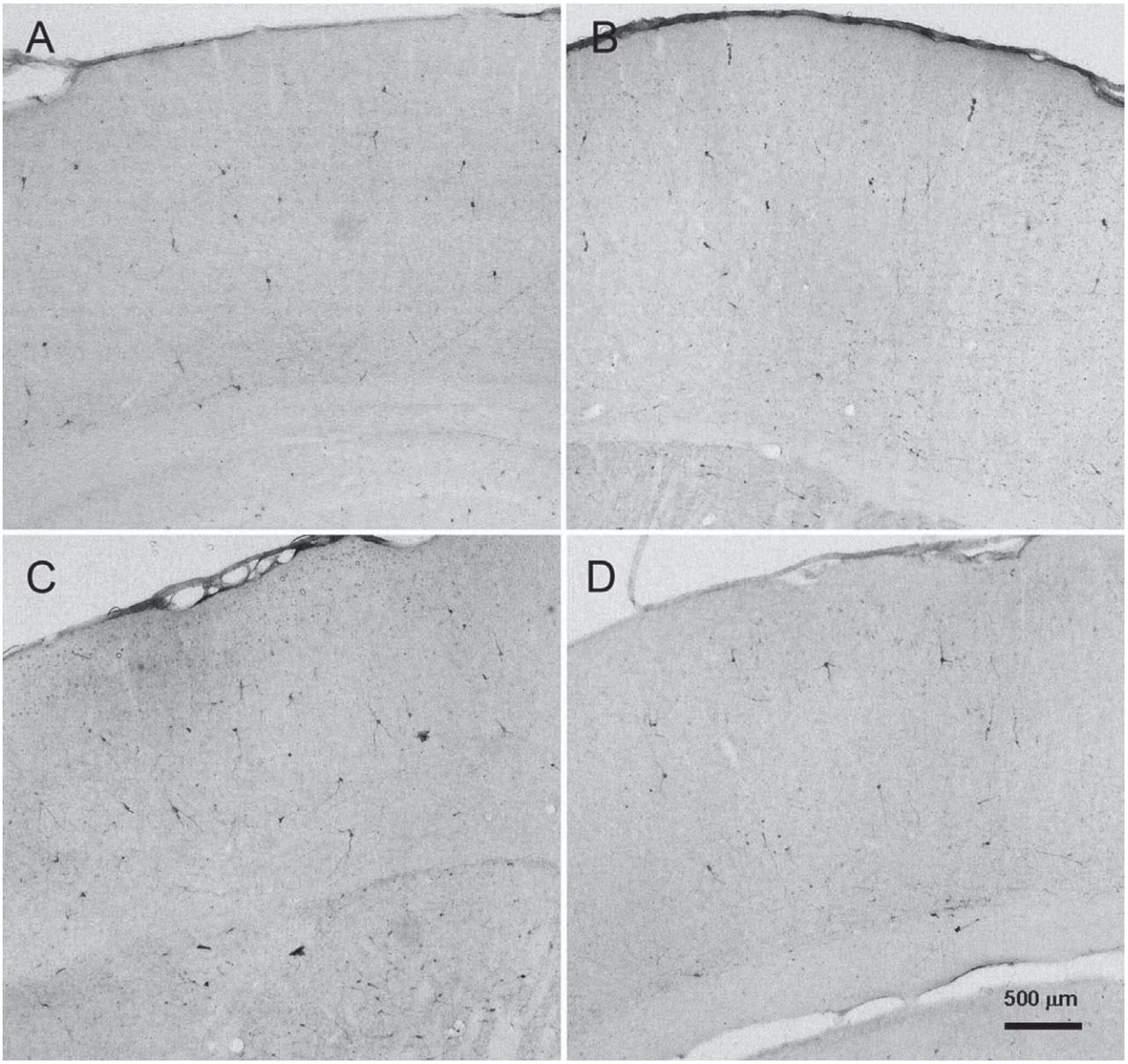

Fig 2.—Effect of L-NAME treatment on cortical spreading depression (CSD)-evoked neuronal nitric oxide synthase (nNOS) expression in the cerebral cortex. CSD-evoked expression on nNOS in cerebral cortex (A). Application of L-NAME minimized the density of nNOS-immunoreactive cells (B). Depleting 5-HT with parachlorophenylalanine treatment increased the density of nNOS-immunoreactive neurons (C). Such increase was also attenuated by L-NAME (D).

predominantly dependent on endothelial NOS (eNOS).[16] Importantly, however, their study was conducted under conditions of normal serotonin levels, which differs notably from the present study.

With regard to the role of NO in nociceptive modulation, mounting evidence indicates that NO drives biphasic modulation of nociception. At a relatively low concentration, an anti-nociceptive effect of NO was observed, whereas at high concentrations NO potentiates nociception. Studies with the NO donor SIN-1 found that intrathecal high-dose administration increased mechanical allodynia, while low doses produced the opposite effect.[17,18] In addition, a study using human patients showed that the transdermal nitroglycerine patch, which releases low concentrations of NO, reduced pain caused by shoulder or

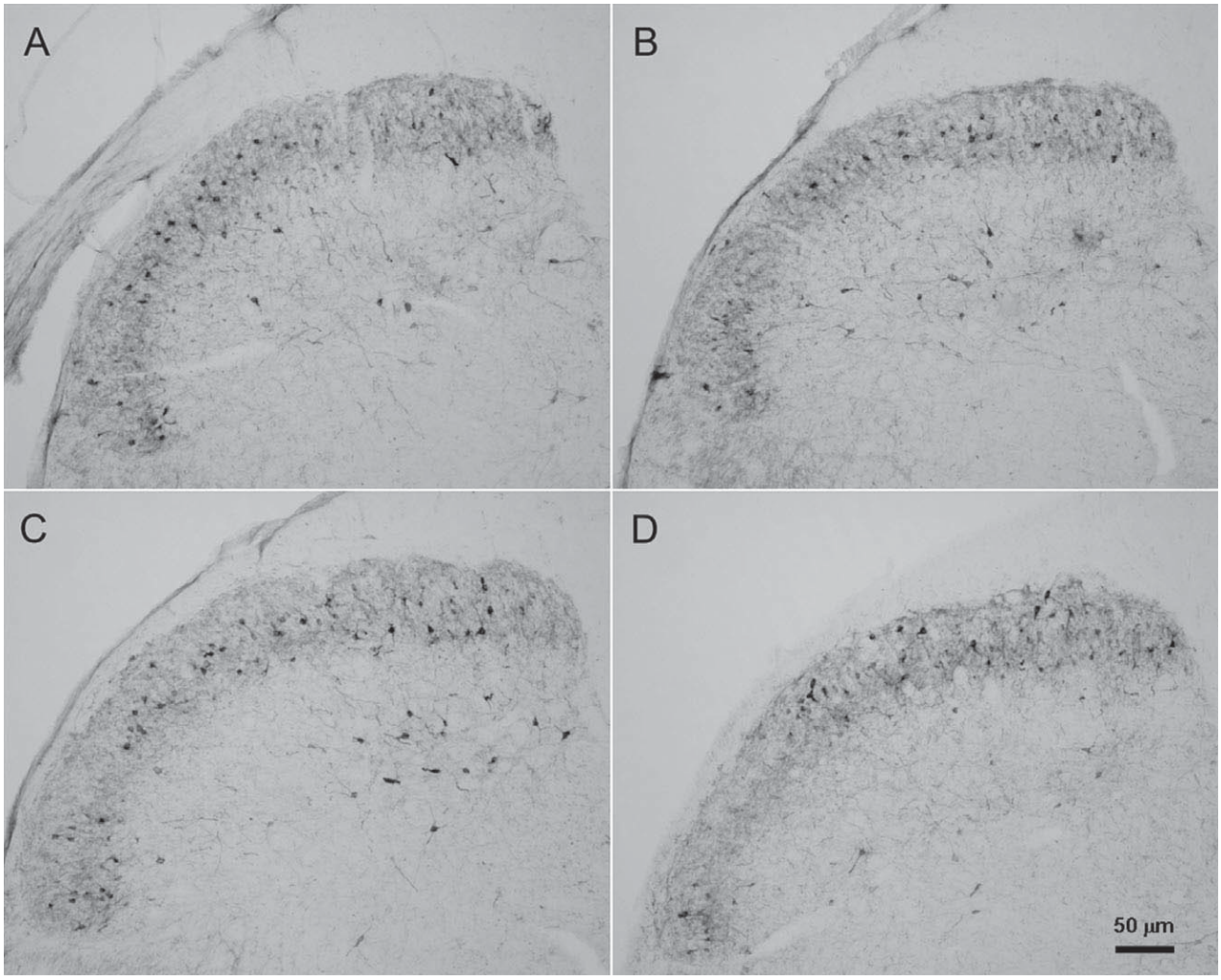

Fig 3.—Effect of L-NAME treatment on cortical spreading depression (CSD)-evoked neuronal nitric oxide synthase (nNOS) expression in the trigeminal nucleus caudalis (TNC). Depleting serotonin with parachlorophenylalanine (PCPA) enhanced nNOS expression in the TNC. CSD-evoked nNOS expression in the TNC (A). The density of nNOS-immunoreactive neurons was attenuated by L-NAME (B). Higher density of CSD-evoked nNOS-immunoreactive cells was observed in the PCPA-treated group (C). More pronounced attenuating effect of L-NAME on CSD-evoked nNOS expression was evident in the PCPA-treated group (D).

elbow injuries.[19,20] On the other hand, hyperalgesia has been reported when a high-dose version of this patch was applied.[21] Interestingly, using the formalin test a dual effect of the cGMP analog 8-bromo-cGRP on nociceptive modulation has also been observed; low doses of 8-bromo-cGMP reduced nociceptive behavior, whereas high doses caused hyperalgesia, which is associated with increased activity of the cGMP-dependent protein kinase I.[22] Together, these findings support the idea that a high concentration of NO via the cGMP signaling pathway is involved in the sensitization of nociception. Therefore, increased NOS expression within the TNC and consequent increased NO production may contribute to sensitization of TNC neurons; this is supported by our finding of increased Fos-IR cells in the serotonin-depleted group.

Based on these findings, we hypothesize that both the activity of nNOS and the production of NO in the cerebral cortex and TNC are increased in the low serotonin state. The resulting high levels of NO in turn increase the excitability of cortical neurons and sensitize the second-order neurons within the TNC, which finally leads to enhanced trigeminal nociception.

Table 2.—Effect of L-NAME on Cortical Spreading Depression-Evoked Neuronal Nitric Oxide Synthase and Fos Expression in Control and Parachlorophenylalanine (PCPA)-Treated Groups

	Control Group			PCPA-Treated Group		
Variable	Saline	L-NAME	% Change	Saline	L-NAME	% Change
NOS-IR in cerebral cortex (cells per 5 mm^2)						
Ipsilateral side	18 ± 3	13 ± 3*	27.8	28 ± 5*	15 ± 4#	46.4
Contralateral side	15 ± 5	12 ± 4	20.0	22 ± 4*	14 ± 4#	36.4
NOS-IR in TNC (cells per section)						
Ipsilateral TNC	25 ± 5	20 ± 3*	20.0	32 ± 5*	21 ± 4#	34.4
Contralateral TNC	16 ± 4	14 ± 3	12.5	21 ± 4*	17 ± 3#	19.0
Fos-IR in TNC (cells per section)						
Ipsilateral TNC	21 ± 3	18 ± 3*	14.3	25 ± 4*	18 ± 3#	28.0
Contralateral TNC	12 ± 4	9 ± 3*	25.0	14 ± 3	12 ± 3#	14.3

*$P < .05$ compared with the saline subgroup in the control group.

#$P < .05$ compared with the saline subgroup in the PCPA-treated group.

Fos-IR = Fos-immunoreactive; NOS-IR = nitric oxide synthase-immunoreactive; TNC = trigeminal nucleus caudalis.

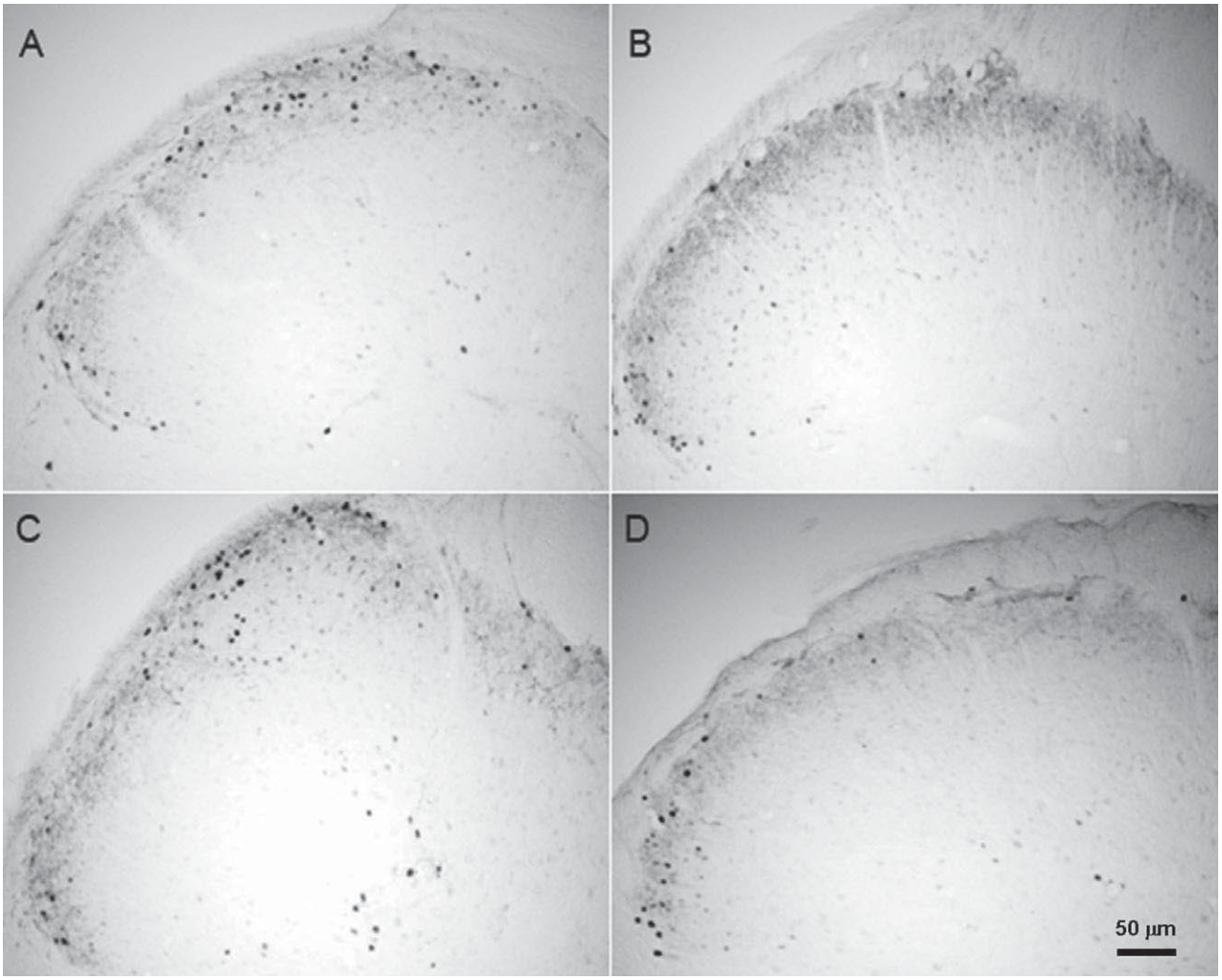

Fig 4.—Triggering cortical spreading depression (CSD) waves induced Fos expression in laminae I and II within the trigeminal nucleus caudalis (TNC). CSD-evoked Fos expression in the TNC (A) and this effect was attenuated by L-NAME (B). The number of Fos-immunoreactive cells was significantly greater in the parachlorophenylalanine-treated group (C). This increase was also attenuated by L-NAME (D).

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STATEMENT OF AUTHORSHIP

Category 1

(a) Conception and Design
Supang Maneesri le Grand; Weera Supornsilpchai; Anan Srikiatkhachorn

(b) Acquisition of Data
Supang Maneesri le Grand; Weera Supornsilpchai; Anan Srikiatkhachorn

(c) Analysis and Interpretation of Data
Supang Maneesri le Grand; Weera Supornsilpchai; Anan Srikiatkhachorn

Category 2

(a) Drafting the Article
Supang Maneesri le Grand; Weera Supornsilpchai; Chonlawan Saengjaroentham; Anan Srikiatkhachorn

(b) Revising It for Intellectual Content
Supang Maneesri le Grand; Anan Srikiatkhachorn

Category 3

(a) Final Approval of the Completed Article
Supang Maneesri le Grand; Weera Supornsilpchai; Chonlawan Saengjaroentham; Anan Srikiatkhachorn

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Effect of the acute and chronic estrogen on anxiety in the elevated T-maze

Sarinee Kalandakanond-Thongsong [a,*], Suwaporn Daendee [b], Anan Srikiatkhachorn [c]

[a] Department of Veterinary Physiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
[b] Inter-disciplinary Program of Physiology, Graduate School, Chulalongkorn University, Bangkok 10330, Thailand
[c] Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

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ABSTRACT

Despite the extensive studies on the influences of estrogen (E_2) on anxiety-like behaviors, there is still conflicting evidence regarding the specific effects of E_2 on anxiety. These discrepancies may be a result of different replacement regimens. The goals of this study were to evaluate anxiety-like behavior in ovariectomized rats (Ovx) using the elevated T-maze (ETM) test for the following variables: (1) the effects of acute versus chronic E_2 dosing, (2) the effects of chronic E_2 at different doses and, (3) the effects of Tamoxifen (Tam) co-administered with E_2. Rats in the acute E_2 dosing group (aE_2) showed reduced inhibitory avoidance responses with prolong escape latencies compared to Ovx; while rats in the chronic E_2 dosing group (cE_2) showed reduced inhibitory avoidance responses only. These results suggest that E_2 contains anxiolytic effects when given once or repeatedly. Moreover, when various doses of E_2 (1–100 μg/kg) were chronically given to the Ovx rats, all doses produced impaired inhibitory avoidance responses compared to Ovx, suggesting that chronic replacement of E_2 had no dose-dependent effect on anxiety-like behavior. Interestingly, in the 3-week delay replacement regimen, the low dose E_2 (1 μg/kg, s.c.) group displayed no anxiolytic effects as their inhibitory avoidance responses in the ETM were not different from their Ovx counterparts. On the contrary, the Ovx group that received Tam + E_2 (Tam 1 mg/kg, PO and E_2 1 μg/kg, s.c.) had reduced inhibitory avoidance responses compared to other groups. These findings indicate that when Tam is co-administered with chronic low dose estrogen, it can act as an estrogen receptor agonist and result in anti-anxiety effects. Therefore, it is likely that the anxiolytic-like behavior relative to generalized anxiety disorder can be conserved when estrogen is given acutely or chronically; while the anxiolytic-like behavior relative to panic disorder can be conserved only when estrogen is given acutely.

1. Introduction

Anxiety disorders are very prevalent in psychiatric illness. Many factors are involved in the etiology of anxiety disorders including genetics, gender, brain chemistry and unmanageable stressful events. Several reports revealed that women were likely to be affected more than men [1,2]. In postmenopausal women, estrogen replacement therapy has been consistently reported to improve symptoms of cognitive function impairment, depression and anxiety [3,4]. This suggests that the decrease in plasma concentrations of the ovarian hormone – estrogen – is associated with an increase in anxiety. To study the anxiolytic effects of estrogen in animals, female rats may be left intact and studied during proestrous, when the level of estrogen is highest. In addition, rats may be ovariectomized (Ovx) and subjected to either acute or chronic systemic estrogen administration. Some studies reported that proestrous female rats had lower levels of anxiety [5,6], while no differences were observed between the phases of the estrous cycle in other studies [7,8]. In Ovx rats, estrogen induced anxiolytic effects when given chronically [9–11] while a single dose of estrogen given to either Ovx or diestrous female rats (with naturally low levels of estrogen) caused anxiolytic effects in some studies [6,12,13] but no effects on anxiety-like behavior in other studies [14,15]. These different results may be related to many factors. In these studies, behavioral testing began at various times after estrogen administration and varied in these studies from as little as 30 min. to as much as 48 h later, and estrogen dosing varied from 2 μg to 100 μg [6,12–15]. In addition, the behavioral responses were detected as early as 10 min. but no longer than 1 h after the administration of estrogen (see reference [16] to revision). Thus, the effects of estrogen on anxiety may depend upon the dose of estrogen and the replacement regimen.

One interesting factor that may confuse the anti-anxiety effects of estrogen is the nature of the behavioral test models. For example, despite utilizing the same model, the elevated plus-maze test (EPM), both anti-anxiety effects and no effects on anxiety were demonstrated in proestrous rats [6,7]. Previously, we reported that proestrous rats and Ovx rats receiving chronic estrogen treatment both demonstrated

* Corresponding author at: Department of Veterinary Physiology, Faculty of Veterinary Science, Chulalongkorn University, Henri Dunant Rd., Bangkok, 10330, Thailand. Tel.: +66 2 218 9533; fax: +66 2 252 0737.
E-mail address: Sarinee.Ka@chula.ac.th (S. Kalandakanond-Thongsong).

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anxiolytic behavior when tested with the elevated T-maze (ETM); however, it is likely that they are exhibiting different forms of anxiety [11]. The ETM, developed by Graeff and colleagues [17], is used to evaluate animal anxiety. It was derived from the EPM. The advantage of this model is that it can evaluate two types of anxiety in the same animal: (1) generalized anxiety disorder (GAD) (learned or conditioned anxiety), represented by inhibitory avoidance behavior, and (2) panic disorder (PD) (innate or unconditioned fear), represented by one-way escape [17–19]. Previous studies reported that inhibitory avoidance was impaired by drugs that were effective in treating GAD [20,21], while one-way escape was increased by chronic treatments that were effective in treating PD [21]. Based on these results, it may be inferred that the inhibitory avoidance task is related to GAD and one-way escape is related to PD [17,19,21]. It also suggests that ETM is a more appropriate test to evaluate GAD and PD, since the results are more consistent than those obtained when using the EPM [22].

Estrogen may mediate its anxiolytic effects through either genomic or non-genomic pathways, so our first experiment was to administer acute or chronic doses of estrogen to the Ovx rats and then test them for anxiety-like behaviors in the ETM. In Experiment 2, the dosages of estrogen were also varied to examine the relationship between estrogen dose and anxiety. Finally, we investigated whether the anxiolytic-like effects of estrogen were specific to estrogen receptors by coadministered tamoxifen, a nonselective estrogen receptor antagonist, in conjunction with estrogen in Experiment 3.

2. Material and methods

2.1. Animals

Female Wistar rats weighing 180–200 g at the beginning of the experiments were obtained from the National Laboratory Animal Center, Mahidol University (NLAC-MU), Thailand. All animals were housed in pairs in shoebox cages (23 cm wide × 40 cm long × 20 cm high) under 12 h light/dark cycles (lights on at 0600 h; light intensity approximately 150 lx) at room temperature (25 ± 2 °C). Standard rat chow and water were supplied *ad libitum*. After a 7-day adaptation period, all rats were bilaterally ovariectomized under anesthesia (Isoflurane; Terrell™, Minrad Inc., Bethlehem, PA, USA). Body weight and amount of food consumed were measured daily. On the day of sacrifice by overdose inhalation of Isoflurane, the uterine weight, which indicates sex hormones deficiency, was determined. Serum estradiol was measured using an enzyme immunoassay test kit (KAP0621; Bio-Source Europe S.A., Belgium). All procedures were done according to the National Institutes of Health Guide for care and use of Laboratory animals under the approval of the Animal Use Committee, Faculty of Veterinary Science, Chulalongkorn University (Protocol numbers 0831055 and 0831056).

2.2. Behavioral evaluation

2.2.1. Elevated T-maze test

The ETM was made of black wood and consisted of three arms with equal dimensions (50 × 10 cm). One arm, enclosed by walls (40 cm high) was perpendicular to two opposed open arms. These three arms were connected by a square (10 × 10 cm). The apparatus was elevated 50 cm above the floor. To prevent rats from falling, the open arms were surrounded by a 1 cm high Plexiglas rim. Each test session consisted of three inhibitory avoidance trials and three escape trials held at 30-s intervals according to the method of Graeff et al. [17]. Between the trials, the animals were placed in the Plexiglas cage. On the first three inhibitory avoidance trials, each animal was placed at the distal end of the enclosed arm facing the center of the maze. The baseline latency was defined as the time(s) required for the rat to leave this arm with all four paws. The same measurement was repeated in two subsequent trials (avoidance 1 and 2). Following avoidance training, the escape trial was done by placed the animal at the end of the right open arm facing the center of the maze. The time the animal took to exit this arm with four paws was recorded and designated as the escape time. For all tasks, a cutoff time of 300 s was established. The behavioral tests were conducted between 0900 am and 1200 pm and testing took place in a dimly illuminated room with the light intensity of 16 lx at the open arm. Between each rat, the maze was carefully wiped with a wet towel.

2.2.2. Open field test

After the ETM session, the animals were tested in the open field for 5 min to measure locomotor activity. The open field test was used in accordance with the methods described by McCarthy et al. [26]. The open field was a black wooden box (76 cm long × 57 cm wide × 35 cm high) with a 48-square grid floor (6 × 8 squares, 9.5 cm per side). The numbers of total crosses that the rat made during the 5 min in this task were recorded as the locomotor activity. The experiments were recorded by a digital camcorder for later analysis. Between each rat, the apparatus was completely cleaned with a wet towel.

2.3. Experiments

2.3.1. Experiment 1 — the effect of acute versus chronic replacement of estrogen

The Ovx rats were randomly assigned into 3 groups: vehicle- (Ovx), acute estrogen- (aE_2) and chronic estrogen- (cE_2) treated Ovx groups. For the cE_2 group, replacement regimens were started 1 day after ovariectomy by daily injections of 17β-estradiol (Sigma, St. Louis, MO, USA) (10 μg/kg in propylene glycol) given subcutaneously into the dorsal region of the neck for 4 weeks. In the Ovx and aE_2 groups, rats received daily injections with an equivalent volume of the propylene glycol; however, on the day of the behavioral test, the aE_2 rats were injected with 17β-estradiol (10 μg/kg) 30 min before the test. The dose of estrogen was based on previous studies [10,11] and is considered a physiological dose as it was able to induce behavioral effects similar to that observed at estrus [23].

2.3.2. Experiment 2 — the effect of different chronic doses of estrogen

The Ovx rats were randomly assigned into 8 groups: vehicle- (Ovx) and estrogen-treated Ovx groups at the doses of 1, 5, 10, 15, 30, 50 or 100 μg/kg. The replacement regimens were started 1 day after ovariectomy by daily injections of 17β-estradiol (1–100 μg/kg in propylene glycol) given subcutaneously into the dorsal region of the neck for 4 weeks. In the Ovx group, rats were injected with an equivalent volume of the propylene glycol. The estrogen doses were selected to incorporate a wide range of doses used in previous studies by several other researchers [6,12–15].

2.3.3. Experiment 3 — the effect of estrogen coadministered with tamoxifen, a nonselective estrogen receptor antagonist

All rats were ovariectomized 3 weeks prior to the beginning of this experiment to ensure the lack of endogenous estrogen before they were randomly assigned into 4 groups: vehicle- (Ovx), tamoxifen- (Tam), estrogen- (E_2) and Tam + E_2 treated Ovx groups. The three-week delay in estrogen replacement was based on a study in our laboratory which indicated that Ovx rats exhibit anxiety after this period of time. In the E_2 and Tam + E_2 groups, the rats were subcutaneously injected with 17β-estradiol (1 μg/kg in propylene glycol); while Ovx and Tam groups were subcutaneously injected with an equivalent volume of the propylene glycol into the dorsal region of the neck. In the Tam and Tam + E_2 groups, the rats were gavaged daily with tamoxifen (Sigma) (1 mg/kg in propylene glycol); while Ovx and E_2 groups received an equivalent volume of the propylene glycol. The treatments lasted for 4 weeks. Tamoxifen was used because it is a non-selective estrogen receptor antagonist that can readily pass the

blood brain barrier, and this dose was chosen based on the previous study on the effects of tamoxifen on food intake and conditioned taste avoidance [24,25].

2.4. Statistical analyses

All data were presented as mean and standard errors of mean (SEM). The avoidance latency in the ETM was analyzed by two-way analysis of variance (ANOVA) with treatment as the independent factor and trials (baseline, avoidance 1 and 2) as the dependent factor. In the case of a significant effect of treatment, data were analyzed by one-way ANOVA followed by the Duncan post hoc test. Other data were submitted to a one-way ANOVA followed by the Duncan post hoc test. Pearson's correlation was used for correlations between parameters. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. The effect of acute versus chronic replacement of estrogen

3.1.1. Body weight, food intake and uterine weight

The initial body weights did not differ among groups (Table 1). Four weeks after ovariectomy, the cE_2 rats demonstrated a significantly lower body weight [$F(2,21)=12.16$; $P=0.0003$] and daily weight gain [$F(2,21)=17.83$; $P<0.0001$] when compared to the Ovx and aE_2 groups (Table 1). Consequently, the percent change of body weight in the cE_2 group was lowered than the Ovx and aE_2 groups [$F(2,21)=13.82$; $P<0.0001$] (Table 1). In addition, the daily food intakes were lower [$F(2,21)=3.71$; $P=0.0416$] in the cE_2 rats compared to the Ovx and aE_2 rats (Table 1). As expected, the uterine weights of the Ovx and aE_2 rats were significantly lower than the cE_2 rats [$F(2,21)=338.97$; $P<0.0001$]. Percentage of uterine-to-body weight ratio (% UW/BW) was also lower in the Ovx and the aE_2 groups than cE_2 group [$F(2,21)=265.80$; $P<0.0001$] demonstrating the effectiveness of ovariectomy and chronic estrogen replacement.

3.1.2. Behavioral tests

The level of anxiety as measured by the ETM is shown in Fig. 1. The inhibitory avoidance trials from the ETM tests revealed a significant effect of treatment [$F(2,63)=3.80$, $P=0.0278$] and trials [$F(2,63)=5.32$, $P=0.0073$]. The Duncan post hoc test showed that the baseline latency was not different among treatments. Significant difference was shown in the avoidance latencies in trial 2, the latency to leave the enclosed arm was decreased in both aE_2 and cE_2 groups compared to the Ovx group [$F(2,21)=4.67$; $P=0.0209$] as shown in Fig. 1A. Moreover, in the escape trial, the escape latency of the aE_2 rats was significantly increased [$F(2,21)=3.81$, $P=0.0387$] compared to Ovx rats (Fig. 1B). In the cE_2 group, although the escape latency was longer than Ovx group and shorter than the aE_2 group, it was not significant different from either group. The locomotor activity of the treated rats as measured by open field test revealed that the total number of lines crossed during the 5 min. test period was not differed among groups [$F(2,21)=2.71$, $P=0.0897$] indicating that it was not affected by treatments (Fig. 1C).

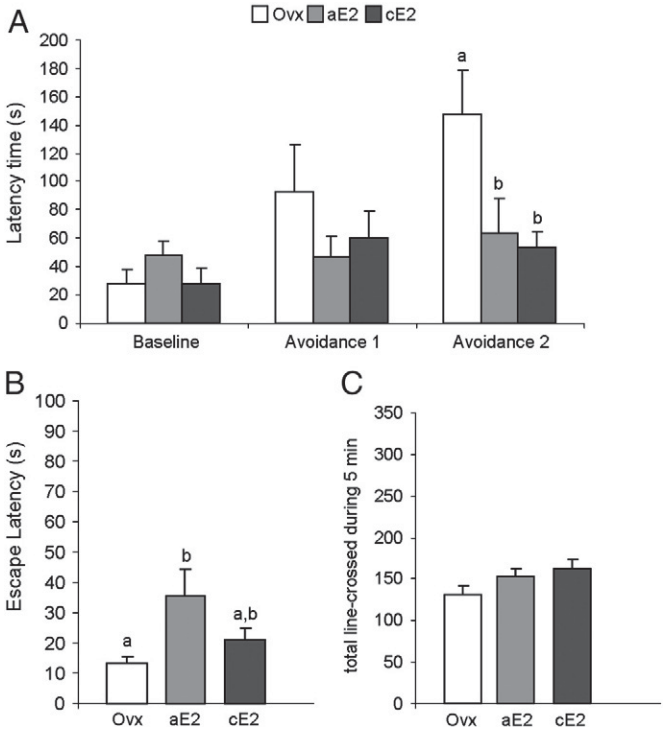

Fig. 1. Effects of acute (aE_2) versus chronic (cE_2) replacement of estrogen compared with Ovx rats on the anxiety-like behavior submitted to inhibitory avoidance (A), one-way escape (B) tasks of the ETM and on the total number of line crossed during 5 min in the open field (C). Data presented as mean ± SEM; [a,b]Different letters indicate statistical differences ($P<0.05$) among groups. $n=8$ for each group.

3.2. The effect of different chronic doses of estrogen

3.2.1. Body weight, food intake, uterine weight and serum estradiol

The body weights at the beginning and at the end of the experiment are shown in Table 2. There was no significant difference in the beginning weight among groups [$F(7,59)=1.73$, $P=0.1201$]. However, 4 weeks after ovariectomy, the body weight of 17β-estradiol treated rats (E_2, 1–100 μg/kg) were less than the Ovx rats [$F(7,59)=5.73$, $P<0.0001$] (Table 2). The daily weight gain and the percent change of body weight from the beginning of the experiment of all groups receiving various doses of estrogen were then lower than those of the Ovx group [$F(7,59)=14.33$, $P<0.0001$ and $F(7,59)=18.93$, $P<0.0001$, respectively] (Table 2). There was no difference in daily feed intake among groups [$F(7,59)=0.97$, $P=0.4639$]. Moreover, significant correlations were found between estrogen dose and the body weight at the end of the experiment ($r^2=-0.45$, $P=0.0020$, $N=67$); daily weight gain ($r^2=-0.45$, $P=0.0001$, $N=67$); and the percent change of body weight ($r^2=-0.46$, $P<0.0001$, $N=67$).

Table 1
Mean body weight, food intake and uterine weight of ovariectomized rats, ovariectomized rats supplemented with acute or chronic estrogen.

Group	BW (g)		DWG (g/day)	% body weight change	DFI (g/day)	UW (g)	UW/BW (%)
	Beginning	End					
Ovx	211.25 ± 3.34	279.69 ± 5.74[a]	2.31 ± 0.16[a]	32.47 ± 2.39[a]	16.40 ± 0.61[a]	0.116 ± 0.003[a]	0.042 ± 0.001[a]
aE_2	207.81 ± 2.24	273.44 ± 7.15[a]	2.23 ± 0.20[a]	31.57 ± 3.13[a]	16.68 ± 1.36[a]	0.109 ± 0.001[a]	0.040 ± 0.001[a]
cE_2	208.75 ± 3.72	240.94 ± 4.77[b]	1.05 ± 0.14[b]	15.52 ± 2.06[b]	13.64 ± 0.27[b]	0.383 ± 0.014[b]	0.160 ± 0.007[b]

Values are mean ± S.E.M. Number of rats was 8 in each group. BW, mean body weight; DWG, daily weight gain; DFI, daily food intake; UW, mean uterine weight; %UW/BW, [UW (g)/BW(g)] × 100.
[a,b]Different superscripts in the same column indicate statistical differences ($P<0.05$) among groups.

Table 2
Mean body weight, food intake, uterine weight and serum estradiol of ovariectomized rats and ovariectomized rats supplemented with different chronic doses of estrogen.

Group	BW (g)		DWG (g/day)	% body weight change	DFI (g/day)	UW (g)	UW/BW (%)	Serum estradiol (pg/ml)
	Beginning	End						
Ovx	197.22 ± 2.61	269.44 ± 6.18^{a}	2.13 ± 0.18^{a}	36.65 ± 2.81^{a}	13.29 ± 0.37	0.122 ± 0.005^{a}	0.045 ± 0.002^{a}	44.40 ± 8.49^{a}
E1	210.00 ± 3.65	253.75 ± 8.46^{a,b}	1.35 ± 0.17^{b}	20.75 ± 2.92^{b}	13.22 ± 0.54	0.412 ± 0.011^{b,c}	0.163 ± 0.008^{b}	40.75 ± 1.11^{a}
E5	204.75 ± 3.52	240.75 ± 5.26^{b,c}	1.10 ± 0.13^{b,c}	17.65 ± 2.00^{b,c}	12.75 ± 0.24	0.385 ± 0.013^{b}	0.161 ± 0.006^{b}	67.40 ± 10.71^{a}
E10	210.50 ± 3.63	240.75 ± 4.07^{b,c}	0.92 ± 0.09^{c,d}	14.47 ± 1.50^{b,c,d}	12.64 ± 0.43	0.436 ± 0.029^{b,c}	0.181 ± 0.012^{b,c}	121.50 ± 51.55^{a}
E15	206.94 ± 2.69	231.94 ± 4.14^{c}	0.76 ± 0.14^{c,d}	12.18 ± 2.14^{c,d}	11.90 ± 0.32	0.462 ± 0.016^{c,d}	0.200 ± 0.009^{c,d}	151.40 ± 17.77^{a}
E30	208.44 ± 3.84	238.13 ± 6.39^{b,c}	0.92 ± 0.10^{c,d}	14.13 ± 1.04^{b,c,d}	12.24 ± 0.44	0.543 ± 0.022^{e,f}	0.230 ± 0.013^{e,f}	184.50 ± 46.97^{a}
E50	210.71 ± 3.96	235.36 ± 4.83^{c}	0.77 ± 0.11^{c,d}	11.77 ± 1.97^{c,d}	12.37 ± 0.35	0.509 ± 0.022^{d,e}	0.217 ± 0.009^{d,e}	318.20 ± 64.42^{a}
E100	208.75 ± 3.72	230.00 ± 5.20^{c}	0.64 ± 0.07^{d,d}	10.14 ± 0.92^{d}	13.13 ± 1.04	0.584 ± 0.019^{f}	0.255 ± 0.009^{f}	1008.80 ± 231.26^{b}

Values are mean ± S.E.M. Number of rats was 6–10 in each group. BW, mean body weight; DWG, daily weight gain; DFI, daily food intake; UW, mean uterine weight; %UW/BW, [UW (g)/BW(g)] × 100.
^{a,b,c,d,e,f} Different superscripts in the same column indicate statistical differences ($P<0.05$) among groups.

Lack of ovarian hormones was confirmed by the reduction in uterine weight in Ovx rats. Various doses of estrogen had uterotrophic effect as shown by increased in uterine weight and ratio of UW/BW [$F(7,58) = 14.33$, $P<0.0001$; $F(7,58) = 52.18$, $P<0.0001$, respectively] (Table 2). Moreover, a positive correlation between the dose of estrogen and the uterine weight was found ($r^2 = +0.62$, $P<0.0001$, $N = 66$).

Serum estradiol levels increased as the dosage of exogenous estradiol was increased; however, the difference was significant only when the highest dose used (100 μg/kg) as shown in Table 2 [$F(7,29) = 12.17$, $P<0.0001$]. The increase in serum estradiol was correlated to the doses of estrogen ($r^2 = +0.84$, $P<0.0001$, $N = 37$).

3.2.2. Behavioral tests

The level of anxiety as measured by the ETM is shown in Fig. 2. The inhibitory avoidance trials from the ETM tests revealed a significant effect of treatment [$F(7,177) = 2.64$, $P = 0.0128$] and trials [$F(2,177) = 26.16$, $P<0.0001$]. The Duncan post hoc test showed that the baseline latency was not different among treatments [$F(7,59) = 1.44$, $P = 0.2067$].

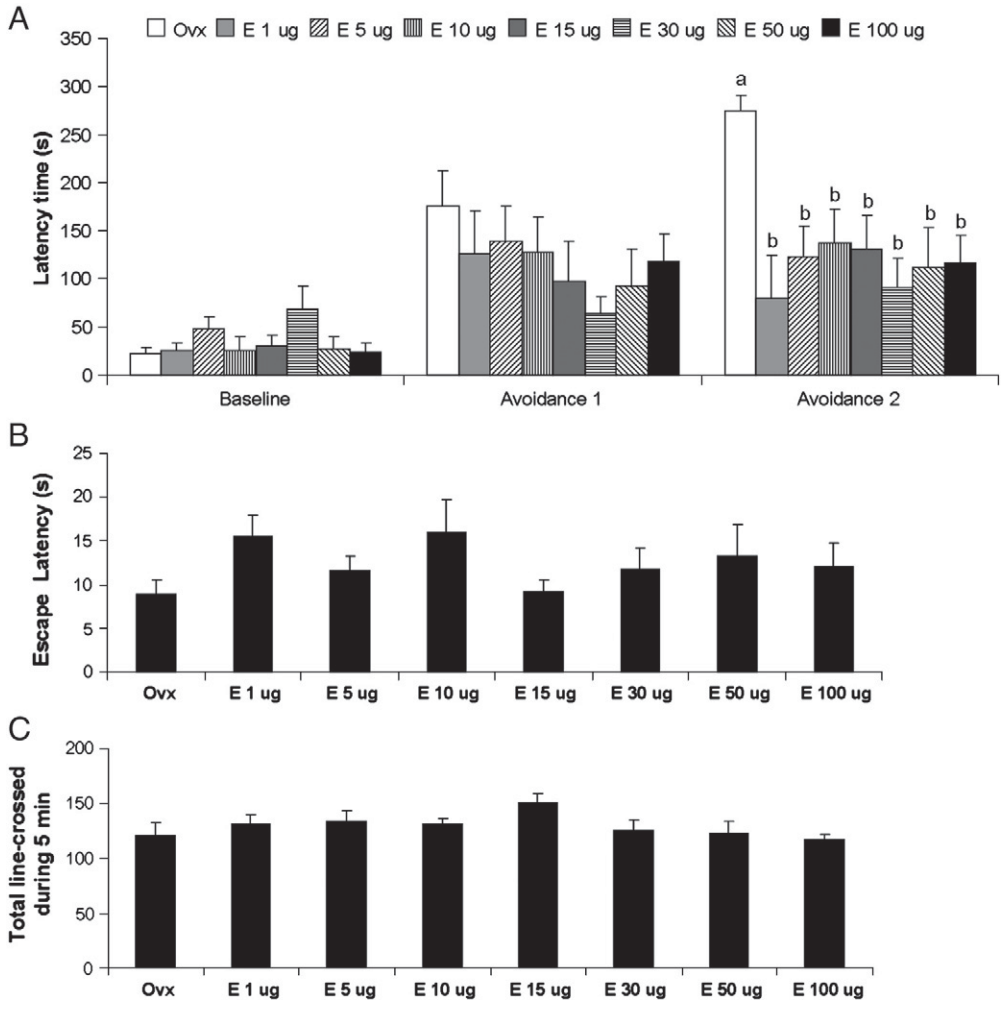

Fig. 2. Effects of chronic various doses of estrogen (1–100 μg/kg) compared with Ovx rats on the anxiety-like behavior submitted to inhibitory avoidance (A), one-way escape (B) tasks of the ETM and on the total number of line crossed during 5 min in the open field (C). Data presented as mean ± SEM; ^{a,b} Different letters indicate statistical differences ($P<0.05$) among groups. n = 6–10 for each group.

Significant difference was shown in the avoidance latencies in trial 2 [$F(7,59)=3.36$, $P=0.0044$] in that the inhibitory avoidance was impaired in the rats treated with estrogen (1–100 μg/kg) (Fig. 2A). The escape latency was not significantly different among treatments [$F(7,59)=1.02$, $P=0.4302$] (Fig. 2B). For the open field test, the total number of crosses in the open field was not different among groups [$F(7,59)=1.42$, $P=0.2140$] as shown in Fig. 2C.

3.3. The effect of estrogen coadministered with tamoxifen, a nonselective estrogen receptor antagonist

3.3.1. Body weight, food intake and uterine weight

The body weights at the beginning of the experiment and at 3 weeks following ovariectomy did not differ among groups (Table 3) [$F(3,23)=0.45$; $P=0.7175$ and $F(3,23)=0.36$; $P=0.7835$, respectively]. However, four weeks after treatment with E_2 and/or Tam, the body weight of E_2, Tam and Tam + E_2 rats demonstrated a significantly lower body weight than Ovx rats [$F(3,23)=9.68$; $P=0.0003$] (Table 3). The percent change in body weight from the beginning of the treatment revealed that E_2, Tam and Tam + E_2 treated rats had significantly lower body weight when compared to Ovx rats [$F(3,23)=42.79$; $P<0.0001$] (Table 3). This corresponded to the lower daily food intakes in these groups compared to the Ovx rats [$F(3,23)=4.91$; $P=0.0088$] (Table 3). Uterine weights and the percentage of uterine-to-body weight ratio (% UW/BW) of the Ovx and Tam + E_2 rats were significantly lower than the E_2 rats [$F(3,23)=5.39$; $P=0.0059$ and $F(3,23)=3.40$; $P=0.0348$, respectively]. In the Tam rats, the uterine weights and the ratio of UW/BW were higher than the Ovx and the Tam + E_2 groups, and lower than the E_2 group; however, the differences were not statistically significant.

3.3.2. Behavioral tests

The level of anxiety as measured by the ETM is shown in Fig. 3. The inhibitory avoidance trials from the ETM tests revealed a significant effect of treatment [$F(3,69)=5.31$, $P=0.0024$] and trials [$F(2,69)=5.94$, $P=0.0042$]. The Duncan post hoc test showed that the baseline latency was not different among treatments. Significant difference was shown in the avoidance latencies in trial 2; the latency to leave the enclosed arm in the avoidance trial 2 was decreased in Tam + E_2 groups when compared to Ovx, Tam and E_2 groups [$F(3,23)=3.30$; $P=0.0384$] as shown in Fig. 3A. For the escape trial, there was no significant difference in the escape latency between treatments [$F(3,23)=1.21$, $P=0.3194$] (Fig. 3B). The total number of crosses in the open field was not different among groups [$F(3,15)=2.20$, $P=0.1306$] indicating that the locomotor activity was not affected by treatments (Fig. 3C).

4. Discussion

The suggestion that acute and chronic estrogen treatments would influence anxiety-like behaviors differently was supported. Utilizing the ETM, an animal model that evaluates innate (panic) and learned (generalized) anxiety, we found that acute (aE_2) estrogen treatment resulted in reduced inhibitory avoidance responses with prolonged escape latencies. Therefore, estrogen given as a single dose is anxiolytic with respect to both GAD and PD. On the other hand, we found that chronic (cE_2) estrogen treatment resulted in reduced inhibitory avoidance responses, but produced no effect on escape latencies. This suggests that estrogen given as a repeated dose is anxiolytic with respect to GAD only. Since the locomotor activity, as measured by the total numbers of lines crossed in the open field arena, were not different among groups, we concluded that the changes in anxiety parameters obtained in the ETM were not due to any alterations in locomotor activity.

Despite different behavioral models, the chronic replacement of estrogen has been reported to consistently reduce anxiety [7–11,27,28]. On the contrary, the acute administration of estrogen has been reported to either produce no effect [7,15] or anxiolytic effects [6,12,13,29,30]. This discrepancy could be due to the site of estrogen administration (systemic versus site-specific injection); time of behavioral test after estrogen administration; dose of estrogen; and the nature of the behavioral tasks. In the current study, the dose of estrogen used in the acute treatment (10 μg/kg) was considered a physiological dose as it was able to induce behavioral effects similar to that observed at estrus [23] and it was used in several other studies [7,12–15]. It is therefore likely that the delay in behavioral testing after estrogen administration is at least partially responsible for these different effects. It is well accepted that estrogen mediates its effects through both genomic and non-genomic pathways. The non-genomic pathway is relatively rapid; it can occur within 10–15 min, lasts for approximately 30–45 min. and is no longer detected 1 h after the administration [see reference 16 to revision]. Therefore, it is possible that studies which waited 3 h after systemic estrogen administration before beginning behavioral testing [7,14,15], waited too long to observe any anxiolytic effects through the non-genomic pathway and did not wait long enough to observe effects activated by the genomic pathway. Additionally, it has been reported that estrogen injected 48 h prior to behavioral testing has induced anxiolytic effects in Ovx rats [12,13]. These results were likely mediated through the genomic pathway, which could be activated in this time frame. Considering this, it is likely that the anxiolytic effects observed in our acute estrogen treatment group occurred through the non-genomic pathway since the estrogen was injected 30 min prior to behavioral testing. However, it is not possible to determine whether the anxiolytic effects observed in our chronic treatment group were mediated solely through the genomic or non-genomic pathway, and, in fact, it is possible that both pathways were involved.

To determine what role the amount of estrogen has on anxiety-like behavior, doses of estrogen ranging from as low as 2 μg/kg [12] to as high as 100 μg/kg [7] were administered to Ovx rats in the second experiment of this study. Behavioral data in the ETM revealed that all doses of estrogen (1–100 μg/kg) were able to impair the avoidance 2 latency time suggesting an anxiolytic effect in terms of GAD-like behavior. Interestingly, there was no correlation between doses of estrogen and the avoidance 2 latency; while the uterine weight, body weight and serum estradiol levels were changed with respect to estrogen dose. It is thus possible that in order to mediate anxiolytic effects, very high levels of estrogen are not required but

Table 3
Mean body weight, food intake and uterine weight of ovariectomized rats, ovariectomized rats treated with estrogen and/or tamoxifen.

Group	BW (g)			% body weight change	DFI (g/day)	UW (g)	UW/BW (%)
	Beginning	3-week post-Ovx	4-week treatment				
Ovx	205.42 ± 4.67	259.17 ± 5.69	281.25 ± 3.97[a]	8.63 ± 1.24[a]	13.12 ± 0.72[a]	0.145 ± 0.029[a]	0.053 ± 0.011[a]
E_2	201.25 ± 6.45	259.58 ± 9.23	250.83 ± 8.96[b]	−3.36 ± 0.64[b]	11.90 ± 0.26[a,b]	0.268 ± 0.027[b]	0.151 ± 0.014[b]
Tam	204.06 ± 4.22	265.94 ± 5.73	253.44 ± 4.93[b]	−4.61 ± 1.30[b,c]	10.90 ± 0.41[b]	0.205 ± 0.020[a,b]	0.123 ± 0.037[a,b]
Tam + E_2	198.58 ± 2.44	258.21 ± 3.96	238.93 ± 3.65[b]	−7.46 ± 0.64[c]	11.45 ± 0.13[b]	0.175 ± 0.003[a]	0.073 ± 0.001[a]

Values are mean ± S.E.M. Number of rats was 6–8 in each group. BW, mean body weight; DWG, daily weight gain; DFI, daily food intake; UW, mean uterine weight; %UW/BW, [UW (g)/BW(g)] × 100.
[a,b]Different superscripts in the same column indicate statistical differences ($P<0.05$) among groups.

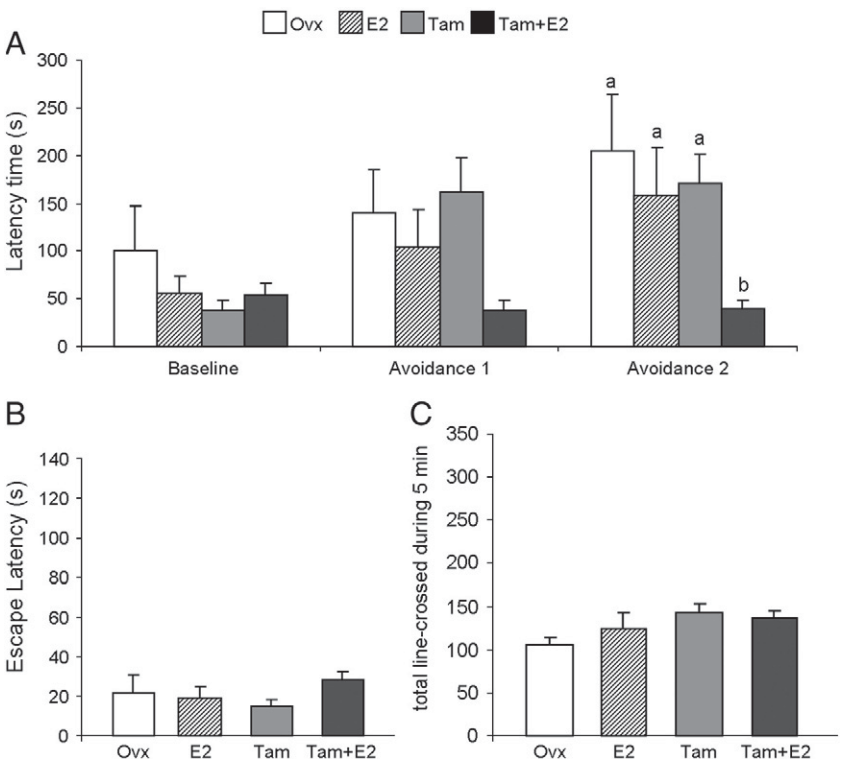

Fig. 3. Effects of estrogen coadministered with tamoxifen (Tam), a nonselective estrogen receptor antagonist on the anxiety-like behavior submitted to inhibitory avoidance (A), one-way escape (B) tasks of the ETM and on the total number of line crossed during 5 min in the open field (C). Data presented as mean ± SEM; [a,b] Different letters indicate statistical differences ($P<0.05$) among groups. n = 6–8 for each group.

rather there is a threshold level. This finding was similar to Hsiao et al. [31] in that they demonstrated there was no correlation between plasma estrogen levels with regard to anxiety in patients with premenstrual dysphoric disorder. On the contrary, Walf and Frye [12,27] demonstrated that estrogen may have a dose dependent effect on anxiety, depressive, sexual and motor behaviors. This controversy may be in part influenced by the study's protocol as Walf and Frye [12,27] used a single dose of estrogen, and the rats were exposed to a restraint stress prior to the behavioral test [12], or were gavaged with a chemical carcinogen [27]. Nonetheless, this study verified that a low dose replacement regimen (1 μg/kg) was indeed sufficient to have anxiolytic-like effects in Ovx rats when given chronically.

In the last experiment, we first hypothesized that the estrogen conserved anxiolytic effects could be countered by tamoxifen (Tam), the non-selective estrogen receptor antagonist; however, we found that estrogen or Tam alone resulted in an insignificantly lower level of anxiety compared to Ovx. Intriguingly, the co-administration of estrogen with Tam resulted in the shortening of avoidance trial 2 latencies compared to other groups suggesting Tam together with estrogen mediated anxiolytic effects with respect to GAD. In this part of experiment, the replacement regimen was different from the first two experiments in that the rats were ovariectomized for 3 weeks before estrogen replacement was started and the estrogen was given for 4 weeks. The delay regimen was done in order to minimize any effects that might have been caused by the lower level of the endogenous estrogen. In this task, low dose estrogen (1 μg/kg) did not conserve the anxiolytic-like effects as seen in the previous experiment. Plausible explanations for this difference include: (1) a 3-week delay in treatment may have affected the neuronal/neurotransmission pathway involved with anxiety, and (2) the stress effect from daily gavage may have influenced the results. However, it is unlikely that the 3-week delay would have irreversible effects on neuronal pathways and data from our laboratory has revealed that this is not the case (data not shown). Therefore, it is possible that the daily gavaging may have induced stress and activated the hypothalamic-pituitary-adrenal axis (HPA). Walf and Frye [12] previously found that restraint stress can attenuate the anti-anxiety and anti-depressive effects of estrogen. There were two interesting points with regard to the actions of TAM. First, Tam-treated rats showed anxiety levels somewhat comparable to E_2-treated rats. Second, when Tam was given in combination with E_2, the effects were very noticeably anti-anxiety. From the uterine weight of the Tam + E_2 group, it can be determined that the uterotrophic effect of E_2 was antagonized by Tam; as the uterine weight was lower than the E_2 group and not different from the Ovx group. This suggests that Tam was acting as an estrogen receptor antagonist in the uterus; however, this effect was not extended to behavior. This may be explained by the fact that Tam had been shown to be a tissue-specific estrogen agonist or antagonist. For example, in breast tissue Tam is an estrogen receptor antagonist while in uterine tissue Tam exhibits estrogen receptor agonist properties [reviewed by 32]. In the brain, Tam may act as an estrogen receptor agonist by produced conditioned taste avoidance [25] or suppress food intake [24,33]; or as an estrogen receptor antagonist by blocking the anti-anxiety behavior produced by estrogen [13]. These diverse findings in the brain can be partly explained by: (1) the replacement regimen (acute [13] versus chronic treatment (as in the current study)); (2) the level of endogenous estrogen. It is worth noting that the action of Tam as a selective estrogen receptor modulator (SERM) is dependent upon the level of endogenous estrogen. In the intact female or in the presence of estrogen, Tam acted as an estrogen receptor antagonist by causing a reduction in uterine weight, as seen in this study and in others [34,35]; while in the absence of estrogen, it acted as an estrogen receptor agonist by increasing uterine weight, as shown here and as seen in postmenopausal women receiving Tam [36]. It is therefore possible that under some circumstances, low dose estrogen may act in concert with Tam which acts as an estrogen receptor agonist, resulting in anxiolytic effects.

In conclusion, the present study suggests that acute estrogen administration produces anxiolytic effects in terms of anti-GAD and anti-PD responses; while chronic estrogen administration produces anxiolytic effects with respect to anti-GAD responses only. Moreover, we also demonstrated that there was no correlation between various doses of estrogen (1–100 μg/kg) and the level of anxiety when given chronically. Intriguingly, tamoxifen, a selective estrogen receptor modulator, may act as an estrogen receptor agonist resulting in anti-anxiety effects when used concurrently with low dose estrogen. These findings provide valuable information which may help to elucidate the complex regulation of anxiety by estrogen.

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Research Submission

Potential Risk Factors for Psychiatric Disorders in Patients With Headache

Chaichana Nimnuan, MD, PhD; Thanin Asawavichienjinda, MD, PhD; Anan Srikiatkhachorn, MD

Background.—Psychiatric comorbidities are common among patients with headache. These can compromise the quality of life of patients and may affect the result of treatment. No available systematic study concerning this problem has been conducted in Thailand.

Objective.—The study aimed to determine the prevalence and risk factors of psychiatric disorders in patients with headache in tertiary care facility.

Methods.—The study was conducted at the Headache Clinic, King Chulalongkorn Memorial Hospital in Bangkok, Thailand. One hundred and thirteen patients were enrolled. Diagnosis of headache was made based on International Classification of Headache Disorders II system. Mental disorders were assessed using Primary Care Evaluation of Mental Disorders. Other possible risk factors were extracted using significant physical symptoms count and accumulated risk for mental disorder.

Results.—Of the 113 samples analyzed, the prevalence of depression, anxiety, and somatoform disorder was found to be 29.2%, 9.7%, and 27.4%, respectively. No definite relationship between headache types and mental disorders was observed. High number of significant physical complaints and health concerns significantly increased the risk for depression (OR = 4.6, 95% CI = 1.6 to 13.5) while the level of possible risk for mental disorder was associated with an increased risk for somatoform disorder (OR = 1.6, 95% CI = 1.2 to 2.2).

Conclusion.—The study confirmed high prevalence of psychiatric comorbidities in patients with headache. The results of this study will raise the awareness of physicians to possible underlying mental disorders in patients with headache and facilitate appropriate treatment or psychiatric referral.

Key words: psychiatric comorbidity, headache, risk factor

Abbreviations: ARMD accumulated risk for mental disorder, PHQ Patient Health Questionnaire, SPSC significant physical symptoms count

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The co-occurrence of various psychiatric symptoms and migraine has long been observed and the comorbidity of migraine is a rule rather than an exception. It usually coincides with medically unexplained physical symptoms such as fibromyalgia[1,2] and other mental conditions. Commonly observed physical conditions associated with migraine include angina, hypertension, colitis ulcer, stroke, asthma, epilepsy, essential tremor, and allergies.[3-5] Symptoms that

From Department of Psychiatry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (C. Nimnuan); Department of Internal Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (T. Asawavichienjinda); Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (A. Srikiatkhachorn).

Address all correspondence to A. Srikiatkhachorn, Department of Physiology, Faculty of Medicine, Chulalongkorn University, Rama IV Road, Patumwan, Bangkok 10330, Thailand, email: anan.s@chula.ac.th

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overlap with psychiatric disorders, including sleep disorder, decreased energy, anhedonia, decreased concentration, and decreased libido, are also well documented.[6]

The comorbidity of migraine with mood and anxiety disorders has long been noted. Data available are consistent and indicate lifetime prevalence of major depression in persons with migraine to be 3 to 5 times higher than those without migraine.[7-9] Psychiatric comorbidities severely impact on individuals suffering from migraine and on society at large. Comorbidity can alter the clinical course of migraine, its prognosis, and the quality of life of those afflicted. Merikangas and colleagues reported that 1-year prevalence of dysthymia and major depression in persons with migraine to be 6.6 and 14.7%, respectively (odds ratio [OR] = 1.8 and 2.2, compared with persons without migraine).[10] Lifetime prevalence of major depression is approximately 3 to 4 times higher in persons with migraine compared with controls.

Breslau and colleagues compared the incidence of first-onset major depression in a 2-year follow-up period in persons with migraine, persons with severe headache awaiting migraine diagnosis, and nonheadache controls.[11] Results showed the incidence of major depression in the migraine group to be 10.5%, in the severe headache group 5.1%, and in the controls 2.0%. Based on these rates, the OR for major depression in persons with migraine was 5.8 (95% confidence interval [CI] 2.7 to 12.3) and 2.7 (95% CI 0.9 to 8.1) in those with nonmigraine severe headache. Preexisting major depression also increased the risk of new-onset migraine. The incidence of new-onset migraine in a 2-year follow-up period in persons with histories of major depression was 9.3%, compared with 2.9% in those without. The OR of migraine associated with prior major depression was 3.4 (95% CI 1.4 to 7.6).

Regarding anxiety disorders, in a population study, men with history of panic disorder had approximately 7 times as high risk to have migraine compared with those without. For women, the risk was lower (relative risk = 3.7).[12] A 14-month follow-up study revealed that patients with migraine at baseline had significantly increased rate of panic disorder (OR = 12.8, 95% CI 4.1 to 39.8).[13] Data drawn from nationally representative sample in the United States revealed that persons with migraine, adjusted for demographic variables, had greater risk of having panic (OR 2.37; 95% CI 1.42 to 3.99) or generalized anxiety disorders (OR 3.13; 95% CI 1.56 to 6.30).[14]

Somatoform disorders in patients with headache have been less well studied than depression or anxiety. Okasha and colleagues found that the prevalence of somatoform pain in patients with nonorganic, nonspecific headache was 43% compared with only 20% in organic headache group.[15] An international study conducted by a group of Italian investigators on tension type headache using structured interview reported that about 22% of subjects had somatoform disorder. In the same study, the investigators also examined the prevalence of psychosocial stress and found that 30% of all subjects had any type of stress, with 14% contributing to occupational stress.[16]

Despite the high prevalence of psychiatric morbidity in patients with migraine, to date there has been no systematic study of psychiatric morbidity of migraine in Thailand. Only one study reports 8% of subjects with migraine as having been diagnosed with mental stress prior to visiting their headache clinics and 42% as having mental stress as a precipitating factor for the migraine they suffer.[17] Specific psychiatric disorders were not identified in this report. Our primary aim is, therefore, to determine prevalence of psychiatric morbidities in patients with headache using standardized measure. The secondary aim is to address potential factors associated with psychiatric comorbidities in patients with headache.

METHODOLOGY

Sample.—All new subjects from the Headache Clinic at the King Chulalongkorn Memorial Hospital were approached. Only Thai-speaking patients presenting at the clinic aged 18 and older were recruited. Those diagnosed with psychotic or organic brain syndromes or who were unable to communicate their consent were excluded. Data collection took place at the end of 2007. The size of the sample was determined based on prevalence study design. The fact that

the prevalence of mental disorders in patients with migraine varies from one study to another made the sample size difficult to estimate. The confidence level required at the end of the study was set at 95%. The maximum allowable difference between the estimate and the true prevalence was set at 10%. Using an approximate reference value of 50%, adjusted to about 20% for missing data, the final sample size was approximately 120.

Measurement.—*Demographic Data.*—This consisted of age, gender, marital status, level of education, and employment status. Demographic variables were collected using a checklist questionnaire.

Disease-Related Variables.—Using the Data Extract form, the following details were collected from the files of patients: headache diagnosis, duration of headaches, and limitation of function. Headaches were diagnosed based on International Classification of Headache Disorders and Cranial Neuralgia (second edition) system.[18] Case notes were also reviewed for investigation results and physicians' final diagnoses approximately 3 months after the initial visits. This was carried out to assure there was no medical diagnosis responsible for physical complaints. With this, diagnosis of somatoform disorder could be made correctly.

Psychiatric Morbidity.—Designed for primary care settings, the Primary Care Evaluation of Mental Disorders (PRIME-MD) is a standardized measure which encompasses a variety of mental disorders. It was designed to assist clinicians as they screen for common psychiatric disorders often associated with unexplained physical complaints that may go unrecognized during regular visits. The PRIME-MD consists of 2 instruments. The first is the Patient Health Questionnaire (PHQ) which assesses somatoform, depression, panic and anxiety, binge eating, alcohol use, social and occupational functions, health concerns, weight concerns, sexual dysfunction, posttraumatic stress disorder, stressful events, psychiatric medication, and menstrual status. The second instrument is the Brief PHQ, a shorter version of the former which excludes the somatoform, eating problems, and alcohol use sections. Since the PHQ was developed, both the original and the brief versions have been extensively used. Strong psychometric properties of the PHQ compared with Structured Clinical Interview for *Diagnostic and Statistical Manual, Fourth Edition* were also evident.[19] In this study, the PHQ, an updated version of the original PRIME-MD, developed by Spitzer and colleagues, was used.[20] The PHQ was translated from English into Thai by one of the investigators (CN) and back-translated by a native English-speaking expert. The translation process was further verified at meetings of the research team and translators.

In this study, we focused on diagnosis of somatoform disorder (PHQ1a-m), depressive syndrome (PHQ2a-i), and anxiety syndrome (PHQ3a-d, 4a-k, 5a-g), which were considerably common in patients with headache. We also included other possible risk factors such as significant physical symptoms count (SPSC) extracted from PHQ1, with taken values 0-1, 2, 3, 4, or higher; accumulated risk for mental disorder (ARMD) from PHQ12d-i, with taken values 0, 1, 2, 3, 4, 5, or higher, and type of stress from PHQ14 which consisted of none, health issue, study/work/finance issue, and relationship issue of concern.

The PHQ was subsequently tested and piloted for content validity by experts and for acceptability by a group of subjects with similar characteristics to our sample. To determine the psychometric property of the Thai-PHQ, the measure was systematically validated through scale evaluation including item analysis and reliability assessment. Acceptability by subjects and descriptions of items and their response distribution, together with inter-item correlations relevant to each subscale, were assessed. Internal consistency, based on values of item-total correlation (0.3 or higher), was determined. The reliability of each subscale was checked and the acceptable value was set at 0.70 at the minimum. Content validity was confirmed by experts in psychiatry. Reliability of scales, namely depression, anxiety and somatoform, was estimated using internal consistency with the Cronbach's alpha value being 0.79, 0.90, and 0.78, respectively.

Data Collection and Management.—After thorough validation, the questionnaires were distributed by the drop-off and pickup data collection method. The general aim of the study was explained. Patients were reassured that all responses would be treated

confidentially and that they would not be disclosed to their doctors. It was also stressed to them that they were under no obligation to participate in the study. The study was approved by the Research Ethics Committee of the Faculty of Medicine at Chulalongkorn University (Protocol no. 17349). The data set was checked and edited for data entry errors. The distribution of continuous variables, such as age and education, was examined visually. Categorical variables were checked to ensure that the frequencies of each category made sense.

Statistical Analysis.—The presence of mental disorders was presented as percentage units using all eligible subjects as denominators. Univariate analysis examined relationships between mental disorders and possible risk factors for each pair. Chi-square value was generally reported with an exception of variables with natural ordering where chi-square test for trend was applied.

Logistic regression analysis was subsequently used to examine relationships among various variables to identify potential confounders. As an exploratory study, many variables could be confounders. Criteria for including variables into the regression models were based on clinical and statistics relevance. All clinically possible confounders, such as sex and age, were systematically included into the models despite nonstatistically significant effects ($P > .05$). Other potential confounders were included based on the results of univariate analysis. Any variables with P values from univariate analysis lower than .20 were considered. Although some were weakly associated with the outcomes, they could be statistically important when taken together in the model.

Modeling was then carried out by iteration, ie, the process of deleting and fitting repeatedly until all relevant variables were reasonably included and the clinically and statistically unimportant variables were excluded. Risk factors in the last model were reported as OR with 95% CI. To increase power to detect an association with an ordered variable, such as age group, SPSC, and ARMD, a trend was estimated to assume linearity by likelihood ratio test. All modeling was performed by STATA 8 software package.[21] All P values calculated were two tailed.

RESULTS

One hundred and twenty-four consecutive new patients at the Headache Clinic were recruited over a period of 12 months. Of these, 3 failed to return their questionnaires, 5 returned it incomplete – with more than 50% of the data missing, and 3 did not have any headache diagnosis. Overall, 113 units were analyzed (Table 1). Most subjects were female (81%) and in the workforce (65%). Migraine was the most common (65%) diagnosis. Over 75% had suffered from chronic headache for longer than 3 months. Most of the sample (70%) had some degree of dysfunction. Approximately 44% (50/113) had at least 1 psychiatric disorder, 17% (19/113) had at least 2 psychiatric disorders, and 5% had 3 disorders (6/113). Among psychiatric disorders, depression was the most common (29%, 33/113). Somatoform disorder, however, was most common among patients with migraine and other primary headaches (Table 2).

Our findings show that the total number of significant physical complaints and the level of risk of mental disorders increase the risk of depression (Table 3). Type of stress was also significantly associated with depression. Results of logistic regression modeling showed that health issues compared with other types of stress was the strongest predictors of depression (OR = 4.6, 95% CI = 1.6 to 13.5). An increase in the level of SPSC was another significant factor associated with the occurrence of depression (OR = 2.0, 95% CI = 1.3 to 3.0) while the level of ARMD was associated with depression only marginally (OR = 1.3, CI = 1.0 to 1.8).

Univariate analysis shows that SPSC, the types of stress, and headache diagnosis were considerably related to anxiety. Logistic regression, adjusting for potential confounders, reveals that only type of stress, that is, health issues compared with none or other types of stress concern, significantly increased risk of anxiety (OR = 13.4, 95% CI = 1.6 to 113.4). The association of SPSC and headache diagnosis with anxiety in univariate analysis was likely to be due to confounding effects.

To examine associations with somatoform disorder, SPSC was excluded from the analysis. The fact that somatoform diagnosis can be made by the

Table 1.—Characteristics of Sample (N = 113)

		n	%
Sex	Male	21	18.6
	Female	92	81.4
Age (years)	15-24	17	15.0
	25-34	27	23.9
	35-44	24	21.2
	45-54	25	22.1
	55-64	13	11.5
	≥65	7	6.2
	Mean = 40.8, SD = 15.01, minimum = 15, maximum = 79		
Marital status	Married/cohabiting	60	53.1
	Single	34	30.1
	Other	19	16.8
Employment status	Full-time – income earning	59	52.2
	Part-time – income earning	14	12.4
	Unpaid occupations (eg, housewives)	26	23.0
	Income earning but not in the workforce (eg, retired)	8	7.1
	Nonincome earning – unemployed	6	5.3
Level of education†	≤Grade 6	28	25.0
	Grades 7-12	30	26.8
	≥College level	54	48.2
Headache diagnosis	Migraine	73	64.6
	Other primary headache	24	21.2
	Secondary headache	16	14.2
Headache duration	≤3 months	28	24.8
	3 months-1 year	17	15.0
	1-2 years	15	13.3
	2-4 years	17	15.0
	4-10 years	29	25.7
	≥10 years	7	6.2
	Mean = 4.3 years, SD = 4.97, minimum = 0, maximum = 20 years		
Limitation of function	None	33	29.2
	Some work interruption	70	61.9
	Off-work	10	8.8
SPSC	0-1	56	49.6
	2	22	19.5
	3	15	13.3
	≥4	20	17.7
	Mean = 2.0, SD = 1.80, minimum = 0, maximum = 8		
Psychiatric disorder	Depression	33	29.2
	Anxiety	11	9.7
	Somatoform disorders	31	27.4
ARMD (0-12)	0	28	24.8
	1	22	19.5
	2	21	18.6
	3	15	13.3
	4	14	12.4
	≥5	13	11.5
	Mean = 2.1, SD = 1.92, minimum = 0, maximum = 8		
Type of stress†	None	17	17.9
	Health related	38	40.0
	Study/work/finance related	26	27.4
	Relationship related	14	14.7

†Number is not 113 due to missing data.
ARMD = accumulated risk of mental disorder; SPSC = significant physical symptom count.

Table 2.—Percentage and Number of Psychiatric Disorders Within Headache Type (% [N])

Headache Type (N)	Depression	Anxiety	Somatoform
All headache diagnosis (113)	29.2 (33)	9.7 (11)	27.4 (31)
Migraine (73)	27.4 (20)	8.2 (6)	30.1 (22)
Other primary headache (24)	29.2 (7)	20.8 (5)	37.5 (9)
Secondary headache (16)	37.5 (6)	0 (0)	0 (0)

presence of physical symptoms per se to include SPSC to univariate or regression analysis, was not theoretically relevant. The finding shows aging, an increased level of ARMD, and types of stress to be significantly associated with somatoform disorders. The effect of an increased age and ARMD at each level remained even after logistic regression modeling (OR = 1.6, 95% CI = 1.2 to 2.3; OR = 1.6, 95% CI = 1.2 to 2.2, respectively).

DISCUSSION

Data from clinical diagnosis for headache were obtained for 113 patients from the Headache Clinic at the King Chulalongkorn Memorial Hospital. Migraine was the most common headache type. Most patients suffered from chronic headache before their first visits to the clinic. Approximately half had at least one of the following psychiatric disorders: depression, somatoform, anxiety, or alcohol abuse. Within each headache type, somatoform disorder was found to be more prevalent, more so than depression and anxiety. The number of significant physical complaints, measured by PHQ and health concerns (ie, hypochondriacal tendency), showed significant associations with depression, while age and level of accumulated risk for mental disorders showed significant associations with somatoform disorder. Only the health concern, compared with none or other types of stress, was significantly related to anxiety.

Our findings show a comparable prevalence of depression in patients with migraine headache (27%) as does that conducted by Saunders et al[22] and Merikangas et al[10] despite differences in population and methodology. Both studies employed community samples and standard structured interviews to determine psychiatric morbidity while ours utilized headache clinic samples at a university hospital and standard self-reported questionnaires. However, the prevalence of anxiety in our study is considerably lower in contrast. The prevalence of anxiety disorders is 10% compared with 18% as reported by Saunders et al and 14% by Merikangas et al. One possible explanation for this difference is that anxiety, determined by PHQ, requires exclusion of medical causes. For this reason, we were strict at excluding cases with potential organic causes, directly related to headache or other accompanying symptoms.

The comorbidity of somatoform disorder with headache has not been studied as extensively as depression or anxiety. Our study used the PHQ to detect somatic symptoms and diagnose somatoform disorder by excluding potential organic causes as results revealed. We ensured strict diagnoses by consulting patients' records 3 months after initial visits, an adequate interval for final diagnoses. Our results show the prevalence of somatoform disorder to be approximately 27%. In previous studies, this varies from 6% to 50%.[15,16,23] This difference in prevalence is most likely due to differences in methodology.

Our study shows that sociodemographics such as educational level and employment status are not significantly associated with psychiatric comorbidities. The findings are in line with the results of Jette et al's study.[24] Our study also shows that headache diagnosis is not significantly associated with depression, anxiety, or somatoform disorder. This is consistent with the findings made by Saunders et al,[22] Merikangas et al,[10] and Beghi et al.[25] A number of significant physical symptoms, however, increase the risk for depression, an observation supported by findings from the study by Maizels and Burchette who also used the PRIME-MD.[26]

Nonetheless, we would advise caution in interpreting our results for the following reasons. Firstly, as this first stage of our study is cross-sectional in design, associations suggested would be only tentative and not reliably determine real risks. Secondly, as our samples are from a clinical population at a tertiary care university hospital. Our subjects may have

Table 3.—Associations Between Demographic and Disease-Related Factor and Psychiatric Disorders (N = 113)

		Depression			Anxiety			Somatoform		
		% Case	Chi-square test, d.f.	*P* value	% Case	Chi-square test, d.f.	*P* value	% Case	Chi-square test, d.f.	*P* value
Sex (113)	Male	38.1	0.99, 1	.321	19.0	2.55, 1	.111	33.3	0.45, 1	.502
	Female	27.2			7.6			26.1		
Age (years) (113)	15-24	17.6	0.47, 1	.493	0	0.41, 1	.520	23.5	4.78, 1	.029
	25-34	37.0			14.8			18.5		
	35-44	29.2			8.3			16.7		
	45-54	24.0			12.0			32.0		
	55-64	23.1			7.7			53.8		
	≥65	57.1			14.3			42.9		
Marital status (113)	Married/cohabiting	28.3	2.08, 2	.353	8.3	0.31, 2	.858	23.3	2.58, 2	.276
	Single	23.5			11.8			26.5		
	Other	42.1			10.5			42.1		
Employment status (113)	Full-time – income earning	28.8	5.60, 4	.231	6.8	3.49, 4	.479	20.3	4.12, 4	.390
	Part-time – income earning	28.6			14.3			28.6		
	Unpaid occupations	19.2			7.7			34.6		
	Income earning but not in the workforce	37.5			25.0			37.5		
	Nonincome earning – unemployed	66.7			16.7			50.0		
Education level† (112)	≤Grade 6	35.7	2.01, 1	.156	10.7	1.86, 1	.173	35.7	1.76, 1	.185
	Grades 7-12	36.7			20.0			30.0		
	≥College level	22.2			3.7			22.2		
Headache type (113)	Migraine	27.4	0.65, 2	.723	8.2	5.28, 2	.071	30.1	2.26, 2	.324
	Other primary headache	29.2			20.8			31.6		
	Secondary headache	37.5			0			14.3		
Headache duration (113)	≤3 months	32.1	0.001, 1	.972	10.7	0.11, 1	.741	28.6	0.44, 1	.508
	3months-1 year	23.5			0			35.3		
	1-2 years	26.7			13.3			26.7		
	2-4 years	29.4			11.8			29.4		
	4-10 years	31.0			13.8			20.7		
	≥10 years	28.6			0			28.6		
Limitation of function (113)	None	27.3	0.64, 2	.727	9.1	1.32, 2	.516	33.3	2.11, 2	.349
	Some work interruption	28.6			11.4			22.9		
	Off-work	40.0			0			40.0		
SPSC (113)	0-1	8.9	20.31, 1	<.001	1.8	7.62, 1	.006	–		
	2	45.5			18.2			–		
	3	40.0			6.7			–		
	≥4	60.0			25.0			–		
ARMD (113)	0	21.4	8.46, 1	.004	10.7	2.03, 1	.154	10.7	8.11, 1	.004
	1	13.6			0			27.3		
	2	28.6			9.5			14.3		
	3	26.7			6.7			46.7		
	4	42.9			14.3			50.0		
	≥5	61.5			23.1			38.5		
Type of stress† (95)	None	17.6	11.03, 3	.012	0	10.14, 3	.017	11.8	8.05, 3	.045
	Health issue	47.4			21.1			31.6		
	Study/work/finance issue	11.5			3.8			15.4		
	Relationship issue	28.6			0			50.0		

†Number is not 113 due to missing data.
ARMD = accumulated risk for mental disorder; SPSC = significant physical symptom count.

shown more severe symptoms and been affected by more potential organic conditions. This may have resulted in a low prevalence of anxiety as we excluded all cases with potential organic causes. Applying our results to other groups could therefore be misleading. Lastly, although our measurement, the self-reported questionnaire, was validated, it is not as thorough as other measures, such as the structured diagnostic interview employed in other investigations. Self-reported questionnaires could have allowed subjects to amplify somatic symptoms as they tended to endorse many of those in the physical symptom list. The prevalence of somatoform disorder could therefore have been unusually high.

Despite these reservations, our research exemplifies a systematic study of psychiatric morbidity of headache in a clinical population in Thailand and provides a precise method of determining somatoform disorder. Few studies have addressed somatoform disorder for this requires the exclusion of medical diagnoses. Our study details how potential organic conditions can be determined over a reasonable time period.

The results of this study will raise the awareness of physicians to possible mental disorders in patients with migraine and other headache types. This will consequently facilitate appropriate treatment or referral for psychological care. Apart from clinical applications, the results of this study can be used for a longitudinal study of psychiatric morbidity of headache and migraine in clinical populations on a nationwide scale.

In conclusion, the present study confirms considerably high prevalence of depression, anxiety, and somatoform disorder in headache clinic patients. Significant associations emerge between a number of significant physical symptom complaints, health concern, and depression. Health concern was also found to be significantly associated with anxiety. The level of risk for mental disorder is seen to be closely related to somatoform disorder. The relationship between headache diagnosis and mental disorders is not evident in this study. The findings may help increase awareness among physicians of the importance of mental disorders in patients with headache.

STATEMENT OF AUTHORSHIP

Category 1

(a) Conception and Design
Chaichana Nimnuan; Anan Srikiatkhachorn

(b) Acquisition of Data
Chaichana Nimnuan; Thanin Asawavichienjinda; Anan Srikiatkhachorn

(c) Analysis and Interpretation of Data
Chaichana Nimnuan; Thanin Asawavichienjinda; Anan Srikiatkhachorn

Category 2

(a) Drafting the Article
Chaichana Nimnuan; Anan Srikiatkhachorn

(b) Revising It for Intellectual Content
Chaichana Nimnuan; Thanin Asawavichienjinda; Anan Srikiatkhachorn

Category 3

(a) Final Approval of the Completed Article
Chaichana Nimnuan; Thanin Asawavichienjinda; Anan Srikiatkhachorn

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Clinical Summary

Migraine: psychiatric comorbidities

Contributors

Chaichana Nimnuan MD PhD, author. Dr. Nimnuan of Chulalongkorn University in Bangkok, Thailand, received honorariums from Invida Pfizer Thailand and Eli Lilly Thailand for speaking engagements.

Anan Srikiatkhachorn MD, author. Dr. Srikiatkhachorn of Chulalongkorn University in Bangkok, Thailand, received honorariums from Janssen-Cilag Thailand and Invida Pfizer Thailand for speaking engagements.

Shuu-Jiun Wang MD, editor. Dr. Wang of the National Yang-Ming University School of Medicine and The Neurological Institute at Taipei Veterans General Hospital received honorariums from Eli-Lilly Taiwan and GlaxoSmithKline Taiwan for speaking engagements; consulting fees from Allergan, Pfizer Taiwan, and MSD Taiwan as a member of advisory committees; and honorariums from Boehringer Ingelheim Taiwan, Eli-Lilly Taiwan, and Pfizer Taiwan for serving as a meeting moderator.

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Key points

- The psychiatric conditions coinciding with migraine can be classified into 3 main categories: mood disorders, anxiety disorders, and personality disorders.
- The onset of depression either preceding or following migraine is common in patients with migraine.
- Shared genetic vulnerability is the most likely explanation for migraine and comorbid depression.

Historical note and nomenclature

The co-occurrence of various psychiatric symptoms and migraine has long been observed. Such observation led to a hypothesis of certain psychological traits; namely depression, anxiety, and social phobia, that predisposed patients to migraine. In 1937 Wolff initiated the concept of the purported “migraine personality” (Wolff 1937). This personality is characterized by a constellation of obsessive-compulsive traits including perfectionism, orderliness, moralistic preoccupation, and rigidity. These entrenched, often interpersonally successful surface qualities may be understood as reaction formations against a considerable amount of anger. The other traits, such as unexpressed dependency, shyness, sensitivity to criticism, sexual inadequacy, and exploitive interpersonal relationships were also mentioned. These clinical studies concluded that unexpressed anger is what is represented in migraine (Harrison 1975). This concept of migraine personality has not been supported by more recent studies with better methodology. Accumulating evidence revealed that the psychiatric symptoms observed in

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persons with migraine are the result of coexisting disorders rather than underlying personality abnormalities.

The term “comorbidity,” introduced by Feinstein in 1970, refers to the presence of any additional coexisting ailment with a particular index disorder (Feinstein 1970). Comorbidity of migraine is a rule rather than exception. It usually coincides with medical symptoms, medically unexplained physical symptoms, and mental conditions. Angina, hypertension, colitis ulcer, stroke, asthma, epilepsy, essential tremor, and allergies are among the physically explained conditions (Merikangas and Fenton 1994; Silberstein 2001; Low and Merikangas 2003). For medically unexplained physical symptoms, fibromyalgia and irritable bowel syndrome were reported as associated with the presence of migraine (Hudson and Pope 1989; Peres et al 2001; Cole et al 2006). Overlapping symptoms with psychiatric disorders are sleep disorders, decrease energy, anhedonia, decreased concentration, and decreased libido (Sheftell and Atlas 2002). Comorbidity of migraine has also been supported statistically by multivariate technique, ie, cluster analysis. Two constellations are evident, 1 with medical conditions, and the other with medically unexplained syndrome and psychiatric disorders (Tietjen et al 2007).

Comorbidity of migraine with mental disorders has long been noted in literature. Consistent reports on this comorbidity appear far too often to be coincidental. A population survey in the U.S. found prevalence of any mental disorder in migraineurs was 1.5 and 3.1 times as high compared to nonmigraine headache and nonheadache groups respectively (Saunders et al 2008). These psychiatric comorbidities have strong impact on an individual suffering from migraine as well as on the society. Comorbidity can alter the clinical course of migraine, its prognosis, and quality of life of the sufferers. Persons with migraine with comorbid depression or anxiety also have significantly higher medical costs than those with episodic migraine (Pesa and Lage 2004).

Top

Clinical manifestations

The psychiatric conditions that coincide with migraine can be classified into 3 main categories, namely mood disorders, anxiety disorders, and personality disorders.

Mood disorders. Both unipolar and bipolar mood disorders have been reported to be comorbid with migraine. Regarding depression, symptoms such as lack of energy, poor concentration, sleep disturbance, loss of appetite, psychomotor agitation or retardation, weight changes, feelings of worthlessness, etc. are commonly observed in persons with migraine. The syndrome of depression can range from mild dysthymia to life-threatening major depression. The Zurich Cohort Study showed that 1-year prevalence of dysthymia and major depression in persons with migraine was 6.6% and 14.7% (Merikangas et al 1990). Comparatively, findings from the National Comorbidity Survey-Replication in the U.S. showed 1-year prevalence of dysthymia and major depression was 8.8% and 18.8%, respectively (Saunders et al 2008). Lifetime prevalence of major depression is approximately 3 to 4 times higher in persons with migraine compared with controls (Breslau and Davis 1992; Breslau et al 2000). A large-sample, case-control study in patients with depression in the UK also showed that the strength of association between migraine with aura and depression was high (OR=5.6, 95% CI=3.5 to 9.0) (Samaan et al 2009). The findings consistently support the idea that the risk of having major

depression is greater in persons with migraine with aura.

The onset of depression can either precede or follow that of migraine. Using survival analysis of the lifetime data, which covered the respondents' history up to the time of the baseline interview, Breslau and colleagues demonstrated that the association between migraine and major depression is bidirectional (Breslau et al 1994; 2000). The sex-adjusted hazard ratio of the first onset of major depression in persons with migraine was 2.35 [95% confidence interval (CI) 1.84 to 3.01], whereas the hazard ratio for the first occurrence of migraine in persons with prior major depression was 2.75 (95% CI 2.17 to 3.48). This bidirectional association was later confirmed by the same investigators using a different approach. In 2003, Breslau and colleagues compared the incidence of first-onset major depression in the 2-year follow-up period across persons with migraine, persons with severe headache not fulfilled migraine diagnosis, and nonheadache controls (Breslau et al 2003). The results revealed the incidence of major depression in the migraine group to be 10.5%, in the severe headache group 5.1%, and in the controls 2.0%. Based on these rates, the odds ratio for major depression in persons with migraine was 5.8 (95% CI 2.7 to 12.3) and 2.7 (95% CI 0.9 to 8.1) in those with nonmigraine severe headache. Preexisting major depression also increased the risk of having new-onset migraine. The incidence of new-onset migraine in the 2-year follow-up period in persons with history of major depression was 9.3%, compared to 2.9% in those without. The odds ratio of migraine associated with prior major depression was 3.4 (95% CI 1.4 to 7.6). It is interesting that, in contrast to migraine, prior depression did not increase risk of having severe headaches that did not fulfill the criteria for migraine diagnosis.

Concerning the impact of comorbid depression on the course of migraine, Breslau and colleagues showed that persons with this comorbidity experienced headache of greater severity than those without (Breslau et al 2003). They were not, however, at greater risk for headache persistence. Frequency of migraine attacks was not related to the presence of comorbid major depression (Breslau et al 2003). A longitudinal study showed that although co-occurrence of migraine, personality changes, and depression in women does not appear to influence the results of treatment at short-term, it seems to be influential on headache history in the long term (Mongini et al 2003).

Comorbid major depression has strong impact on patients' quality of life. Based on a population-based, case-control study, Lipton and colleagues showed that patients with migraine and depression had lower scores on health-related quality of life as measured by the Short Form (SF)-12. Lower quality-of-life scores were observed in both mental health component scores (MCS-12) and physical health component scores (PCS-12) (Lipton et al 2000). Frequency of migraine attacks and severity of depressive symptoms emerged as a predictor for poor quality of life in patients suffered from chronic migraine (Canuet et al 2008). Persons suffering from migraine with aura and coexisting major depression had higher rates of suicidal attempts and suicidal ideation compared with patients with neither migraine nor major depression (Breslau et al 1991). A study in Taiwan also showed that migraine with aura itself increases suicide risk score in adolescents with chronic daily headache, even after being adjusted for depression and anxiety disorders (Wang et al 2007). The same investigators also showed that comorbidity with migraine was associated with more somatic symptoms in patients with major depression, and migraine was a strong and independent

predictor for the somatic symptoms of this condition (Hung et al 2009).

Apart from major depression, association between migraine and bipolar disorder has also been documented. Merikangas and colleagues reported in their longitudinal study the association between migraine and bipolar spectrum at odds ratio of 2.9 (95% CI 1.1, 8.6) (Merikangas et al 1990). Another longitudinal study conducted by Breslau and colleagues showed sex-adjusted odds ratio was up to 4.7 (95% CI 1.4, 15.4) (Breslau et al 1991). A cross-sectional hospital-based study of 102 patients with major depression or mania found that, as compared to the patients without migraine (n = 49), the patients with comorbid migraine (n = 53) had a higher frequency of bipolar II disorder (43% vs. 10%), a lower frequency of bipolar I disorder (11% vs. 33%), and an approximately equal frequency of unipolar depressive disorder (45% vs. 57%) (Fasmer and Oedegaard 2001). Among the patients with bipolar disorders, prevalence of migraine was higher in persons with bipolar type II (77%) than those with bipolar type I (14%) (Fasmer 2001). Association between migraine and bipolar disorders has been evident consistently, especially for bipolar type II. The overall prevalence of migraine in patients with bipolar disorder is about 25% to 40% (Low et al 2003; Ortiz et al 2010). Patients with bipolar disorder with migraine were younger, higher educated, more likely to be in work or study, and had fewer hospitalizations; the initial presentation for psychiatric treatment was more often depression, and these patients more likely to have a family history of migraine or psychiatric disorders (Low et al 2003). Ortiz and colleagues also found that bipolar patients with migraine had more psychiatric morbidity such as social phobia, obsessive-compulsive disorder, panic disorder, and generalized anxiety disorder than those without migraine (Ortiz et al 2010). The population survey also confirmed the high prevalence of migraine in persons with bipolar disorders (Hirschfeld et al 2003; McIntyre et al 2006). A cross-sectional community survey conducted in Canada using lifetime WHO-CIDI defined-measures showed that individuals with bipolar disorder had prevalence of migraine twice as high as those without (24.8% vs. 10.3% respectively). Despite consistent findings of bipolar disorder and migraine, comorbid association with sex, education, and health care service use were inconclusive (Low et al 2003; McIntyre et al 2006).

Anxiety disorders. Anxiety disorder is more prevalent in patients with migraine than in the general population (Breslau et al 1991). Saunders and colleagues reported that 1-year prevalence of any anxiety disorder in migraineurs was 44.5%, which was higher than that of any mood disorder (24.7%) in the same household survey (Saunders et al 2008). In a population study, men with a history of panic disorder had approximately 7 times higher risk to have migraine compared to those without. For women, the risk was lower (RR = 3.7) (Stewart et al 1989). In a follow-up study of the same sample, subjects with a history of panic disorders had a higher rate of health service use for headache, which suggested comorbid panic disorder increased the likelihood of seeking health care. A 14-month follow-up study revealed that patients with migraine at baseline had a significantly increased rate of panic disorder (odds ratio = 12.8, 95% CI 4.1 to 39.8) (Breslau and Davis 1993). Data drawn from a nationally representative sample in the United States revealed that persons with migraine, adjusted for demographic variables, had greater risk of having panic (odds ratio 2.37; 95% CI 1.42 to 3.99%) or generalized anxiety disorders (3.13;95% CI 1.56 to 6.30)

(McWilliams et al 2004).

It should be noted that association between panic disorder and headache syndromes is not specific to migraine. In 2001, Breslau and colleagues showed that lifetime prevalence of panic disorder was significantly higher in persons with migraine as well as persons with other severe headaches. Both migraine and other severe headaches were associated with an increased risk of first onset of panic disorder [hazard ratios = 3.55 (95% CI 2.18 to 5.76) and 5.75 (95% CI 2.70 to 12.27), respectively]. Preexisting panic disorder was also associated with an increased risk of first onset of migraine and for first onset of other severe headaches, although the influence on this direction was lower [hazard ratios = 2.10 (95% CI 1.44 to 3.08) and 1.85, (95% CI 0.71 to 4.48) respectively] (Breslau et al 2001). In patients with coexisting migraine, treatment of panic disorder has shown to improve migraine in most cases (58%) (Yamada et al 2011). Association between panic disorder and migraine may vary depending on age group. For example, a population-based study in Sweden showed no significant association between migraine and panic disorder in women aged 40 to 74 years. (Mattsson and Ekselius 2002).

Coexistence of anxiety and mood disorders are frequently observed in persons with migraine. In a clinical sample of patients with migraine, 24% and 42%, respectively, had current and lifetime diagnoses of both mood and anxiety disorders (Ortiz et al 2010). In the Detroit study, 88% of persons with a history of migraine and major depression also reported at least 1 anxiety disorder (Breslau et al 1991). Data from a prospective longitudinal study in Zurich indicated that age of onset of anxiety disorders generally preceded that of migraine and that the onset of affective disorders in the majority of comorbid subjects followed that of the onset of migraine (Merikangas et al 1990; 1993). The results implied a strong relationship between migraine and anxiety or depression, rather than their representing discrete manifestations.

Personality disorders. Several personality traits have been reported to be more prevalent in persons with migraine. Among those, neuroticism measured by Eysenck's personality questionnaire showed a considerable degree of association with migraine, which suggested that migraine sufferers might be more vulnerable to psychopathology (Brandt et al 1990; Breslau 1995; Silberstein et al 1995). No significant difference among subtypes of migraine has been documented. The association between migraine and neuroticism remained significant when sex, history of major depression, and history of any anxiety disorder were controlled. Elevated rates of neuroticism and somatization, as measured by the Freiburg Personality Inventory and the Symptom Checklist 90, have been observed in persons with migraine (Merikangas et al 1994). Abnormal personality profiles are more prevalent in persons with either chronic headache, chronic migraine, or migraine with analgesic rebound headache. A case-control study showed that persons with chronic migraine as well as analgesic rebound headache had higher score in multiple subscales of MMPI compared to persons with episodic migraine. These subscales included hypochondriasis, depression, schizophrenia, and social introversion (Bigal et al 2003).

Significant headache is a common complaint in patients with borderline personality disorder. Based on information gathered from 112 patients visiting a psychiatric clinic, Hegarty reported overall prevalence of severe headache was 60.4% in those with borderline personality disorder. Fifty percent of females and 24% of males

were diagnosed as having migraine (Hegarty 1993).

Top

Clinical vignette

A 52-year-old married female was referred to a neurologic clinic for management of headache. She suffered from recurrent headaches since the age of 15 years. The attacks were characterized by recurrent, severe, unilateral or bilateral throbbing headache with accompanying nausea and sometimes vomiting. The frequency of headache attacks was 2 to 3 times a month, and each attack usually lasted 1 to 2 days. The headache was triggered by menstruation, mental stress, and lack of sleep. Her general physician advised her to avoid trigger factors and take painkillers when attacks occurred. No prophylactic medication was prescribed. After the age of 47 years, her headache attacks became more frequent and eventually occurred in a daily basis for 6 months. The pain was diffuse, nonthrobbing, and pressure-like in character. She took analgesics every day to control her symptoms. Her sleep became fragmented. She felt depressed and had asthenia. Her symptoms fulfilled depression criteria, and her depression was eventually diagnosed and treated.

Top

Etiology

Ample evidence has indicated that the association between migraine and psychiatric comorbidities is not a matter of chance. Two explanations for this association are possible (Merikangas and Stevens 1997; Silberstein 2001). First, migraine and depression or anxiety are causally related, either migraine causing depression or anxiety or the reverse. In this model, an index disorder causes or predisposes to the development of the comorbid disorder. Therefore, this association must be unidirectional in nature. However, epidemiological and clinical evidence confirms that the association between migraine and psychiatric disorders, especially major depression, is bidirectional.

The second explanation is that migraine and depression or anxiety share common underlying pathologic mechanisms. The index and comorbid disorders may represent alternative manifestations of the same underlying factor or factors, or different stages of the same disease. Franchini and colleagues reported that mood disorder and migraine familiarity in first-degree relatives was significantly related to the risk for comorbidity (Franchini et al 2004). Clinical evidence also supports this common underlying hypothesis. Drugs that act on serotonin such as SSRIs and SNRIs can be used effectively, both in the treatment of depression and in the prophylaxis of migraine (Tarlaci 2009). In 1989, Hudson and Pope proposed a hypothesis of an “affective spectrum disorder” to explain the comorbidity among 8 disorders that included fibromyalgia, irritable bowel syndrome, major depression, panic disorder, obsessive compulsive disorder, bulimia, cataplexy, migraine, and attention deficit disorder with hyperactivity (Hudson and Pope 1989). They suggested that all these disorders exhibited shared phenomenology, family history, and treatment response to antidepressant medications (Merikangas et all 1988). This model is in accordance with the observed bidirectional association. This might be the result of a common, albeit unknown, pathophysiology.

Psychosocial factors have also been mentioned. External locus of control is associated with higher level of depression, poor pain coping strategies, and greater disability. Concept of locus of control,

which was introduced by Rotter, describes an individual's approach to interpreting and attributing events (Rotter 1966). Scharff and colleagues found that external locus of control was significantly related to headache intensity as well as to the patient's perception of the extent to which pain interfered with many domains of their lives (Scharff et al 1995). Variances explained that headache-related disability is accounted for independently by locus of control and self-efficacy belief (French et al 2000). Concept of learned helplessness has also been proposed. Such helplessness caused by uncontrolled migraine attacks brought about chronic recurrent migraine and also depression (Sheftell and Atlas 2002).

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Pathogenesis and pathophysiology

In the “shared mechanism model,” pleiotropic effects of the same genes could lead to different clinical manifestations (migraine, depression, or anxiety) depending on the background genes and the intrinsic and extrinsic environment in which they are expressed (Merikangas and Stevens 1997). Shared genetic vulnerability is the most likely explanation for migraine and comorbid depression. A study of 758 monozygotic and 306 dizygotic female pairs showed that heritability was estimated to be 58% (95% CI 48% to 67%) for depression and 44% (95% CI 32% to 56%) for migraine (Schur et al 2009). Using bivariate structural equation modeling, the authors estimated that 20% of the variability in depression and migraine headaches was due to shared genes, and 4% was due to shared unique environmental factors. The shared genetic factor hypothesis is also supported by a study in genetic isolate population. Stam and colleagues investigated the contribution of shared genetic factors in migraine and depression by comparing heritability estimates for migraine with and without adjustment for symptoms of depression, and by comparing the heritability scores of depression between migraineurs and controls (Stam et al 2010). The results showed that the heritability estimates were significant for all migraine (0.56), migraine without aura (0.77), and migraine with aura (0.96) and decreased after adjustment for symptoms of depression or use of antidepressant medication, especially in migraine with aura. Comparison of the heritability scores for depression between patients with migraine and controls showed a genetic correlation between Hospital Anxiety and Depression Scale score and migraine with aura.

Derangement in aminergic activity, especially serotonin and dopamine, in the central nervous system is the most likely hypothesis for explaining the comorbidities. It is known that this central aminergic system plays a pivotal role in controlling various behaviors, ie, sleep-wake, feeding, emotional level, etc. Change in the amine system in persons with migraine with comorbid depression has been documented. In 1995, Merikangas and colleagues reported lower tyramine sulfate excretion values among persons with migraine and depression compared to those in migraine alone or depression alone (Merikangas et al 1995). Based on these findings, the authors suggested that comorbid migraine with depression may represent a more severe form of migraine than migraine alone.

Serotonin and its subtypes play major roles in pathogenesis of depression, anxiety, and migraine. Decreased central serotonergic activity is a main theory in pathogenesis of depression. Depletion of platelet serotonin and increased level of urinary 5-hydroxyindole acetic acid have been reported during the attack of migraine. It is

interesting that the reduction of serotonin concentration is more prominent in persons with migraine complicated with medication overuse headache, the condition in which the risk of having comorbid depression is greater (Srikiatkhachorn and Anthony 1996). The hypothesis of hyposerotonin is supported by the clinical observation that drugs that enhance serotonergic function, such as tricyclic antidepressants or selective serotonin reuptake inhibitors, are effective in treatment of both migraine and depression.

Dopamine has been noted with its effect on yawning, mood change, nausea, and vomiting, which are common in the prodromal phase of migraine. Moreover, antidopaminergic compounds are used effectively in helping to relieve these symptoms. Patients with migraine have an increased density of dopamine receptors on peripheral lymphocytes, which reflects hypofunction of the dopaminergic system (Barbanti et al 2000). Migraine with aura, anxiety disorder, and major depression can be components of a distinct syndrome associated with allelic variations within the *DRD2* gene (Peroutka et al 1998). However, 1 genetic study does not support the role of the dopaminergic system in migraine and comorbid panic disorder. Stochino and colleagues showed that the allele frequencies of *DRD1*, *DRD3*, *DRD5*, and *DRD2* in persons with migraine with aura with comorbid panic disorder did not differ from that of parental nontransmitted chromosomes (Stochino et al 2003). GABA was another neurotransmitter found to be associated with both migraine and depression. Vieira and colleagues examined CSF GABA levels and found that chronic migraine patients with depression had significantly lower CSF GABA levels than those without (Vieira et al 2006).

Association between mitochondrial dysfunction or depression and migraine comorbidity, together with irritable bowel syndrome were demonstrated by Burnett and colleagues (Burnett et al 2005). The investigators recruited 166 families with at least 1 member who had been diagnosed with mitochondrial disease by a physician. Probable maternal inheritance group and probable nonmaternal inheritance group were identified and compared. The results showed that those from the former group had a higher prevalence of depression, migraine, and bowel dysmotility with statistical significance. The hypothesis that mitochondrial dysfunction is a significant common factor underlying the association of these 3 conditions in the general population was proposed (Burnett et al 2005).

In summary, mechanism of association has been so far discarded in the hypothesis of chance or direct causation, leaving the common factors hypothesis to be verified further. Common biological factors are by far the most likely explanation of the association between migraine and psychiatric disorders. However, the exact mechanism of such factors is still far from clear.

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Epidemiology

Comorbidity between migraine and psychiatric disorders has been reported from several countries worldwide, including the United States, Switzerland, Greece, Turkey, Taiwan, and Italy. The information obtained from these countries is rather consistent and shows the lifetime prevalence of major depression in persons with migraine to be 3 to 5 times higher than in those without (Guidetti et al 1998; Breslau et al 2000; Kececi et al 2003). A case-control study revealed strong association between depression and migraine with aura with odds ratio of 5.6 (Samaan et al 2009). A nationwide survey in Canada showed that migraine was associated with major

depressive disorder, bipolar disorder, panic disorder, and social phobia. The lifetime prevalence of these psychiatric disorders was more than twice as high in those with migraines compared with those without. Migraine was not associated with drug, alcohol, or substance dependence. The higher prevalence of psychiatric disorders in migraineurs was not related to sociodemographic variables (Jette et al 2008).

Risk of having comorbid depression is higher in the persons suffering from chronic migraine and those with analgesic overuse headache (Mitsikostas and Thomas 1999). Mathew and colleagues reported that 46% of patients with transformed migraine who visited Houston Headache Clinic had clinical depression (Mathew et al 1982). Subsequent study from the same clinic showed that persons with transformed migraine had higher scores in Zung Depression Scale, Beck Depression Inventory, and Type A Behavioral Pattern compared with those with episodic migraine (Mathew et al 1987). The prevalence of depression is higher in persons with migraine with chronic substance-induced headache (odds ratio, 8.7; 95% CI 1.78 to 42.9) compared to persons with migraine without analgesic overuse (Radat et al 1999). A study from Taiwan showed that 78% of persons with transformed migraine had psychiatric comorbidity, namely: major depression (57%), panic (30%), dysthymia (11%), and generalized anxiety disorder (8%). The authors also suggested that women and patients with transformed migraine were at higher risk of psychiatric morbidity (Juang et al 2000). However, Magnusson and Becker reported contradictory findings, which showed that depression scores obtained from persons with transformed migraine and episodic migraine were not significantly different (Magnusson and Becker 2003). Psychological distress and impaired quality of life are associated with frequent headache and frequent disability but not with severity of headache (Marcus 2000).

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Prevention

Factors increasing psychiatric comorbidity include migraine with aura, chronicity of migraine, female gender, analgesic overuse, and coexisting painful conditions. Higher risk of having comorbid depression and anxiety disorders (panic or generalized anxiety disorders) has also been identified in migraine patients who also suffer from other pain syndromes such as arthritis or back pain (McWilliams et al 2004). Other painful conditions in patients with migraine, therefore, need to be identified and properly treated to prevent possible comorbid anxiety and depression later on.

Psychiatric comorbidities are more prevalent in persons with frequent migraine attacks with or without analgesics or ergot overuse. The higher risk of having depression may be the result of chronicity of the disease. Early and effective treatment of migraine is then highly recommended. Comorbid mood disorders should be looked, for and prompt treatment should be employed.

Several drugs used in aborting or preventing the attacks of migraine may influence the risk of having comorbid psychiatric symptoms. Clinical depression can be uncovered or worsened by some prophylactic medications, such as beta-adrenergic blockers or calcium channel blockers. Caffeine-containing compounds, which are used as abortive medications, can cause palpitation and trigger the panic attack. Therefore, physicians should prescribe those with caution, especially in the persons with higher risk.

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Differential diagnosis

Mood changes can be observed during the prodromal period of migraine. Such mood changes should not be diagnosed as depressive or manic episodes.

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Diagnostic workup

Both migraine and its comorbid psychiatric conditions so far have no specific biological markers. The diagnoses are made solely on clinical symptoms. Psychological tests for depression or anxiety are useful in epidemiological study but are less beneficial in clinical situations. Laboratory investigations including neuroimaging studies are useful in excluding the secondary causes that can mimic migraine--for example, vascular lesions such as arteriovenous malformation. They can also identify and exclude lesions in subcortical structures, such as the thalamus, that can cause depression.

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Prognosis and complications

Major complications of migraine with psychiatric comorbidities are substance abuse and suicide. The rate of attempted suicide has been reported to be highest in persons with migraine with aura with comorbid major depression. The risk of suicidal attempt in this group is 38.5 per 100 cases, which is more than double that of the risk of depression alone (16.5 per 100 cases). Female migraineurs of any subtypes have higher rates of attempted suicide than their male counterparts (Breslau et al 1991).

Sleep disturbances are common in depression and anxiety. Therefore, persons with these conditions are at risk of sedative or hypnotic abuse.

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Management

The fact that migraine and psychiatric disorders often occur together but its mechanism has not yet been well established poses a challenge in treatment. To provide effective management of patients with migraine and psychiatric disorders, therefore, requires a comprehensive approach. First and foremost, physicians must be aware of the high prevalence of such comorbidities. Physicians should elicit from the history any mood and anxiety symptoms when patients present their symptoms of migraine. Further exploration into past history is also beneficial. Second, before prescribing specific medication, history of multiple medical treatments or drug used should be explored. Generally, tricyclic antidepressants such as amitriptyline, nortriptyline, or doxepin work well in treating migraine and depression. Dosage of these medications should be high enough to reach antidepressant levels, which are usually higher than those for controlling migraine attacks. With the higher dosages, some adverse effects, especially anticholinergic effects, can be severe and limit the use of these medications. A selective serotonin reuptake inhibitor, such as fluoxetine, can be an alternative. This group is useful in treating depression and has fewer side effects (Sheftell and Atlas 2002; Punay and Couch 2003). Education about side effects of antidepressants is crucial, and emphasis on the benefits of regular and long term use of the medication is necessary. Explaining to patients that antidepressants take time to show their effects and

that patients must tolerate the drugs' side effects at the beginning of treatment helps increase compliance. Third, supporting psychotherapy (which stresses an increasing internal locus of control, healthy lifestyle, and adhering to medication) benefits patients with long-term suffering from both migraine and mood disorders. Cognitive behavioral therapy has a prophylactic efficacy to migraine and treatment efficacy to mood disorders. The combination of behavioral therapy with prophylactic medication creates a synergistic effect, increasing efficacy beyond either type of treatment alone. Cognitive behavioral therapy has earned an important place in the comprehensive treatment of patients with episodic migraine (Lake 2001; Lemstra et al 2002). Finally, in patients that are refractory to treatment, liaison with a psychiatrist is recommended.

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Pregnancy

Not applicable.

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Anesthesia

Not applicable.

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ICD codes

ICD-9:
Anxiety state unspecified: 300.00
Depression NOS: 311
Migraine unspecified: 346.9

ICD-10:
Anxiety disorder, unspecified: F41.9
Depressive episode, unspecified: F32.9
Migraine, unspecified: G43.9

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Associated disorders

None identified

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Epidemiology of headache
Headache guidelines
Migraine
Migraine and epilepsy
Psychological headache
Sleep and headaches
Sleep disorders associated with mental disorders

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Differential diagnosis

depressive episodes
manic episodes

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Demographics

For more specific demographic information, see the Epidemiology, Etiology, and Pathogenesis and pathophysiology sections of this clinical summary.

Age
0-01 month
01-23 months
02-05 years
06-12 years
13-18 years
19-44 years
45-64 years
65+ years

Population
None selectively affected.

Occupation
None selectively affected.

Sex
female>male, >2:1

Family history
None

Heredity
heredity may be a factor

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**References especially recommended by the author or editor for general reading.

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Clinical Summary

Epidemiology of headache

Contributors

Chaichana Nimnuan MD PhD, author. Dr. Nimnuan of Chulalongkorn University in Bangkok, Thailand, received honorariums from Invida Pfizer Thailand and Eli Lilly Asia Inc. (Thailand Branch) for speaking engagements.

Anan Srikiatkhachorn MD, author. Dr. Srikiatkhachorn of Chulalongkorn University in Bangkok, Thailand, received honorariums from Janssen-Cilag Thailand and Invida Pfizer Thailand for speaking engagements.

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Former author(s)

Mario F P Peres MD (original author)

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Key points

• Primary headaches are a common problem in the general population and one of the most common complaints in neurology clinics.
• Migraine has a prevalence of approximately 10% worldwide and contributes around 40% to 50% of the overall headache burden.
• Tension-type headache is the most prevalent primary headache in the population and contributes 60% of the total headache burden.
• Chronic daily headache (including 4 subtypes: chronic migraine, chronic tension-type headache, new daily persistent headache, and hemicrania continua) is the most common headache disorder seen in headache clinics; many patients with chronic daily headache overuse abortive headache medications.
• Psychiatric conditions, especially depression and anxiety, are common co-morbidities.

Introduction

Headache is one of the most common public health concerns worldwide. Its prevalence is high, though the exact figure of prevalence is difficult to methodologically determine. Headache mostly affects people in their productive years, ie, late teens to 50s, and the cost of lost productivity and work hours due to headache is enormous. Headache is also the most common reason for neurologic consultation. Despite such magnitude in pain, disability, and cost, headache is under-diagnosed and under-treated. One study showed that approximately half of those suffering from migraine had been

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seen by doctors, and most of them received neither correct diagnosis nor effective treatment (Lipton et al 2007); this may be because of clinical and sociological barriers as most headaches are episodic, non-fatal, and non-contagious. The condition may, therefore, be perceived as less serious and not a high priority (World Health Organization 2004; Stovner et al 2007).

To broaden our views on the disorder, we need reliable information from various epidemiological studies. Epidemiology is a science aiming to examine patterns of disease occurrence in human populations and determinants of these patterns (Lilienfeld and Lilienfeld 1980). Epidemiological studies tend to address causation and various public health issues. In this clinical summary, we will explore population frequency of headache as well as headache risk factors, co-morbidities, course, and resultant disability. We will focus more on primary headache due to its higher prevalence and the availability of comprehensive studies. But first, we will address methodological factors to be considered when determining the epidemiology of headache.

Methodological considerations

Studies of headache progress generally from cross-sectional to longitudinal, from clinical to population-based, and from clinical case definition to standardized diagnostic criteria. Each study design has its own merits and flaws. Cross-sectional study is comparatively cheap and simple compared to its longitudinal counterpart. Cross-sectional studies have point-prevalence as the primary outcome measure. The prevalence can be incorrectly estimated in chronic and intermittent conditions, such as frequent tension-type headache or other chronic daily headache (Haut et al 2006). When cross-sectional study is applied to examine the determinants of the disease, its results lead to problems in determining cause and effect from any observed associations. This is because the risks and outcomes are measured simultaneously. With these limitations, studies of headache have been conducted longitudinally, for example, Zurich cohort study (Merikangas et al 1990), Danish follow-up study (Lyngberg et al 2005), and American Migraine Prevention and Prevalence (AMPP) study (Stewart et al 2008).

Longitudinal study has incidence as the main outcome. Additionally, this type of study can determine the temporal sequence between risk factors and the occurrence of the disease. With this design, individuals are assessed at the beginning of the study and then reassessed after predetermined time intervals. Only longitudinal design can reliably determine the onset, the rate of remission, or relapse. For instance, a longitudinal study conducted by Stewart and colleagues found that cumulative lifetime incidence of migraine was approximately 3 times the prevalence (Stewart et al 2008). This can be explained by a considerably high rate of migraine remission.

Although longitudinal study offers a promising result in terms of the progress of the disease, it still requires cautious interpretation. Notably, ageing and period effect can confound the longitudinal results as seen in patients with migraine whose symptoms change when they are older. Changes in socioeconomic or environmental factors can also change individuals' pattern of disease. Longitudinal studies also have other limitations, such as a relatively high attrition rate (which may cause nonresponse bias) and high cost (which may compromise the way we conduct the study).

Samples selected for the study also influence the results. Generally, clinical-based studies constitute biased samples and can

give a deceptively high prevalence of disease. However, if the aim of the study is to focus on rare diseases, such as cluster headache, it is hardly possible to get a reliable result from a population-based study. Clinical-based studies have, therefore, an advantage in investigating rare phenomena. Apart from such reasons, population-based studies have been almost always preferable in determining the prevalence or incidence of a disease or disorder. When comparing the magnitude of disorders, selection of samples must be considered.

Determining whether or not patients have headache is not simple, especially in primary headache and even more so for individuals in research. Until 1988, there was no consensus on the research definition of headache; the studies conducted before 1988 were not comparable. The International Classification of Headache Disorders (ICHD), following the first edition in 1988, has provided specific criteria for diagnosis of headaches for both clinicians and researchers (Headache Classification Subcommittee of the International Headache Society 2004). Most criteria are explicit; thus, interobserver reliability can be achieved. By using ICHD criteria, we are able to consistently compare results from various studies (Headache Classification Subcommittee of the International Headache Society 2004).

All primary headaches are, however, defined based on symptom-based criteria, similar to the way mental disorders are defined (American Psychiatric Association 1994). These subjective criteria cannot provide adequate information for accurate diagnosis of a postulated disease. Thus, they lack specificity, and to distinguish one condition from another becomes difficult (Hyams 1998). For instance, primary headache has been found to overlap other symptom-based conditions, such as irritable bowel syndrome, fibromyalgia, chronic fatigue syndrome, depression, and anxiety (Yunus et al 1989; Breslau and Davis 1993; Prescott et al 1993; Saunders et al 2008).

Although case definition is well operationalized, case-finding instruments are still problematic. As most instruments are in forms of interview or self-completion questionnaires, they are prone to interviewer bias, recall bias, and acquiescence bias. Samples with and without disease condition tend to respond to measures differently. This may result in over- or under-estimation of any associations being studied. We should, therefore, interpret results of epidemiological studies of headaches with caution.

Classification

Classification is a major issue in the epidemiological study of headaches. Without a clear classification system and criteria, an epidemiological study is almost impossible, and information from different studies cannot be compared. In acknowledgement of this problem, the International Headache Society (IHS) established a Headache Classification Subcommittee in late 1980. In 1988, IHS published the first edition of the International Classification of Headache Disorders (ICHD-I). This classification system was revised, and the second edition (ICHD-II) was published in 2004 (Headache Classification Subcommittee of the International Headache Society 2004).

The ICHD classification system has been universally accepted since its first edition. The existence of the operational diagnostic criteria described in this system has strong impact on both clinical and epidemiological studies of headache. In the ICHD system, headache disorders are primarily divided into 3 parts, namely (1) primary

headaches, (2) secondary headaches, and (3) cranial neuralgias, central and primary facial pain, and other headaches. The headache disorders are classified into major groups, and each group is then subdivided into headache types, subtypes, and subforms. A major change in the second edition is that all secondary headaches are described as “attributed to” another disorder whereas the first edition used the term “associated with.” This change reflects the well established causal link between the underlying disorders and the headache disorders. A new chapter for “Headache attributed to psychiatric disorder” has been included as one of the secondary headaches. New entities such as chronic migraine, hypnic headache, primary thunderclap headache, and hemicrania continua are also added in the latest revision. Soon after distribution, the section on “medication-overuse headache and its subforms” of the ICHD-II was revised. The major changes are: (i) elimination of the headache characteristics; and (ii) a new subform (8.2.6 medication overuse headache attributed to combination of acute medications) that takes into account patients overusing medications of different classes but not any single class (Silberstein et al 2005).

Epidemiology of primary headaches

Generally, primary headaches are far more common than secondary headaches. Due to vast variety of their etiology, accurately determining prevalence of secondary headaches is not possible. Therefore, this review focuses on the primary headaches of migraine, tension-type headache, chronic daily headache, and cluster headache; other primary headaches, such as primary stabbing headache and primary exertional headache are also described.

Migraine. Migraine is common. A large study in the United States showed that the prevalence of migraine in men and women was 6% and 18% respectively (Lipton et al 2001). Similar figures were evident in Norway and Danish studies (Rasmussen et al 1991; Hagen et al 2000). A longitudinal 12-year follow-up population-based study showed that the incidence rate was 8 per 1000 person-years, with a female-to-male ratio of approximately 6 to 1 (Lyngberg et al 2005a). No significant age or sex differences were noted between migraine subtypes. Similar findings were seen in the United States. The American Migraine Prevention and Prevalence (AMPP) cohort study also reported that incidence peak was 18/1000 person-years in females of 20 to 24 years of age compared to 6/1000 person-years in males of 15 to 19 years of age. The median age of onset for both sexes was 25 to 34 years of age (Stewart et al 2008).

Unlike tension-type headache, migraine prevalence has remained stable across different regions and time periods. Tension-type headache was observed in approximately 6% of males and 14% of females globally. The lowest prevalence was in Africa (5% for both sexes) and the highest in Europe (15% for both sexes) (Stovner et al 2007). Overall, it ranged from 1% to 25%. Compared to tension-type headache (see below), the range was quite narrow taking into account the different methodologies employed in each study.

Migraine also affects children and adolescents. A study from the United Kingdom of children aged 5 to 15 found that around 10% of both boys and girls suffered from migraine within the last year (Abu-Arefeh and Russell 1994). In adolescents, prevalence in males was lower--4.8% in males versus 9.1% in females (Zwart et al 2004). Migraine seems to decline with age. That is, the incidence of approximately 14/1000 person-years in the 25 to 34 years age

group was down to less than 5/1000 in the 55 to 64 years age group for both sexes (Lyngberg et al 2005a). However, female incidence was predominant in adulthood; even in the postmenopause group, incidence was around 2% to 3:1% (Lipton et al 2007).

Lyngberg and colleagues studied risk factors of migraine and found that young age, no vocational education, family history of migraine, and coincidence with frequent tension-type headache increased the likelihood of migraine occurrence (Lyngberg et al 2005a). From the same study population, the results showed prognosis of migraine was rather favorable. They found that 42% of subjects with migraine at baseline were in remission at follow-up. Only 20% experienced poor outcome, ie, having more than 14 days of migraine attack per year. Younger age of onset and high migraine frequency at baseline were among factors associated with poor outcome (Lyngberg et al 2005b).

Migraine has been found to overlap with other physical and mental disorders. Migraine was associated with an increased risk of ischemic stroke (adjusted RR 2.2 95% confidence interval (CI) 1.7-2.9) and transient ischemic attack (2.4 95% CI 1.8 – 3.3) (Becker et al 2007). With other symptom-based conditions, migraine was found to be associated with pain conditions, especially conditions involving the musculoskeletal system (Peres et al 2001; Hagen et al 2002; Von Korff et al 2005). The comorbidity of migraine and psychiatric disorders has long been consistently evident (Merikangas et al 1990; Breslau et al 1991; 1994; 2000; 2003; Saunders et al 2008). Major depression, bipolar disorder, panic disorder, and social phobia were all at least twice as prevalent in migraine (Jette et al 2008). Of those, anxiety disorder was the most common mental disorder and affected approximately 44.5% of migraine sufferers; compare this to major depressive disorder, which affected only 18.8% (Saunders et al 2008).

Apart from anxiety and depression, association between migraine and bipolar disorder has also been documented. A longitudinal study conducted by Breslau and colleagues showed that patients with migraine had an increased risk of bipolar disorder at an odds ratio of up to 4.7 (95% CI 1.4, 15.4) (Breslau et al 1991). A cross-sectional hospital-based study found that, as compared to the patients without migraine, the patients with comorbid migraine had a higher frequency of bipolar II disorder (43% vs. 10%) and lower frequency of bipolar I disorder (11% vs. 33%) (Fasmer and Oedegaard 2001).

The nature of the relationship between migraine and mental disorder, especially depression, was examined by Breslau and colleagues, who demonstrated that the association was bidirectional (Breslau et al 1994; 2000). In persons with migraine, the hazard ratio for the onset of major depression was 2.35 (95% CI 1.84 to 3.01), whereas the hazard ratio for the first occurrence of migraine in persons with prior major depression was 2.75 (95% CI 2.17 to 3.48).

Migraine affects individuals, families, and also the public. Most patients with migraine experience severe distress and impairment during migraine attacks. They have a substantially reduced health-related quality of life, comparable to hypertension, diabetes, and coronary heart disease (Turner-Bowker et al 2003). A review of global burden of headache suggested that migraine contributed around 40% to 50% of the total burden (Stovner et al 2007). The total annual cost for migraine was around 14 billion dollars. Of that, 13 billion dollars was accounted for by indirect cost, which was secondary to missed workdays (Hazard et al 2009).

Tension-type headache. Tension-type headache is the most prevalent primary headache disorder. The largest American study demonstrated a 1-year prevalence of episodic tension-type headache to be 36.3% in men and 42% in women. The prevalence of chronic tension-type headache was much lower, at 1.4% in men and 2.8% in women (Schwartz et al 1998). In 2006, Russell and colleagues studied the prevalence of tension-type headache in 33,764 Danish twins 12 to 41 years of age (Russell et al 2006). The 1-year prevalence was 83.5% (78.9% in men; 92.5% in women). A longitudinal 12-year follow-up population-based study showed that the incidence rate was 14.2 per 1000 person-years, with a female-to-male ratio of approximately 3 to 1 (Lyngberg et al 2005a). Although the prevalence of tension-type headache has been acceptably high, the range varies across regions from 20% to 87%. Tension-type headache prevalence is less consistent than that of migraine. It was suggested that the prevalence was lower in Asia than in other regions, such as Europe or the United States (Stovner et al 2007). Illness behavior, that is, perception and attitude towards minor health conditions may explain this variation.

A recent study in children aged 5 to 12 years showed the prevalence of infrequent episodic tension-type headache to be 2.3% of the sample whereas prevalence of frequent episodic tension-type headache was 1.6% (Arruda et al 2010). Chronic tension-type headache is rare in early adolescence. Most studies confirm prevalence of tension-type headache to be highest in the fourth decade of life. The prevalence is much lower in the younger age groups. Tension-type headache seems to decline with age; that is, the incidence of approximately 23/1000 person-years in the 25 to 34 years age group was down to less than 5/1000 in the 55 to 64 years age group for both sexes (Lyngberg et al 2005a).

Lyngberg and colleagues studied risk factors of tension-type headache and found that poor general health, deficient sleep, and difficulty with relaxation after work increased the likelihood of tension-type headache occurrence (Lyngberg et al 2005a). From the same study population, the results showed the prognosis of tension-type headache was rather favorable. They found that 45% of subjects with frequent tension-type headache or chronic tension-type headache at baseline were in remission at follow-up. Only 16% experienced poor outcome, ie, onset or continued chronic tension-type headache. Being single, having poor sleep habits, and suffering from migraine were among factors associated with poor outcome (Lyngberg et al 2005b).

Tension-type headache has been found to overlap with other physical and mental disorders, especially those diagnosed by symptom-based criteria. Tension-type headache overlaps with other physical conditions, such as temporomandibular disorder, fibromyalgia, irritable bowel syndrome, and chronic fatigue syndrome (Feinmann and Harris 1984; Yunus et al 1989; Prescott et al 1993; Glaros et al 2007). A review of tension-type headache and psychiatric disorders showed that chronic tension-type headache had a higher frequency of psychiatric comorbidity than episodic tension-type headache (Heckman and Holroyd 2006). Anxiety disorder was the most common mental disorder and affected approximately 48% compared to 30% for depressive disorder in chronic tension-type headache. Most studies under review were, however, clinically-based rather than population-based, and different measures were used. Therefore, comparison of the results had some limitations.

Tension-type headache is less disabling but more common than

migraine. Because tension-type headache has higher prevalence, the burden of this headache is larger than that of migraine. A review of global burden of headache suggested that tension-type headache contributed around 50% to 60% of total burden compared to 40% to 50% by migraine (Stovner et al 2007). Individuals with tension-type headache also account for 4% to 12% of the missed work in 1 year; this was 3 times higher than the work hours lost because of migraine (Rasmussen et al 1992; Schwartz et al 1997).

Trigeminal autonomic cephalalgias. Trigeminal autonomic cephalalgias refer to the group of headache disorders associated with autonomic features, especially cranial parasympathetic overactivity. This headache group comprises cluster headache, paroxysmal hemicrania, and short-lasting unilateral neuralgiform headache attacks with conjunctival injection and tearing (SUNCT). Cluster headache is the most common and best-studied of the trigeminal autonomic cephalalgias. A meta-analysis of population-based studies showed the lifetime prevalence of 124 per 100,000 (confidence interval 101, 151) and a 1-year prevalence of 53 per 100,000 (confidence interval 26, 95) (Fischera et al 2008). The overall male-to-female ratio was 4.3. Interestingly, the ratio was higher in chronic cluster headache (15.0) compared with episodic cluster headache (3.8). The overall ratio for episodic versus chronic cluster headache was 6.0. A 10-year follow-up study showed that about 13% of the baseline episodic cluster headache patients evolved to the chronic form (Manzoni et al 1991). Predictors of progression included onset of cluster headache from the third decade of life onwards, greater than one annual cluster period, and short duration of remission periods (Torelli et al 2000). Compared to Western series, Asian patients were different in several aspects, including the very rare occurrence of chronic cluster headache and a low prevalence of restlessness and aura (Lin et al 2004). Racial and geographical factors might contribute to the differences.

The information regarding the comorbidities of cluster headache is limited due to the low prevalence of this condition. The documented comorbidities include sleep disorders, exposure to second-hand smoke, and history of head injuries. Association between cluster headache and sleep disorders, especially obstructive sleep apnea, has been observed. Using polysomnography, around 80% of cluster headache patients had an apnea–hypopnea index indicating at least mild obstructive sleep apnea (Graff-Radford and Newman 2004). Results from the United States Cluster Headache survey, the largest survey ever done of cluster headache, suggested that cluster headache can result from secondhand cigarette smoke exposure during childhood. The study showed that more than 60% of nonsmoking cluster headache patients had parents who smoked, compared to only 25% in the United States population as a whole. Second hand exposure to cigarette smoke appeared to initiate cluster headache at an earlier age (Rozen 2010). Few posttraumatic cluster headaches have been reported, but the cause-effect relationship is still unclear (Lambru et al 2009).

Chronic daily headache. Chronic daily headache refers to headaches that occur 15 or more days per month for more than 3 months. The nomenclature of chronic daily headache has not been included in the ICHD-II. The 4 main subtypes of primary chronic daily headache of long duration (more than 4 hours per day) are chronic migraine, chronic tension-type headache, hemicrania continua, and new daily persistent headache.

Many population-based studies demonstrate the prevalence of chronic daily headache to be between 3% and 5% (Scher et al

1998; Castillo et al 1999; Wang et al 2000; Henry et al 2002). The prevalence is approximately 3 times higher in women (Robbins and Lipton 2010). The prevalence remains consistent from mid-adolescence through adulthood and in the elderly. The majority of chronic daily headache sufferers have either chronic migraine or chronic tension-type headache. A recent systematic review showed the prevalence of chronic migraine to be 0% to 5.1% (Natoli et al 2010). Women had higher chronic migraine prevalence than men (1.39% vs. 0.15%). A long-term follow-up study of a community-based adolescent cohort showed that presence of migraine, chronic daily headache onset at younger than 13 years of age, duration of more than 2 years, and medication overuse at baseline predicted poorer outcome (Wang et al 2009).

Other forms of chronic daily headache are less prevalent compared to chronic migraine and chronic tension-type headache. Prevalence rates of new daily persistent headache in the general population have been reported at 0.03% to 0.1% (Castillo et al 1999; Grande et al 2009). This condition is more prevalent in younger age groups. New daily persistent headache has been estimated to occur in 1.7% to 10.8% of adults with chronic daily headache (Bigal et al 2002; 2004) and in 13% to 35% of children with chronic daily headache (Gladstein and Holden 1996; Koenig et al 2002). Similar to other primary headaches, new daily persistent headache is more common in females, with the female-to-male ratio in adults at 2.5:1 and in children and adolescents at 1.8:1 (Kung et al 2009).

Several studies have been conducted to determine the risk factors of chronic daily headache. Comorbid psychiatric conditions, especially depression and medication overuse, have been consistently reported to be associated with chronic daily headache. These factors are also independent predictors of persistence of chronic daily headache in adolescence (Wang et al 2007). Over-consumption of abortive treatments may interfere with the endogenous pain control system and lead to chronic daily headache. A longitudinal population-based study showed that nausea, daily use of acute headache medications, and coexistent tension-type headache and migraine were significant predictors of chronic headache (Ashina et al 2010). A population-based study showed that individuals using barbiturate-containing compounds (OR 2.06, 95% CI 1.3 to 3.1) and opiates (OR 1.98, 95% CI 1.4 to 2.2) were at the greatest risk of chronic daily headache (Bigal et al 2008). Lower socioeconomic status has been reported to be a risk factor for chronic daily headache whereas age, gender, and race were not found to be risk factors (Scher et al 2003). Obesity is also an independent risk factor for incident chronic daily headache. Chronic daily headache prevalence was higher in obese (OR 1.3, 95% CI 1.1 to 1.6) and morbidly obese (6.8% OR 1.8, 95% CI 1.4 to 2.2) individuals (Bigal and Lipton 2006). Fibromyalgia is present in 35% of chronic migraine patients, and it is associated with depression and insomnia (Peres et al 2001). Hypertension prevalence is also higher in patients with chronic daily headache compared to those with migraine and episodic tension-type headache. However, its causal effect as a predictor of headache chronification is still unclear (Gipponi et al 2010).

Chronic daily headache has strong impact on quality of life. A population-based study showed a significant decrease in each health-related concept of the short form-36 (SF-36) as compared with healthy subjects. The highest decreases were seen for physical role, bodily pain, vitality, and social functioning (Guitera et al 2002). Among chronic daily headache patients, the quality of life was lower

in those with migraine features (Autret et al 2010).

Other primary headaches. According to ICHD-II, other primary headaches consist of primary stabbing headache, primary cough headache, primary exertional headache, primary headache associated with sexual activity, hypnic headache, primary thunderclap headache, hemicrania continua, and new daily persistent headache. Among these groups, primary stabbing headache is the most prevalent. Its prevalence in the population and in headache clinics was 2% to 35.2% and 13% respectively. The prevalence was higher in women, with a female-to-male ratio between 1.5 and 2.3 (Sjaastad et al 2002; Fuh et al 2007; Wang and Fuh 2010). The mean age of onset was around 28 years in the community-based study (Sjaastad et al 2002) and 48 years old in the clinic-based study (Fuh et al 2007). This condition was observed more often in patients with migraine.

The second most prevalent headache in this group is primary exertion headache. The reported prevalence in the population is 12.3% in adults (Sjaastad et al 2002) and 30.4% in adolescents (Chen et al 2009b). Its prevalence tends to decline with increasing age. Female predominance has been demonstrated in the population-based studies whereas the opposite was reported in the hospital-based study (Pascual et al 1996).

Primary cough headache and primary headache associated with sexual activity have comparable prevalence of around 1% (Frese et al 2007; Pascual et al 2008; Chen et al 2009a). Both conditions are male predominant. Primary headache associated with sexual activity was 3 to 4 times more common in men. The mean age of onset was between the 30th and 40th years of life. Orgasmic headache was more common than pre-orgasmic subtype.

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ICD codes

ICD-9:
Headache: 784.0
Headache, cluster: 346.2
Headache, migraine: 346.9
Headache, migraine type: 346.9
Headache, psychogenic:307.81
Headache, tension-type: 307.81
Migraine, atypical: 346.1
Migraine (idiopathic): 346.9
Migraine with aura: 346.0

ICD-10:
Headache: R51
Headache, cluster: G44.0
Headache, migraine: G430
Headache, tension-type: G442
Migraine with aura: G431

Associated disorders

Chronic daily headache
Cluster headache
Migraine
Tension-type headache

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Related summaries

Activity-related headache
Chronic daily headache
Functional neuroimaging in primary headache disorders
Headache guidelines
Hemicrania continua
Idiopathic stabbing headache
Medication overuse headache
Migraine
Neuroimaging of headache
Psychological headache
Sleep and headaches
Status migrainosus
Tension-type headache

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Demographics

Age
0-01 month
01-23 months
02-05 years
06-12 years
13-18 years
19-44 years
45-64 years
64+ years

Population
None selectively affected

Occupation
None selectively affected

Sex
female>male, >1:1

Family history
Family history may be obtained

Heredity
Heredity may be a factor

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CHRONIC DAILY HEADACHE (SJ WANG, SECTION EDITOR)

Pathophysiology of Medication-overuse Headache: Implications from Animal Studies

Saknan Bongsebandhu-phubhakdi · Anan Srikiatkhachorn

Abstract Recent animal experiments have shown that chronic medication exposure profoundly affects the function of several areas in the nervous system related to headache pathogenesis. These changes include upregulation of calcitonin gene–related peptide, substance P, and nitric oxide synthase in trigeminal ganglia; expansion of receptive field and decreased nociceptive threshold of central trigeminal neurons; decrease in diffuse noxious inhibitory control; and increased susceptibility to develop cortical spreading depression (CSD). These changes indicate an increase in excitability of cortical and trigeminal neurons. The neuronal hyperexcitability may be the result of derangement of a central, possibly serotonin (5-HT)-dependent, modulating control system. Experiments with animals with low 5-HT showed that the processes of CSD and trigeminal nociception are enhanced in this condition. Derangement in the central 5-HT–dependent modulating system as a result of chronic medication use may underlie the chronification of headache as observed in patients with medication-overuse headache.

Keywords Analgesics · Calcitonin gene–related peptide · Cortical spreading depression · Ergot · Headache · Medication overuse headache · Migraine · Nitric oxide · Nociception · Serotonin · Substance P · Tension-type headache · Trigeminal system · Triptans

S. Bongsebandhu-phubhakdi · A. Srikiatkhachorn (✉)
Neuroscience Unit, Department of Physiology,
Faculty of Medicine, Chulalongkorn University,
Rama IV Road, Patumwan,
Bangkok, Thailand 10330
e-mail: anan.s@chula.ac.th

Introduction

Medication overuse is a major factor that contributes to an increase in frequency of headache in patients with primary headaches, especially migraine and tension-type headache. According to the second edition of the International Classification of Headache Disorders, medication-overuse headache (MOH) refers to the frequent (15 days per month or more) headache condition that occurs in patients with primary headaches who regularly use one or more acute and/or symptomatic drugs for more than 3 months [1]. This condition is common. Population-based studies report the 1-year prevalence rate of MOH to be 1–2% [2]. The relative frequency is much higher in secondary and tertiary care centers [3]. This disorder strongly affects the patients' quality of life and causes substantial economic burden.

The mechanism by which chronic exposure to abortive drugs leads to MOH remains unclear. However, some clinical features of MOH may indicate possible pathogenesis. First, MOH occurs mostly in patients with primary headaches. It also occurs in headache-prone patients who regularly take analgesics for other indications. This observation implies that MOH results from an interaction between an excessive use of abortive medication and a susceptible patient. Second, although migraine and tension-type headache are two different disorders with distinct pathogeneses, both conditions can be induced by medication overuse to become MOH. This finding implies that mechanism underlying MOH is likely to be the alteration of some physiological processes that are common in both conditions. Finally, all classes of symptomatic medications, namely ergots, triptans, and analgesics, are able to cause MOH if they are used excessively. Despite differences in pharmacological effects, the clinical features of MOH caused by these abortive agents are quite similar. This information reflects that

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all drugs may share a common mechanism, unrelated to their direct pharmacological effects, causing MOH.

This article reviews the recent animal studies to demonstrate the effect of chronic medication on neural structures that relate to headache pathogenesis. This review may provide some insight into the pathogenesis of MOH.

Possible Mechanisms

Understanding the possible impact of medication overuse on headache transformation requires an understanding of the pathogenesis of the underlying primary headaches. The current hypothesis focuses on the hyperexcitability of neurons in either the cerebral cortex or the trigeminal system, or both. The trigeminal system is a principle pathway for processing the nociceptive information arising from cranial structures. Therefore, activation of this system is an essential step in generating headaches in all forms of primary headaches. Differences among disorders lie on the structures that elicit the nociceptive impulse. For migraine, it is acknowledged that headache is generated from activation of primary afferents that innervate cranial vessels and meninges. On the other hand, activation of those innervating pericranial myofascial structures is responsible for generating headache in tension-type headache. The mechanism by which these trigeminal nociceptive afferents are activated in primary headaches is still uncertain.

Primary trigeminal afferents terminate by making synapse with neurons in the trigeminal nucleus caudalis (TNC). These second-order neurons then convey the impulse to the ventral posteromedial nucleus of the thalamus, where nociceptive information is interpreted. Apart from the thalamus, ascending trigeminal fibers also terminate in several brainstem areas (eg, the periaqueductal gray, brainstem reticular formation, and nucleus raphe). These brainstem structures form a complex network of endogenous modulating system. The descending projection from these nuclei has a strong influence on nociceptive perception, while its ascending projection can modulate function of several cortical and subcortical areas.

Based on these mechanisms, alteration in various steps is responsible for an increase in headache frequency occurring in MOH. These include changes in excitability of cortical neurons, an increase in sensitivity of the peripheral and central trigeminal nociceptive systems, and derangement of the central endogenous control system.

Medication-overuse Headache and Alteration of Cortical Neuronal Excitability

Clinical and experimental evidence support the role of the cerebral cortex in pathogenesis of aura in migraine. The creeping pattern of transient neurological deficit occurring during the aura phase implies the gradual propagation of abnormal activity of cortical neurons. The rate of progression corresponds perfectly with the propagation velocity of an electrical phenomenon of the cerebral cortex called cortical spreading depression (CSD).

Several evidences confirm the relationship between CSD and vulnerability of headache attacks. For instance, frequency of CSD in experimental animals can be reduced by migraine preventive agents [4, 5]. Transgenic animals carrying mutant *CACNA1A* gene, a gene that causes familial hemiplegic migraine, has higher susceptibility to CSD. The increase in CSD susceptibility also has been demonstrated in animals with low serotonin (5-hydroxytryptamine [5-HT]). This finding may explain an increase in headache frequency in chronic daily headache, the condition in which 5-HT level is low [6]. The lower threshold for CSD was reported in female mice [7]. Sex hormones alter cortical excitability, and thus, may contribute to an increased prevalence of migraine in women.

Experiments in animals revealed that chronic analgesic exposure affects the development of CSD. In rats, a 30-day course of acetaminophen led to an increase in frequency of CSD, CSD-evoked expression of Fos, and 5-HT_{2A} serotonin receptor in cerebral cortex. The number of Fos-immunoreactive cells in TNC also was increased, reflecting the facilitation of trigeminal nociception [8••]. Such findings indicate that chronic analgesic exposure enhances the excitability of cortical neurons. The neuronal hyperexcitabilty may render the cerebral cortex more susceptible to developing CSD and facilitate the trigeminal nociceptive process. The alteration in cortical activity also has been reported in patients with MOH. A functional magnetic resonance imaging study also showed that the right supramarginal gyrus, the right inferior cortex, and the superior parietal cortex of these patients were hypoactive [9]. Because these structures are parts of the lateral pain system, the alteration in their activity would indicate the modification of the pain network in patients with MOH.

An increase in susceptibility to CSD may explain the mechanism by which medication overuse causes clinical deterioration in migraine patients with MOH. However, this hypothesis could not explain how overuse of symptomatic medication leads to MOH in patients with tension-type headache. Other mechanisms are required to explain the transformation of headache induced by medication overuse in this condition.

Medication-overuse Headache and Alteration of the Trigeminal Nociceptive System

Similar to other chronic pain syndromes, an increase in sensitivity of trigeminal afferents (so-called peripheral

sensitization) and central trigeminal neurons (or central sensitization) has a significant role in pathogenesis of primary headaches. Peripheral sensitization refers to the state in which peripheral nociceptors increase their responses to suprathreshold stimuli and become responsive to subthreshold stimuli. Several mediators released during tissue injury and inflammation (eg, prostaglandins, bradykinin, and calcitonin gene–related peptide [CGRP]) can sensitize nociceptors by means of neuronal protein phosphorylation. Phosphorylation of sensory neuron–specific tetrodotoxin-resistant sodium channel as well as calcium channels will lower the threshold of nociceptor membrane. As a result, the nociceptor becomes more ready to fire, and some inactive or "silent" nociceptors are turned on. Therefore, low-intensity stimuli, such as touch and vascular pulsation, can result in the painful sensation. This peripheral sensitization underlies the development of throbbing headache and cutaneous allodynia, which occur during the attack of migraine.

Recent studies have revealed the substantial impact of chronic abortive medication on trigeminal afferents. Rats receiving repeated intermittent injections or continuous infusion of triptans over 6 days exhibited time-dependent and reversible cutaneous tactile allodynia that was maintained throughout and transiently after drug delivery. Chronic triptan exposure increased the number of CGRP-positive dural afferent neurons in the trigeminal ganglion. Sumatriptan exposure also increased the number of trigeminal ganglion cells that co-expressed CGRP and substance P. This upregulation persisted long after discontinuation of triptan exposure. Exposure to triptans also increased CGRP in the blood after challenge by nitric oxide (NO) donor. These results indicate that chronic exposure to triptans can alter the trigeminal system and results in a state of latent sensitization, which may increase sensitivity to migraine triggers [10••]. Interestingly, upregulation of CGRP has been observed in dorsal root ganglion after prolonged exposure to morphine [11, 12]. Sustained morphine exposure also induces a spinal dynorphin–dependent enhancement of excitatory transmitter release from primary afferent fibers [13].

One animal study reported an alteration in the NO system in the trigeminal ganglion. Chronic exposure to triptans induced a long-lasting increase in expression of neuronal nitric oxide synthase (nNOS) in trigeminal ganglionic cells innervating dura. It was also shown that NXN-323, a selective nNOS inhibitor, can reverse cutaneous allodynia observed during the period of triptan administration. The nNOS inhibition also prevented environmental stress–induced hypersensitivity in the period after triptan administration. These findings support the role of NO in controlling the sensitivity to environmental stress [14••]. The increase in function of the NO system may increase sensitivity to stress and susceptibility to headache, as experienced in patients with MOH.

In addition to the change in trigeminal afferents, animal studies also demonstrate plasticity of central trigeminal neurons. Expansion of cutaneous receptive field sizes and lower threshold of dura-sensitive medullary dorsal horn neurons were observed in rats receiving sustained infusion of morphine [15••]. These changes indicate that chronic medication can sensitize central trigeminal neurons.

The concept of peripheral and central sensitization can explain several clinical features of MOH. Progression from unilateral to bilateral headache implies the recruitment of nociceptive neurons of higher level (eg, thalamus) in the process of pain transmission. Allodynia in the form of scalp tenderness reflects the decrease in nociceptive threshold, which is the key feature of sensitization.

Medication-overuse Headache and Alteration of Central Modulating System

It is unlikely that MOH is caused by a mechanism specific for each headache type or drug. As mentioned above, medication overuse can alter excitability and protein expression in the cerebral cortex, trigeminal ganglia, and central trigeminal pathway. It is known that MOH can be developed in several types of primary headaches and can be triggered by various classes of symptomatic medications. The more plausible mechanism is the derangement of the system, which has widespread modulating effects on the central nervous system function. The dysfunction of the central modulating system, such as the aminergic system, can explain several clinical features in patients with MOH. Dysfunction of cortical projection may underlie the increase in CSD susceptibility, resulting in the increase in headache frequency, as well as other psychiatric symptoms such as mood disorders (especially depression, anxiety, and irritability, among others). Effects on the reticular system may cause sleep disorders. Dysfunction of descending projections to the trigeminal system may foster the process of central sensitization, leading to the increased headache susceptibility.

Alteration in the 5-HT system may facilitate the nociception and underlie some behavioral symptoms, such as insomnia, in patients with MOH. Plasticity of the central 5-HT system has been reported in animals on chronic medication. A 15-day course of acetaminophen led to an increase in platelet 5-HT concentration. This change was accompanied by a downregulation of the 5-HT_{2A} receptor and an upregulation of the 5-HT transporter in the frontal cortex. These effects of analgesics on the 5-HT system declined after a more prolonged administration of the drug. These changes coincided with the decrease in analgesic

efficacy [16]. Upregulation of the 5-HT_{2A} receptor in the cerebral cortex has been demonstrated in rats treated with 30-day course of acetaminophen. The increase in CSD susceptibility observed in rats with chronic acetaminophen exposure was blocked by 5-HT_{2A} receptor antagonist [17••]. Alteration of the 5-HT receptor and transporter expression in several subcortical areas, including the periaqueductal grey and the locus coeruleus, also has been reported in animals with chronic triptan exposure [18, 19]. Both areas have important roles in regulating the nociception and other vegetative functions such as alertness and autonomic functions, among others.

Serotonin plays a pivotal role in the pathogenesis of primary headaches, both migraine and tension-type headache. Tryptophan depletion increases nausea, headache, and photophobia in migraineurs [20]. Decreases in 5-HT levels also were reported in patients with MOH [21]. Animal experiments show that dysfunction of this transmitter system leads to several changes in the cerebral cortex and trigeminal system, which can increase the susceptibility of headache attacks. Animals with low 5-HT show increase in CSD susceptibility and CSD-evoked Fos expression in the TNC [6]. Inhibition of NO production can attenuate this cortical hyperexcitability [22]. Low 5-HT state may subsequently upregulate the expression of the excitatory 5-HT_{2A} receptor in the cortex and trigeminal system. Activation of this pronociceptive receptor can activate NO production and increase susceptibility to CSD [23]. The hypothesis is supported by the findings that increased CSD susceptibility in rats with chronic medication was attenuated by 5-HT_{2A} receptor antagonist [17••].

In addition to the effect on the cerebral cortex, decrease in the function of the 5-HT system also affects the trigeminal nociception. The expression of Fos and phosphorylation of the N-Methyl-D-aspartate (NMDA) receptor in the TNC neurons evoked by meningeal inflammation is increased in animals with low 5-HT [24]. This finding indicates that the process of sensitization of central trigeminal neurons is facilitated in low 5-HT condition. An experiment in animals with low 5-HT also shows the increase in CGRP-immunoreactive cells in the trigeminal ganglia [25]. As mentioned, increase in CGRP expression has been observed in rats chronically treated with triptans [10••].

These evidences show that central modulating control has strong influence on the function of the trigeminal

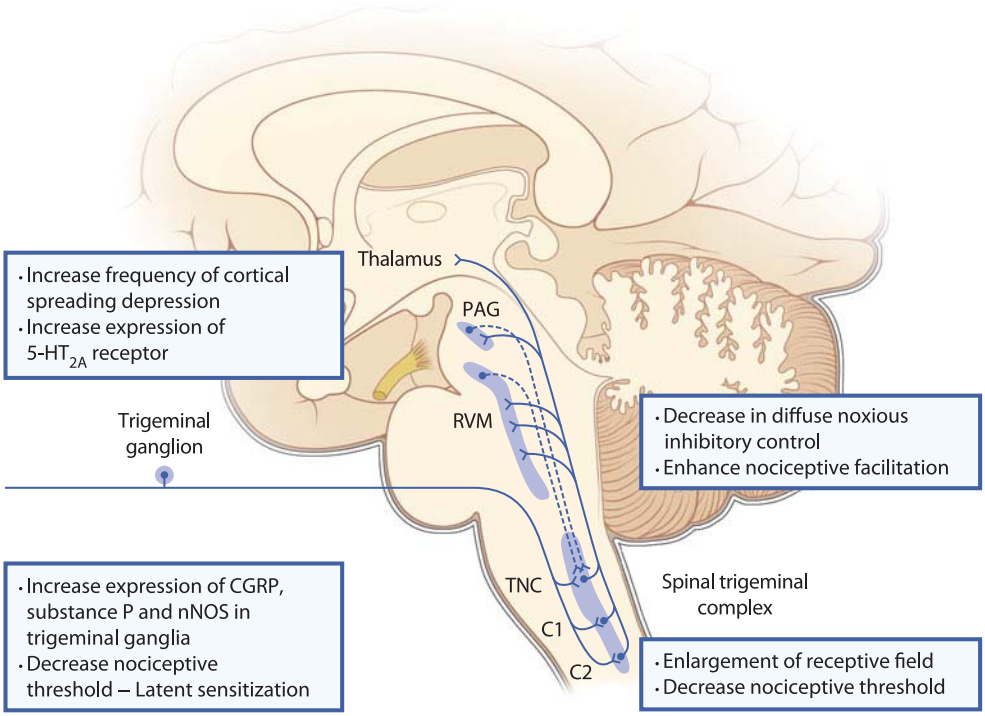

Fig. 1 Diagram showing effect of chronic medication on cerebral cortex, trigeminal, and central modulating system. Chronic medication increases expression of calcitonin gene–related peptide (CGRP), substance P, and neuronal nitric oxide synthase (nNOS) in the trigeminal ganglia as well as decreases its nociceptive threshold. In the central trigeminal pathway, exposure to chronic medication can sensitize central neurons in brainstem. Chronic medication exposure can increase excitability of cortical neurons and increase the susceptibility to develop cortical spreading depression. These changes may be the result of dysfunction of central, possibly 5-hydroxytryptamine (5-HT)–dependent, modulating system in the rostral brainstem. *PAG* periaqueductal grey; *RVM* rostroventral medulla; *TNC* trigeminal nucleus caudalis

system. Derangement of this control system, either decreased nociceptive inhibition or increased nociceptive facilitation, may enhance the process of central sensitization. Several experiments have demonstrated the impairment of the central control system in animals with chronic medication exposure. Okada-Ogawa et al. [15••] showed that diffuse noxious inhibitory controls (DNICs) were impaired in rats receiving chronic morphine administration. The loss of DNICs can be restored by inactivation of the rostral ventromedial medulla with 4% lidocaine, indicating that descending facilitation from the rostroventral medulla is responsible for the loss of DNICs in morphine-treated animals. The dysfunction of DNICs is also evidenced in patients with MOH. Perrotta et al. [26] showed that the temporal summation threshold of the nociceptive withdrawal reflex was markedly reduced in patients with MOH. These neurophysiological abnormalities corresponded with the psychophysical measurements, which revealed enhanced pain perception after single and repeated stimulation and increased temporal summation of pain. The patients with MOH also exhibited a reduced ability to activate the DNICs triggered by cold pressor test. These findings can be explained by the presence of widespread abnormal spinal cord pain processing in patients with MOH, which may be caused by defective functioning of the supraspinal control of pain. Derangement in central nociceptive processing can be implied by several neurophysiologic findings. Fusco et al. [27] demonstrated that the temporal summation of the second pain, the psychophysical correlate of the excitatory pain circuits, was greater in patients with MOH than those with migraine or episodic tension-type headache. Other neurophysiologic abnormalities observed in this condition include decreased critical flicker frequency and increased P3 latency of the event-related potential [28, 29]. All of these neurophysiological variables were normalized after drug discontinuation.

Conclusions

The results of the animal studies demonstrate that chronic symptomatic medication can alter the cerebral cortex and trigeminal system. In the cerebral cortex, chronic medication alters the expression of the 5-HT receptor and enhances the excitability of cortical neurons. In the trigeminal nociceptive system, chronic medication may facilitate the process of peripheral and central sensitization (see Fig. 1). These changes may be secondary to the derangement of the central, especially 5-HT–dependent, modulating system. Medication overuse may further derange this system by inducing a low level of 5-HT. The relative depletion of 5-HT subsequently upregulates the 5-HT_{2A} receptor and changes the intracellular signaling. Increased expression of the cortical 5-HT_{2A} receptor may increase the susceptibility of developing CSD. Reduction of nociceptive inhibitory control may facilitate the process of central sensitization, activate the nociceptive facilitating system, or promote kindling. Thus, derangement in central modulating, as a result of chronic medication use, may increase sensitivity to pain perception and foster or reinforce MOH.

One major issue to be considered for interpretation of this data is that all studies were conducted in normal animals. On the contrary, MOH occurs only in susceptible patients. Further studies using animal models of primary headaches (eg, *CACNA1A* transgenic mice) would provide better understanding of the pathogenesis of this condition.

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Disclosures No potential conflicts of interest relevant to this article were reported

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Involvement of AMPA receptors in CSD-induced impairment of LTP in the hippocampus

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Manuscript type:	Research Submissions
Key Words:	cortical spreading depression (CSD), long-term potentiation (LTP), AMPA receptor, hippocampus
Area of Expertise:	Pathophysiology, Chronic migraine, Mechanism

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Involvement of AMPA receptors in CSD-induced impairment of LTP in the hippocampus

Montree Maneepark, Anan Srikiatkhachorn, Saknan Bongsebandhu-phubhakdi

Department of Physiology, Faculty of Medicine, Chulalongkorn University, Patumwan, Bangkok, Thailand

Correspondence should be addressed to: Saknan Bongsebandhu-phubhakdi, Ph.D., Department of Physiology, Faculty of Medicine, Chulalongkorn University, 1873 Rama IV Rd, Patumwan 10330 Bangkok, Thailand. E-mail: saknan@live.jp

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Keywords: cortical spreading depression (CSD); long-term potentiation (LTP); AMPA receptor; hippocampus

Abbreviations: ACSF - artificial cerebrospinal fluid, CSD - cortical spreading depression, DC - direct current, fEPSP - field excitatory postsynaptic potential, I/O - input-output, LTP - long-term potentiation, PPF - paired-pulse facilitation, SD – spreading depression, TGA - transient global amnesia.

Abstract

Objective: To investigate the alteration of hippocampal long-term plasticity and basal synaptic transmission induced by repetitive cortical spreading depressions (CSDs).

Background: There is a relationship between migraine aura and amnesic attack. CSD, a phenomenon underlying migraine attack, may be responsible for hippocampus-related symptoms. However, the precise role of CSD on hippocampal activity has not been investigated.

Methods: Male Wistar rats were divided into CSD and control groups. Repetitive CSDs were induced *in vivo* by topical application of solid KCl. Forty-five minutes later, the ipsilateral hippocampus was removed, and hippocampal slices were prepared for a series of electrophysiological studies.

Results: Repetitive CSDs led to a decrease in the magnitude of long-term potentiation (LTP) in the hippocampus. CSD also reduced hippocampal synaptic efficacy, as shown by a reduction of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor responses. In contrast, postsynaptic *N*-methyl-D-aspartate (NMDA) receptor responses remained unchanged. In addition, there were no changes in paired-pulse profiles between the groups, indicating that CSD did not induce any presynaptic alterations.

Conclusion: These findings suggest that a reduction of postsynaptic AMPA receptor responses is the mechanism responsible for impaired hippocampal LTP induced by CSD.

Introduction

Spreading depression (SD) is a transient, reversible phenomenon, which is expressed as a self-propagating depolarization of neurons and glia, followed by a depression of neuronal bioelectrical activity for a period of minutes and accompanied by complex and variable changes in vascular caliber, blood flow, and energy metabolism.[1, 2] Although SD has been most extensively studied in the cortex (i.e., cortical SD), the phenomenon can be induced in most grey matter regions, including the hippocampus and the cerebellum, in a variety of species.[3] CSD was originally linked to the aura phase of a migraine.[4] However, some evidence also suggests a link between CSD and migraine pain as well as the associated signs and symptoms of migraine, such as sexual arousal, yawning and drowsiness, nausea and vomiting, and amnesia.[1]

Transient global amnesia (TGA) is a type of memory disorder that is characterized by sudden episodes of severe anterograde and retrograde amnesia that last for several hours and then resolve completely and spontaneously within 24 hours.[5, 6] Many studies have reported a relationship between TGA and migraine. TGA attacks that are accompanied by migraine headache have been described in many case reports.[7-9] In some cases, an amnesic attack occurs only during migraine attack.[10] Migraine is a risk factor and a precipitating event for TGA.[11] Migraine and TGA also share common precipitants.[7]

These associations between TGA and migraine support the hypothesis that both diseases might share a common pathophysiology like SD, as proposed by Olesen and Jorgensen.[12] In clinical study, diffusion-weighted magnetic resonance imaging showed an increased signal intensity, which is compatible with SD, in the hippocampal region of TGA patients during attack.[13] Bartsch and coworkers further demonstrated that an increased signal intensity during attack is selectively found in the hippocampal CA1 area of TGA patients.[14] In animal study, hippocampal injection of potassium chloride produced a deficit in retention

of conditioned suppression learned 24 hours before injection.[15] Bilateral hippocampal or cortical SD evoked immediately after acquisition of a passive avoidance reaction elicited a partial amnesia.[3]

Although CSD may be related to memory disorder and perturbation of hippocampal function, the effect of CSD on hippocampal plasticity has not been fully investigated. A study of *in vitro* cortical-hippocampal combined slices showed that CSD can, depending on the propagation of SD into the hippocampus, facilitate or depress hippocampal LTP.[16] However, the mechanism that underlies this phenomenon is unknown.

It has been well established that activation of postsynaptic NMDA receptors is involved in the induction of LTP in the CA1 region of the hippocampus.[17] In addition to NMDA receptors, AMPA receptors play a pivotal role in LTP induction. Jia and coworkers showed that knockout of AMPA receptor subunits affected LTP induction.[18] Furthermore, pharmacological modulation of AMPA receptors influenced synaptic plasticity in the hippocampus.[19]

The aim of this study was to investigate the effect of CSD on hippocampal long-term plasticity and basal synaptic transmission. The knowledge obtained from this study provides a better understanding of the mechanisms that underlie the perturbation of hippocampal synaptic plasticity induced by CSD and the pathogenesis of hippocampus-related symptoms that occur during a migraine attack.

Materials and Methods

Study Design. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University (No. 012/2010). Adult male Wistar rats (weighing 200-350 g; National Animal Center, Mahidol University, Thailand) were divided into CSD and control groups. In the CSD group, *in vivo* CSDs were induced in the parietal cortex for 45 minutes by topical application of solid KCl. Solid NaCl was applied in the control rats. Cortical direct current (DC) potentials were recorded. Following recordings, the ipsilateral hippocampus was removed, and transverse hippocampal slices were prepared for *in vitro* electrophysiological experiments. The electrophysiological experiments included LTP, paired-pulse facilitation (PPF), and input-output (I/O) relationship of NMDA receptor responses and of AMPA receptor responses.

Surgical Procedures and Induction of CSD. Each rat was anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally), and fitted with an intratracheal tube. Additional doses of the anesthetic were given as required to maintain surgical anesthesia (based on response to tail pinch). Each rat was placed in a stereotaxic apparatus. A surgical incision was made on the midline, and the skin and soft tissue overlying the skull were removed. For induction of CSD, a 2-mm diameter craniotomy was made in the right parietal bone (6 mm posterior to the bregma and 2 mm lateral to the midline). The bone was drilled carefully using a slow speed, saline-cooled technique to minimize surgical irritation of the neurons. The dura mater was opened using a microneedle. Multiple waves of CSD were elicited by topical application of solid KCl (3 mg) onto the exposed surface of the parietal cortex. In the control group, NaCl was used instead of KCl.

Extracellular Cortical Activity Recording. For DC recordings, a cranial window (2 mm in diameter) was created in the right frontal bone (3 mm anterior to the bregma and 2 mm lateral

to the midline). After careful removal of the dura mater, a recording glass microelectrode for detecting negative DC potential was inserted into the frontal neocortex using a hydraulic micromanipulator (Narishige, Scientific Instrument Laboratory, Tokyo, Japan) to a depth of 500 µm. A Ag/AgCl reference electrode was placed on the skin at the back of the rat. The electrical signal was amplified using a microelectrode amplifier (Nihon Kohden, Tokyo, Japan). Analog data were converted into a digital format using an MP100 data acquisition system (Biopac Systems Inc., CA, USA). The converted data were then analyzed using the AcqKnowledge acquisition software (Biopac Systems Inc.). The variables that were measured included the amplitude and the area under the curve (AUC) of each CSD wave as well as the number of CSD waves that occurred within a 45-minute period.

In another set of experiments, DC was recorded in the CA1 region of the hippocampus instead of the neocortex. A craniotomy 2 mm in diameter was made in the right parietal bone (4 mm posterior to the bregma and 2 mm lateral to the midline). A recording glass microelectrode was inserted into the CA1 area of the hippocampus to a depth of 2.2 mm. The results showed that negative DC potential waves also appeared in the hippocampus after CSD induction.

Hippocampal Slice Preparation. Forty-five minutes after elicitation of CSD waves, each rat was decapitated, and the entire ipsilateral hippocampus was quickly removed from the brain. Transverse hippocampal slices (400 µm thickness) were cut using a Vibratome tissue slicer (Vibratome, IL, USA) in ice-cold artificial cerebrospinal fluid (ACSF). The ACSF contained the following (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1.0 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose, and 0.1 picrotoxin, a $GABA_A$ receptor antagonist. The ACSF was bubbled continuously with carbogen (95% O_2/5% CO_2). Fresh slices were placed in a humidified interface-type holding chamber and recovered for at least 1.5 h before electrophysiological experiments were conducted.

Electrophysiological Recording. An individual slice was transferred into a recording chamber, fixed with a nylon net, and submerged in carbogen-saturated ACSF, which was continuously perfused at a rate of 1.5–2.0 ml/min. To avoid epileptiform activity from the CA3 region, a cut was made to separate the CA1 region from the CA3 region. All experiments were performed at room temperature (25 °C).

A bipolar tungsten stimulating electrode was placed in the stratum radiatum. Square-pulse stimuli of 0.2 ms duration at 0.1 Hz (1 pulse every 10 s) were delivered to the slice through the bipolar tungsten electrode to activate the Schaffer-collateral pathway that projects to CA1. A glass microelectrode with 2-8 MΩ resistance containing 3 M NaCl was positioned parallel to the stimulating electrode in the stratum radiatum, 200–300 μm from the stimulating site, to record presynaptic fiber volleys that were followed by field excitatory postsynaptic potentials (fEPSPs). To avoid changes in fEPSP properties that could be attributed to electrode positioning, an attempt was made to maintain a similar orientation of the electrodes relative to the pyramidal cell layer and dentate gyrus in all experiments. For baseline recordings, stimulus strength was adjusted to evoke fEPSPs with slopes of 0.12 to 0.20 mV/ms. Only slices that produced fEPSP amplitudes of more than 1 mV and were stable for at least 30 min were included in this study. All slices that failed to stabilize within 90 min were rejected.

An EPC-10 amplifier with Patchmaster software (HEKA Instruments Inc., NY, USA) was used for all *in vitro* electrophysiological recordings. The signals were amplified, digitally sampled at 10 kHz, and stored for offline analysis.

LTP. The LTP protocol consisted of a high-frequency stimulation (tetanus). After recording stable baseline fEPSPs for at least 30 min, LTP was elicited by applying tetanic stimulation of 100 Hz for 1 sec at the same stimulus intensity as that used for the baseline values. Post-tetanic responses were recorded for 60 min after tetanic stimulation. The magnitude of LTP

was calculated by dividing the average slope of the 50–60 min post-induction responses by the average slope of the 0–30 min pre-induction baseline responses.

I/O. The I/O protocol consisted of multi-step stimulation intensities that were adjusted to evoke threshold to maximal fEPSP responses. After recording stable baseline fEPSPs for at least 15 min, I/O protocol was conducted, and 5 responses were recorded for each stimulation intensity. During the recording of NMDA receptor responses, NMDA receptor-mediated fEPSPs were isolated by bath application of a potent AMPA receptor antagonist, CNQX (10 μM), in low magnesium (0.1 mM) ACSF. During the recording of AMPA receptor responses, AMPA receptor-mediated fEPSPs were isolated by bath application of a highly selective NMDA receptor antagonist, APV (25 μM). To provide an indication of differences in receptor-mediated responses to stimuli of a given intensity, the slopes of the NMDA receptor or AMPA receptor-mediated fEPSPs were plotted against the stimulus voltages.

PPF. PPF has been shown to be presynaptic in origin and does not induce any changes in postsynaptic effectiveness. The PPF protocol included 4 two-pulse pairs with decreasing inter-pulse intervals (400, 200, 100, and 50 ms). After recording stable baseline fEPSPs for at least 15 min, paired-pulse stimulations at 0.1 Hz were delivered, and 10 responses for each inter-pulse interval were recorded. The ratio of facilitation was computed by dividing the fEPSP slope evoked by the second pulse with the fEPSP slope evoked by the first pulse.

Statistical analysis. All data were expressed as the mean ± SEM. Statistical analysis was performed using Student's t-test or repeated-measures ANOVA, where applicable. Probability values of less than 0.05 were considered to be statistically significant.

Results

CSD Induction

After application of solid KCl (3 mg) on the exposed surface of the parietal cortex, a series of negative depolarization shifts characterized as CSD was recorded in the surface of the frontal cortex. The total number of CSD waves that occurred within 45 min was 9.23 ± 0.87. The average amplitude, duration, and area under the curve of each wave were 34.62 ± 3.03 mV, 69.67 ± 8.77 s, and 712.35 ± 93.88 mV·s, respectively. The interval between each wave was 5.10 ± 0.67 min. No depolarization shift was observed in the control group.

Effect of CSD on Hippocampal Excitability

In all electrophysiological protocols, stimulus intensity was adjusted to evoke fEPSPs of 0.12 to 0.20 mV/ms slopes as the baseline responses. Evoked fEPSP slopes obtained from baseline recordings in the CSD slices were comparable with those recorded in the control slices (0.159 ± 0.007 and 0.157 ± 0.005 mV/ms, respectively; $P = 0.828$, n = 10 each group; Fig. 1A). However, the stimulation intensities needed to evoke the same fEPSP responses were higher in slices obtained from the CSD rats than those from the control rats (0.327 ± 0.012 and 0.264 ± 0.014 V, respectively; $P = 0.003$; n = 10 each group; Fig. 1B). These data suggest that CSD had a significant effect on evoked neuronal excitability in the hippocampus.

Effect of CSD on LTP

In both groups, tetanic stimulation induced a rapid, stable and lasting enhancement of the slope of the evoked potential (Fig. 2A). The potentiation rose within 1–2 min and stabilized within another 18–22 min after the stimulus. However, LTP magnitude in the hippocampus was influenced by repetitive CSD induction. In the CSD group, the LTP magnitude significantly decreased (P = 0.009, Student's t-test; Fig.2B). The levels of LTP in the CSD and control groups were 122.7 ± 4.2 and 140.8 ± 4.1 %, respectively (n = 7 each group).

Effect of CSD on Paired-Pulse Facilitation

The effects of CSD on hippocampal presynaptic modifications were studied by examining PPF. PPF is a form of plasticity in which the second of two pulses, delivered within 25-250 ms of one another, evokes a larger synaptic response than the first pulse. PPF is thought to be presynaptic in origin and attributable to a residual increase in the concentration of internal calcium within the presynaptic terminal.[20] The results showed that no overall PPF profile change was observed between the CSD and control slices (P = 0.651 by one-way repeated measures ANOVA, n = 10 each group; Fig. 3). This result indicates that presynaptic responses at hippocampal SC-CA1 synapses were not altered in the rats that received CSD.

Effect of CSD on Input-Output Relationship of AMPA Receptor Responses

The effects of CSD on hippocampal postsynaptic AMPA receptor responses were studied by examining I/O profile of AMPA receptor-mediated fEPSPs. The results showed that the overall I/O curve of the AMPA receptor responses was significantly reduced in hippocampal slices obtained from the CSD rats (P = 0.012, one-way repeated measures

ANOVA, n = 7 each group; Fig. 4). The slices obtained from the CSD rats showed a significant reduction in evoked AMPA receptor-mediated fEPSPs across a range of stimuli intensities. This result indicates that AMPA receptor responses at SC-CA1 synapses were depressed in CSD-induced rats.

Effect of CSD on Input-Output Relationship of NMDA receptor Responses

Examination of the I/O relationship of NMDA receptor-mediated fEPSPs showed no significant differences in the overall response between CSD and control slices (P = 0.256, one-way repeated measures ANOVA, n = 7 each group; Fig. 5). This result indicates that repetitive CSDs did not alter NMDA receptor function at SC-CA1 synapses.

Discussion

This study demonstrated that CSD could affect hippocampal synaptic transmission and plasticity. Repetitive CSD induction resulted in a reduction of excitability as well as a reduction of LTP in the CA1 region of the hippocampus. Our results are in contrast to those of previous studies.[16, 21] Experiments in human neocortical slices demonstrated that CSD led to a long-lasting increase of fEPSP. In addition, induction of LTP in the slice was enhanced following propagation of CSD.[21] Wernsmann and coworkers demonstrated that a single CSD elicited in a neocortical-entorhinal-hippocampal combined slice resulted in increased hippocampal excitability.[16] The result from a previous study showed an augmentation of hippocampal fEPSP in an abortive CSD slice (i.e., a slice in which CSD did not propagate into the hippocampus) as well as an increase in tetanus-induced LTP.

The above observations suggest that alteration of synaptic efficacy could develop in both directions, depending on the characteristics and intensity of stimulation.[22] In our *in vivo* model, topical application of solid KCl induced repetitive CSDs. In another set of experiments, DC shifts were also present in the hippocampus during CSD induction. Thus, our study suggests that repetitive CSDs induced by solid KCl application propagated to the hippocampus and suppressed hippocampal excitability and caused a reduction in tetanus-induced LTP.

According to the results of our study, the changes in synaptic response and plasticity induced by repetitive CSDs are likely to be postsynaptic in origin. Our study showed no alteration of PPF (which is sensitive to changes in presynaptic release probability) in CSD rats. These data suggest that neurotransmitter release is likely to be normal in the CSD rats. This finding is consistent with the data from Hsu and coworkers who showed that exposure of a hippocampal slice to low extracellular magnesium, a condition that induces seizure-like activity and SD[23], impaired hippocampal plasticity without any significant changes in PPF

and postsynaptic NMDA receptor responses.[24] Our results suggest that both the reduction of excitability and LTP suppression by repetitive CSDs are mediated via a postsynaptic process rather than by a presynaptic process.

Our present study showed that CSD impaired AMPA receptor responsiveness as examined by the I/O relationship. The mechanism by which CSD decreases AMPA receptor function remains unclear. The mechanism could be mediated by a change in single channel properties of AMPA receptors or by a decrease in AMPA receptor trafficking to synapses. Reduction of AMPA receptor transcription and translation after SD episodes may be responsible for fewer available AMPA receptors and a decrease in AMPA receptor response. Evidence for this hypothesis has been shown in an ischemic model, which shares similar phenomena with our model (e.g., excessive glutamate release), and also induces SD from the ischemic core out toward the margins of the ischemic zone.[25] Induction of 2 min of ischemia caused a decrease in AMPA receptor subunit transcript levels in the CA1 region of the hippocampus.[26] A reduction in the protein expression levels of GluR1 and GluR2 subunits of the AMPA receptor was observed after 10 consecutive CSD waves.[27] Systemic administration of 4-aminopyridine, a potassium channel blocker that elicits SD and convulsion, diminished the efficacy of glutamatergic transmission.[22, 28] Interestingly, this reduced glutamatergic transmission was consistent with decreased GluR1–4 levels in the cortical layers.[22] It is unclear whether SD affects AMPA receptor trafficking or channel properties. However, some evidence suggests an alteration in a protein kinase related to AMPA receptor modulation. In the ischemic rat model, a 3-min ischemia prevented the autophosphorylation of CaMKII at threonine 286.[29] The inhibition of phosphorylation may influence AMPA receptor function and trafficking. Our results and these data indicate that AMPA receptor transmission is impaired after an SD episode, possibly via these mechanisms.

The present electrophysiological study revealed a reduction of AMPA receptor function as well as LTP suppression in hippocampal slices obtained from rats that received CSD. It is unknown if the decrease in AMPA receptor response was responsible for LTP impairment. It has been well established that mechanisms of LTP induction involve AMPA receptor trafficking and potentiated receptor response by phosphorylation.[30, 31] Thus, any changes in the properties of AMPA receptors are likely to affect LTP. Furthermore, the positive AMPA receptor modulators CX516 and CX546, which prominently enhance synaptic transmission, can facilitate LTP in hippocampal slices.[19] These data suggest that the impairment of LTP induced by repetitive CSDs in the present study may result from a reduction of AMPA receptor response.

We have shown that NMDA receptor function remains intact in rats that received CSD. However, our results could not exclude a role of NMDA receptors in LTP suppression. It is possible that the excessive glutamate release and overstimulation of NMDA receptors that occur during an SD episode affect the subsequent induction of LTP. Much evidence supports the hypothesis by which prior NMDA receptor activation suppresses LTP induction. It has been shown that repetitive activation of NMDA receptors by a weak tetanus (30 Hz, 0.15 s) or by applying NMDA directly inhibits any subsequent LTP induction in the CA1 region of the hippocampus.[32] In addition, GLT-1 knockout mice, whose phenotype is attributed to an increased glutamate level in the synaptic cleft, show an impairment of LTP induction.[33] Furthermore, exposure of hippocampal slices to low extracellular magnesium, a condition that induces seizure-like activity and SD, disrupts the subsequent induction of LTP.[23, 24] The mechanism underlying this phenomenon remains to be determined; however, alteration of CaMKII properties during excessive glutamate release may present a possible explanation. It has been shown that the Ca^{2+}-independent activity and autophosphorylation of CaMKII in cultured rat hippocampal neurons decreases after exposure to high concentrations

of glutamate. These reductions are prevented by pretreatment with NMDA receptor antagonists but not by other types of glutamate receptors.[34] These data suggest that NMDA receptors may be involved in disrupted LTP induction even though SD does not affect their function.

The present study demonstrated that repetitive CSDs could alter hippocampal synaptic plasticity, as shown by a reduction in LTP magnitude. The results indicate that the underlying mechanism may involve a reduction of postsynaptic AMPA receptor responses, while postsynaptic NMDA receptor function and presynaptic processing remain unchanged. The findings from this study may explain hippocampus-related symptoms such as amnesic attack, which occur during migraine attacks.

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Figure legends

Figure 1. Neuronal excitability in the hippocampus was reduced by CSD induction. ***A*,** Evoked baseline fEPSPs were not significantly different between the CSD and control groups. Inset: representative traces of fEPSPs. Calibration: 20 ms, 0.2 mV. N.S.: not significant. ***B*,** Stimulation intensities required to evoke baseline fEPSPs were higher in the CSD group. * $P< 0.05$, Student's *t*-test. Bar and whisker plots indicate the mean ± SEM.

Figure 2. CSD induction affected LTP in hippocampal slices. ***A*,** Hippocampal LTP in the control and CSD groups. LTP was induced by tetanic stimulation (100 Hz for 1 s) applied at time 0. Inset: Representative traces recorded immediately prior to LTP induction and 60 min after LTP induction. Calibration: 20 ms, 0.2 mV. ***B*,** Data plot of the LTP magnitude. The magnitude of potentiation was calculated by dividing the average EPSP slope values from 50 to 60 min with those from 0 to 30 min. LTP was significantly reduced in hippocampal slices obtained from the CSD rats. * $P< 0.05$ Student's *t*-test. Bar and whisker plots indicate the mean ± SEM.

Figure 3. Paired-pulse profiles of the hippocampal slices were not affected by CSD. Closed circles (CSD) and open circles (control) showed the facilitation ratios of the two groups. No significant differences in PPF profiles were observed.

Figure 4. I/O relationship of AMPA receptor-mediated fEPSPs in the hippocampal slices obtained from CSD and control rats. CSD significantly suppressed the AMPA receptor component of fEPSP (P = 0.012, repeated-measures ANOVA). Closed circles (CSD) and open circles (control) showed evoked fEPSPs in hippocampal slices. Representative traces showed AMPA receptor responses to stimuli at 0.275, 0.300, 0.325, 0.350, 0.400, 0.450, 0.500, and 0.550 V. Calibration: 20 ms, 0.4 mV. * $P < 0.05$, Student's t-test.

Figure 5. I/O relationship of NMDA receptor-mediated fEPSPs in hippocampal slices obtained from CSD and control rats. CSD did not significantly alter the NMDA receptor component of fEPSP (P = 0.256, repeated-measures ANOVA). Closed circles (CSD) and open circles (control) showed evoked fEPSPs in hippocampal slices. Representative traces showed NMDA receptor responses to given stimuli. Calibration: 50 ms, 0.4 mV.

Illustrations

Figure 1.

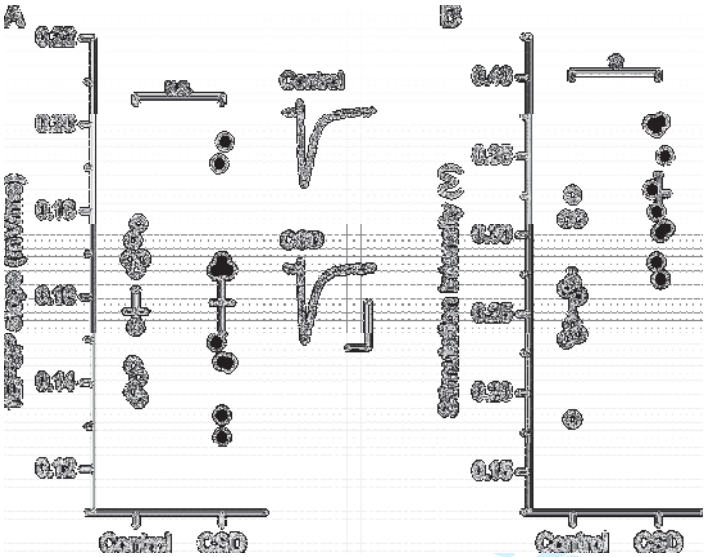


Figure 2.

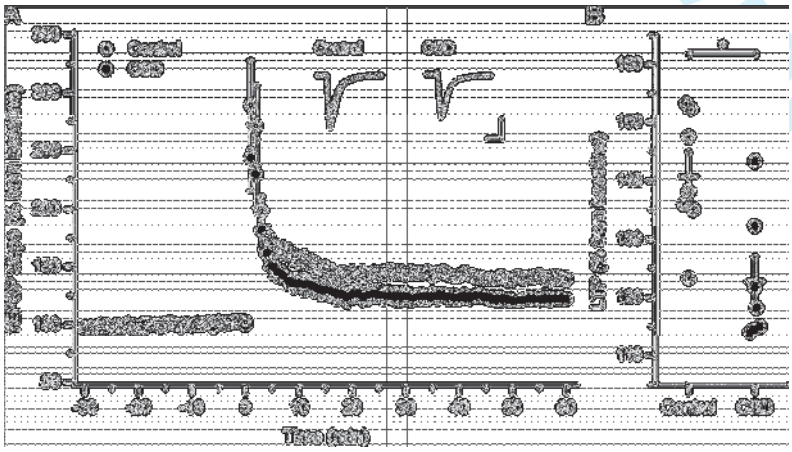


Figure 3.

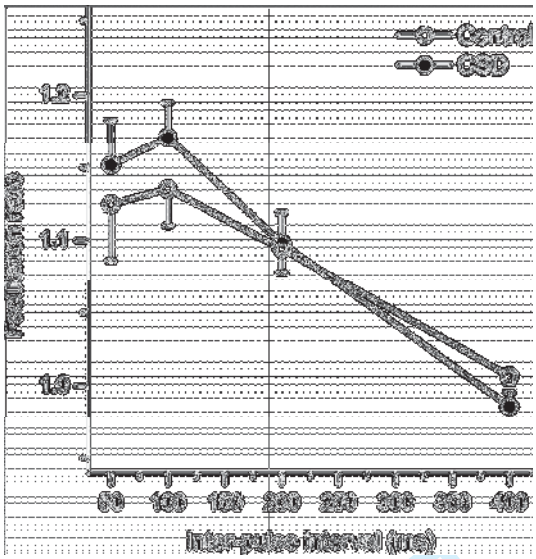


Figure 4.

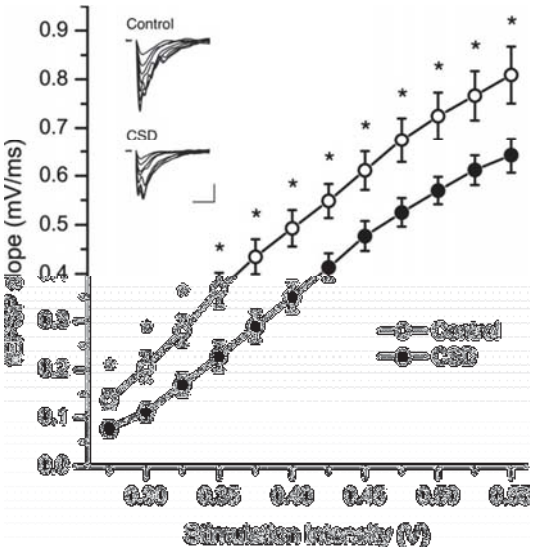
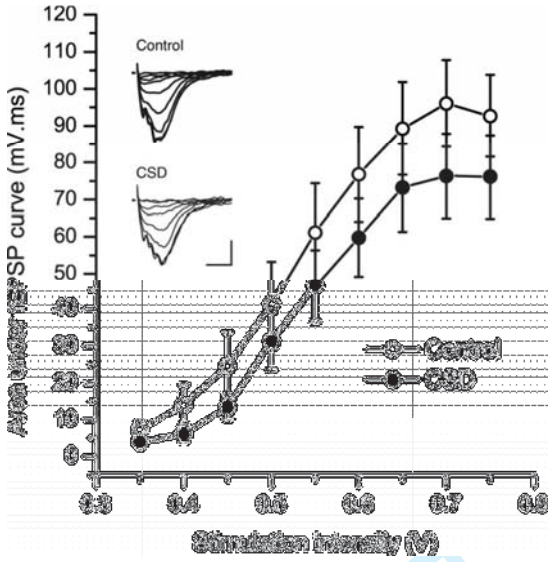
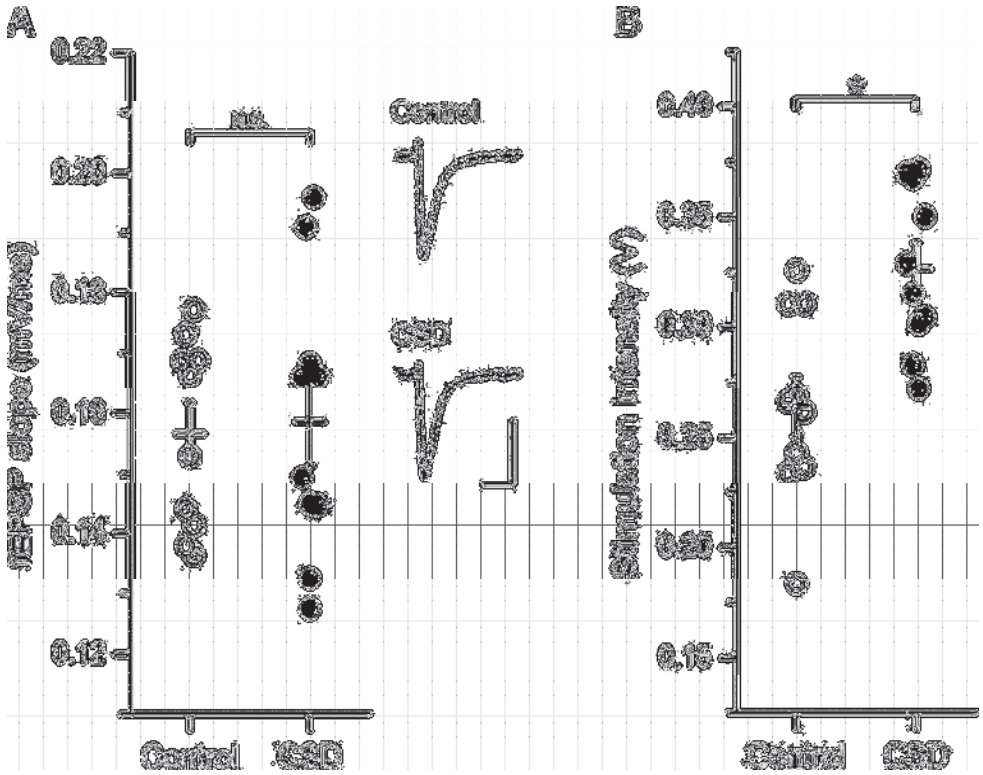
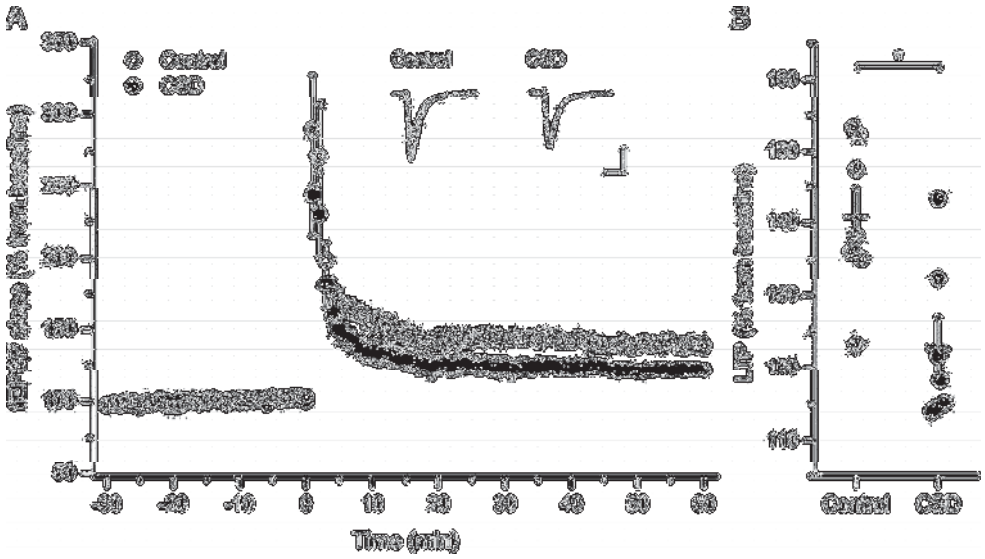


Figure 5.

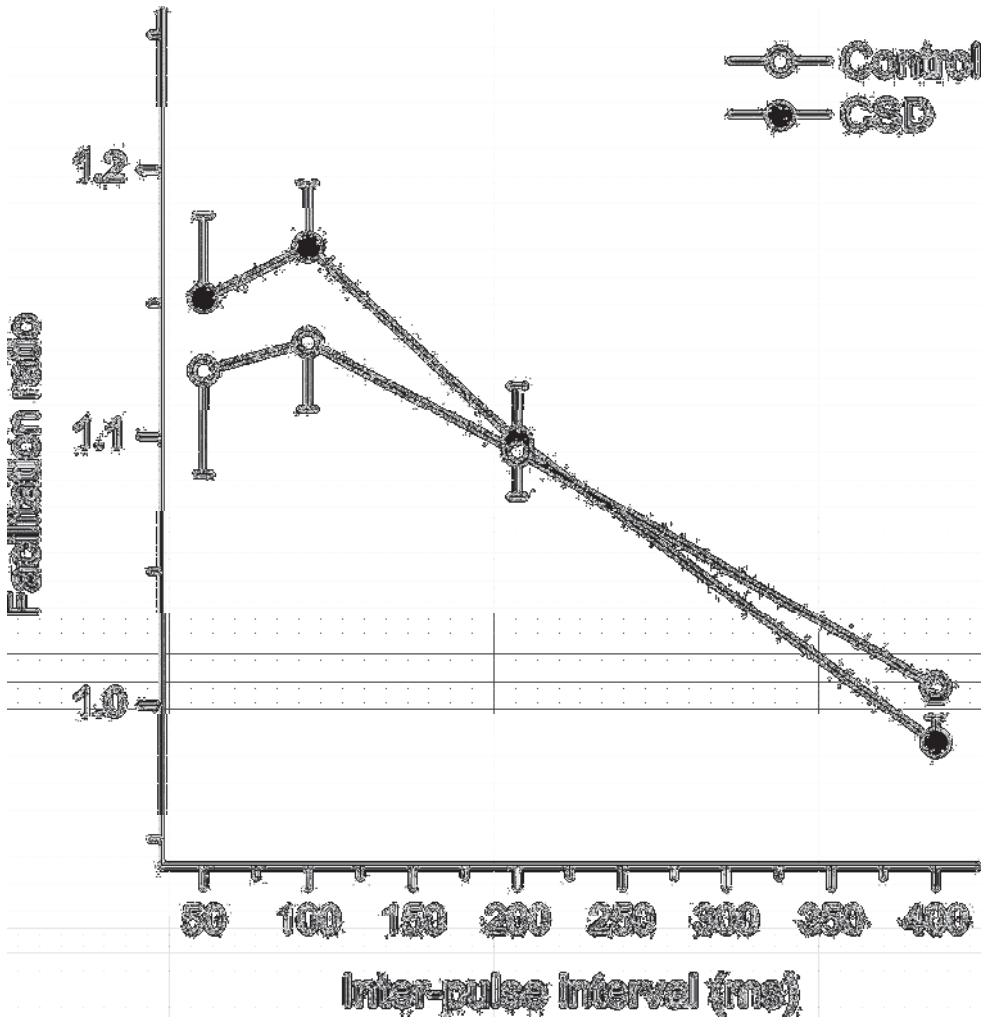




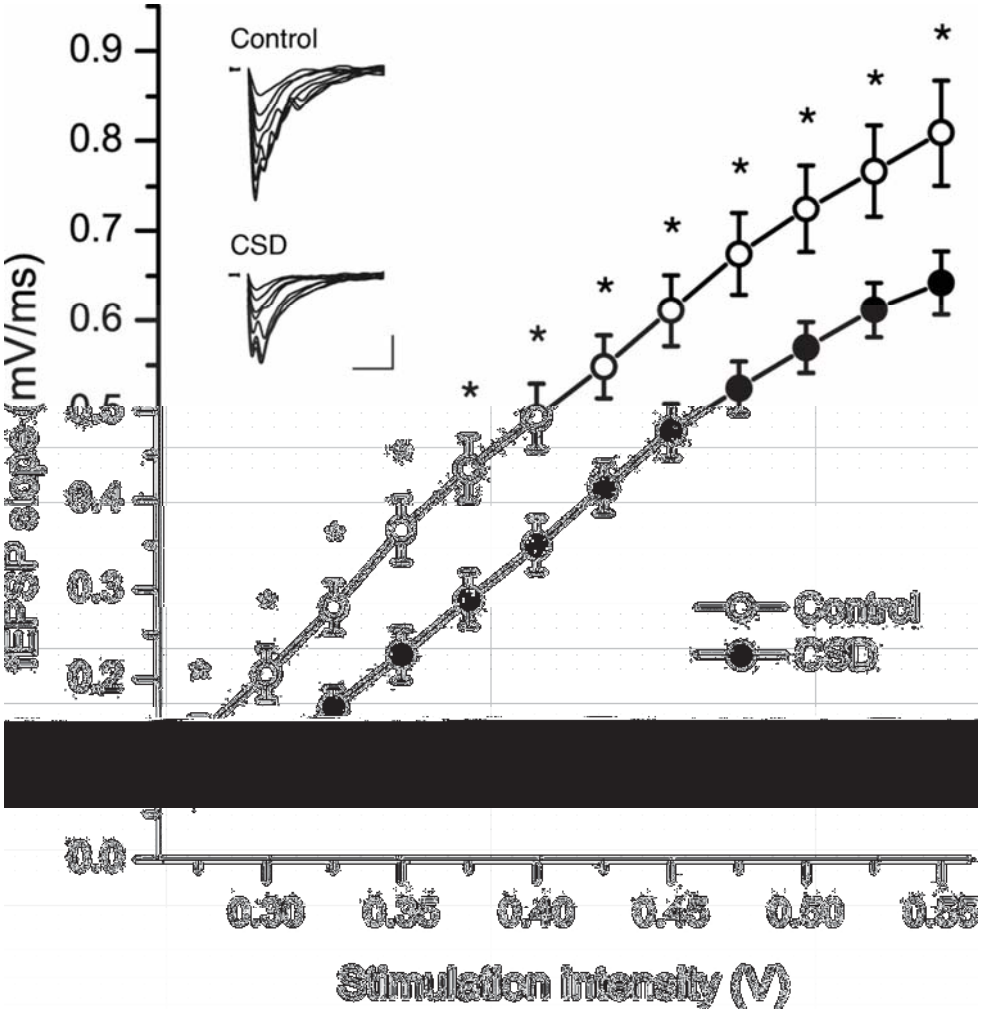
Neuronal excitability in the hippocampus was reduced by CSD induction. A, Evoked baseline fEPSPs were not significantly different between the CSD and control groups. Inset: representative traces of fEPSPs. Calibration: 20 ms, 0.2 mV. N.S.: not significant. B, Stimulation intensities required to evoke baseline fEPSPs were higher in the CSD group. * P< 0.05, Student's t-test. Bar and whisker plots indicate the mean ± SEM.
84x65mm (300 x 300 DPI)



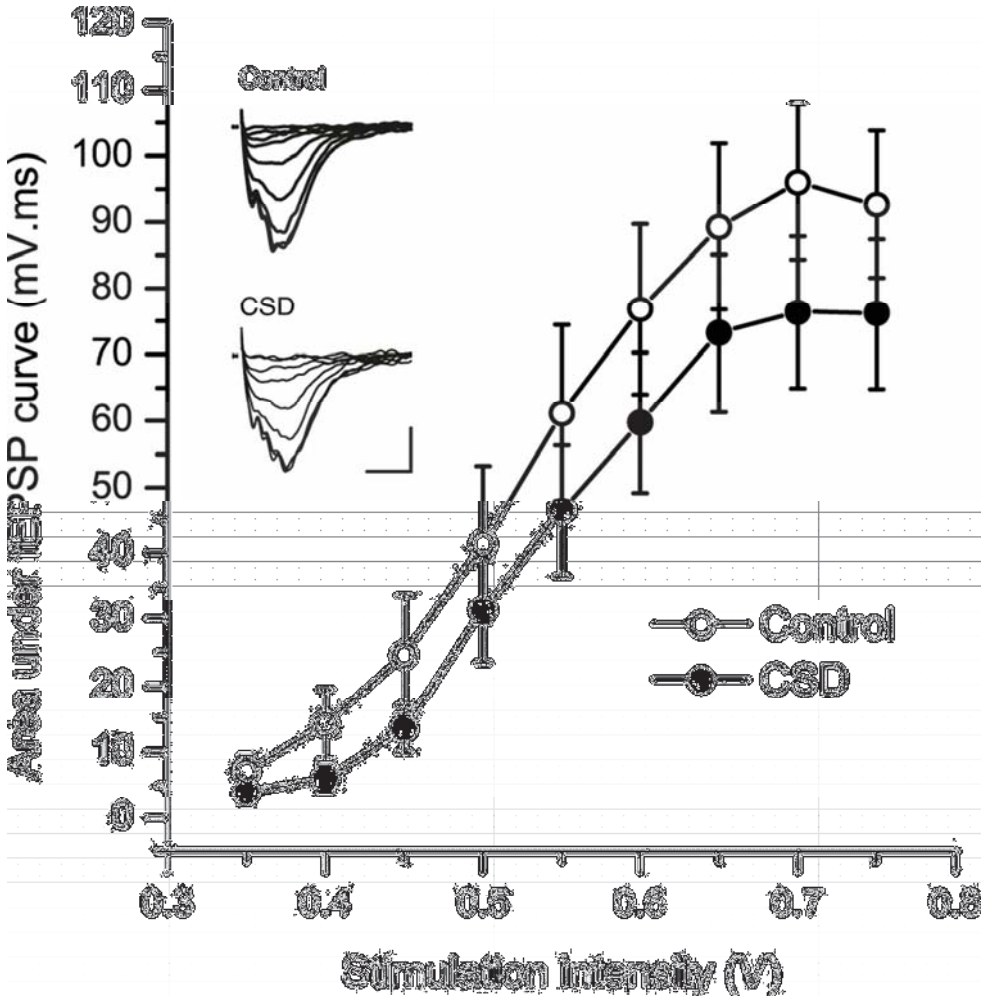
CSD induction affected LTP in hippocampal slices. A, Hippocampal LTP in the control and CSD groups. LTP was induced by tetanic stimulation (100 Hz for 1 s) applied at time 0. Inset: Representative traces recorded immediately prior to LTP induction and 60 min after LTP induction. Calibration: 20 ms, 0.2 mV. B, Data plot of the LTP magnitude. The magnitude of potentiation was calculated by dividing the average EPSP slope values from 50 to 60 min with those from 0 to 30 min. LTP was significantly reduced in hippocampal slices obtained from the CSD rats. * P< 0.05 Student's t-test. Bar and whisker plots indicate the mean ± SEM.
113x63mm (300 x 300 DPI)



Paired-pulse profiles of the hippocampal slices were not affected by CSD. Closed circles (CSD) and open circles (control) showed the facilitation ratios of the two groups. No significant differences in PPF profiles were observed.
76x79mm (300 x 300 DPI)



I/O relationship of AMPA receptor-mediated fEPSPs in the hippocampal slices obtained from CSD and control rats. CSD significantly suppressed the AMPA receptor component of fEPSP (P = 0.012, repeated-measures ANOVA). Closed circles (CSD) and open circles (control) showed evoked fEPSPs in hippocampal slices. Representative traces showed AMPA receptor responses to stimuli at 0.275, 0.300, 0.325, 0.350, 0.400, 0.450, 0.500, and 0.550 V. Calibration: 20 ms, 0.4 mV. * P < 0.05, Student's t-test.
75x78mm (300 x 300 DPI)



I/O relationship of NMDA receptor-mediated fEPSPs in hippocampal slices obtained from CSD and control rats. CSD did not significantly alter the NMDA receptor component of fEPSP (P = 0.256, repeated-measures ANOVA). Closed circles (CSD) and open circles (control) showed evoked fEPSPs in hippocampal slices. Representative traces showed NMDA receptor responses to given stimuli. Calibration: 50 ms, 0.4 mV.
78x79mm (300 x 300 DPI)

Advances in Biomedical Procedures Developed for Nerve Injuries Treatment

Saknan Bongsebandhu-phubhakdi[1], Naruphan Pukalanant[2], Tanunya Visessonchok[2], Anan Srikiatkhachorn[1], Boonrat Lohwongwatana[3]

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[1]Neuroscience Unit, Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

[2]Nanoengineering program, International School of Engineering, Chulalongkorn University

[3]Department of Metallurgical Engineering, Faculty of Engineering, Chulalongkorn University

Correspondence should be addressed to: Saknan Bongsebandhu-phubhakdi, Ph.D., Department of Physiology, Faculty of Medicine, Chulalongkorn University, 1873 Rama IV Rd, Patumwan 10330 Bangkok, Thailand. E-mail: saknan@live.jp

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One of a present-day medical profession's unsolved challenges is the regeneration of damaged nerves. Being a fragile structure, it is very difficult to restore signal transmission between brain and the limbs after nerve injury. Direct repairs, the traditional manners are attempted by directly suturing the nerve ends or fasciculi. Only later these procedures have been developed to connect the distal end to the epinurium of a donor nerve. Although direct repairs can realize the full recovery, they cannot make up for the loss caused by injury gap. Nerve grafting techniques can compensate the gap by the implantation of the patient's sacrificable site or a nerve from the donor. Recent advancements in nerve conduits, combined with application of polymers and micropatterning biomaterials, have resulted in a new biomedical procedure that can control almost all of nerve profiles. Such procedure will allow the improvement of biomaterials which can be conditioned in various ways for the application in nerve injury.

The nervous system, consisting of central and peripheral nervous systems, accounts for movements, thoughts, and sensations of the human body utilizing electrochemical signals. Each electrochemical impulse is sent along the axon of neuron. Minor to moderate injuries will most often impair peripheral nerves, which are responsible for connecting the spinal cords with the limbs.

Peripheral nerve injury is common in both military and civil accidents and approximately 100,000 patients undergo peripheral nerve surgery yearly in Europe and the United States. Nerves are fragile and can be damaged by pressure, cutting, or even stretching. Nerve fibers that carry information can break and seize to transmit due to pressure and stretching injuries. However, when a nerve is injured by a cut, both the insulation layer and the nerve itself are broken. All nerve injuries can result in the lost of signal transmission between the brain and the limbs causing morbidity and paralysis in specific areas.

Defined by symptoms, nerve injuries can be categorized into three main groups; autonomic nerve damage, motor nerve damage, and sensory nerve damage. Autonomic nerve damage may produce symptoms such as constipation, bladder dysfunction, sexual dysfunction, angina or heart attacks. Motor nerve damage may include symptoms such as paralysis, twitching, and muscle atrophy. Sensory nerve injuries could cause numbness, burning sensations, or even problems with positional awareness. Regular symptoms for peripheral nerve injury are sensory and motor nerves termination leading to paralysis of limb or neuropathic pain(Matsuyama et al. 2000).

In addition to frequent peripheral nerve injuries, matured nerve cells have almost no ability to regenerate themselves because there are barely any cellular

replication activities, therefore, our primary goal in nerve repair is to provide restoration techniques for functions of sensory, motor, and autonomic axons. To do so, it is essential to comprehend all disadvantages and to have total control over the factors that are affecting the upshots and outcomes of nerve repair. Our review will focus briefly on all available nerve repair techniques and will mainly concentrate our attention on artificial nerve conduit for peripheral nerve regeneration.

Direct repair

Direct repair of nerves involve direct coaptations or the direct connection of two nerve surfaces allowing recoveries of a torn or damaged nerve. However this method is confined to a small nerve gap of only a few centimeters where the ends of the nerve have minimal tension. Dvali and Mackinnon found that for the optimal nerve regeneration to occur, nerve ends must be accurately aligned, tension-free, and also with minimal number of sutures (Dvali and Mackinnon 2003). The two modes of direct repair are discussed below.

End-to-End Repair

End-to-End Repair is the applied to the patient's injury site by directly connecting the nerve ends by sutures. The sutures that unite proximal and distal nerve ends will act as an anchorage holding the ends together.

Mainly, there are two procedures concerning end-to-end repair; epineural repair and fascicular repair. Epineural repair is preferred for injuries that do not have nerve tissue loss and have fascicle alignment. Through both the external and internal epineural shealth, the suture is sewn and behaves as anchorage for both ends of the nerve. This is intended to facilitate fascicular alignment and prevent discontinuity of the nerve ends. All 16 children, who were afflicted with tears in the median nerve,

were able to fully recover functions of their hands through primary epineural repair (Hudson et al. 1997).

Fascicular repair on the other hand, is similar to epineural repair, but differs because in this procedure, sutures are sewn into each fasciculus; a tract of nerve fibers. This process is being less frequently used due to the high number of sutures required, which influences the increase of scar tissue. However, the few cases which require this technique of nerve repair are when the injury only partially damages the nerves. This repair allows better fascicular alignment which decreases the misdirection of regenerating axons. However, this will need a higher number of sutures that will in turn, increase the scarring of neuron and cause an interference of blood flow.

End-to-Side Repair

End-to-Side Repair has indeed been proposed and used since the beginning of the last century, but its results have been disappointing and it therefore was not recommended again until recent years (De Sa et al. 2004). Although having past reputations of failure, recent researches revealed that end-to-side repair could offer enhancement of axonal growth and nerve regeneration for sensory and motor nerves. This process of nerve repair is implemented when one end of the nerve is lost or destroyed. This can be done by sewing up the proximal end of the distal end to the epinerium of an adjacent and healthy donor nerve (Geuna et al. 2006). This procedure allows collateral sprouting from the axons of the donor nerve, which is responsible for the repopulation of the distal nerve end. In particular, end-to-side repair is constructive in brachial plexus (A network of nerves located in the neck and axilla) and nerve reanimation of paralyzed face. 80% of patients that have gone through

facial reanimation using the end-to-side method showed outstanding results (Manni et al. 2001).

Nerve grafting

One of the widely accepted methods for repairing nerve injuries is nerve grafting. Normally, nerve is taken from an outer part of the leg to replace the damaged part. Nerve grafting techniques include autograft and allograft. The two techniques employ different philosophies but produce the same repairing results:

Autografts

Nerve autografting where autologous nerve is taken from the patient's sacrificable site, normally the sciatic nerve and transplanted into the injured site. This technique results in optimal nerve generation as it gives the best long-term preservation of function. However, autografting is limited to injuries where tension-free suturing is possible. For cases where there is a loss of nerve tissue resulting in significant gaps, nerve autografting is inapplicable.

Disadvantages regarding this technique are also present with donor site morbidity being the biggest concern. This can lead to secondary sensory deformities, neuroma formation and pain. In addition, nerve autografting is often limited by graft availability, and likely differences in nerve diameter and structure which may lead to poor functional recovery.

Allografts

For decades nerve autografts has been the preferred method for peripheral nerve repair. However, its side effects and limitations have been pushing researchers to thrive for alternatives and shift their attention to nerve allografting.

Nerve allografting involves the implantation of a nerve from the donor into the patient's injured site, which would prevent further damage to the patient's nervous system. In addition, practically limitless length of nerve tissue is available for transplantation. However, it has some restrictions of its own. Nerve allografts require immunosuppressive agents for them to be effective. This limitation, nonetheless is only required until the patient's own Schwann cells and axons have repopulated and recovered the allograft to a self-sustaining level (Myckatyn et al. 2004).

Within a short injury defect of about 3 cm or less, it has been seen in peripheral nerve allografts of primates immunsuppressed with cyclosporine that there was a great recovery across the graft (Bain 1998). However, if the injury defect of gap between the nerves is large, as for sheep peripheral nerve of 8 cm graft, the regeneration of nerve was unsatisfactory in comparison with the autograft trials (Strasberg et al. 1996).

Nerve allograft operates by providing regulation and sufficient feasible Schwann cells allowing host axon regeneration. This intern grants patients with its advantages; lack of donor site morbidity and unlimited extents of nerve tissue required for transplant. In addition, the patient's injured nerve can be directly replaced with the identical nerve type from the donor allowing better recovery initiation compared with a different nerve grafting procedure (Chu et al. 2008; Moradzadeh et al. 2008).

With its few drawbacks, nerve allografts prove to be an adequate alternative for patients with small nerve injuries and also a promising procedure that could be enhanced.

Nerve conduits

The main influence that inspired the advancement of nerve guiding conduits is the disadvantage of nerve injury gap repair with autografts such as donor site morbidity and extended surgical intervals. Successful models of nerve conduits are able to offer appropriate surroundings that will promote axon and Schwann cell growth in addition with neurotrophic stimulation. However, this entubulation development is only limited to only the repairing of small nerve injuries that is no bigger than a few centimeters wide.

Biological Conduits

Biological conduits comprise of veins and arteries, which are channels responsible for the transportation of fluids in our body. Brunelli *et al.* were able to create and merge conduit where muscles provided the internal linings of the vein graft (Brunelli et al. 1993). This increased the stability of the vein graft and consequently averted graft collapse. Muscles incorporated into the vein graft were also able to provide guidance and adhesion for nerve fibers. This was possible due to the sufficient amount of basal lamina, which resembles the Schwann cell adhesion role. Other methods such as built-in collagen-coated metal spiral supports have also been used to resolve the limitation of stability problems (Smahel and Jentsch 1986). Extensive studies showed vein grafting was particularly promising in sensory nerve repairs (Risitano et al. 2002).

Acellular nerve grafting is also a means of peripheral nerve repair. It has been proposed as peripheral nerve substitutes when used as non-autologous biological materials (acellular nerve allografts) (Risitano et al. 2002). The procedure of acellular nerve grafting requires the process of decellularization that affects the extracellular matrix scaffold. Overall, decellularization determines the host response to the material (Gilbert et al. 2006). In the study of sensory nerve defects of hands, it was established

that decellularized nerve allografts were able to recover moderate sensation in nerve gaps of 0.5 – 3 cm without any rejection or infection (Karabekmez et al. 2009). Since customarily clinical nerve conduits are able to restore function to nerve injuries of no more than 2 centimeters, acellular nerve grafting could influence and progress to become an effective way for patients with more severe nerve impairment.

Artificial Conduits

Artificial nerve conduits propose alternative treatments to nerve injuries especially for large defects and the approach is currently the focus of interest. From Ichihara *et al.*, three main mechanical properties are required for artificial nerve conduits (Ichihara et al. 2008).

Firstly, the conduit must be able to support physical guidance and regeneration of axons. This includes factors such as mechanical strength, surface topology and surface morphology. The conduit must be able to withstand the pressure from adjacent tissues, prevent invasion of fibrous tissues, and at the same time prevent axons from escaping into the surroundings.

Secondly, the conduit must be semi-permeable to facilitate axonal survival and growth. The permeability of conduits allow the exchange of nutrients and waste products across the conduit wall; diffusion of nerve growth factors produced in the external environment; and accumulation of nerve growth factors released by nerve terminals.

Thirdly, the conduit should be able to degrade with a rate relative to the axonal growth rate, whereas non-degradable conduits require surgical removal due to their long term implications. Other desired characteristics of artificial nerve conduits for clinical applications include good biocompatibility, low antigenicity, minimal inflammatory stimulus, ability to sustain nerve regeneration throughout the length of

the conduit, easy manufacturing, economical, and easy for technical handling (Meek and Coert 2002).

Various materials have been used for artificial conduits, synthetic and natural; biodegradable and non-biodegradable materials.

The first clinical application of artificial conduits was with silicone conduits as reported by Lundborg *et al*., (Lundborg et al. 1991). Silicone conduits are only clinically used for repairing median nerve, ulnar nerve and radial nerve defects, but they are utilized widely for study with different guide fillers. Silicone conduit is the most frequently used non-biodegradable conduit due to its good biocompatibility, inertness and elasticity. For nerve injuries with gap of less than 3 cm, the outcomes of the repairs are satisfactory with relatively good restoration of motor and sensory function. Other non-biodegradable materials such as Polytetrafluoroethylene (PTFE) have also been employed with good results. However, most cases have reported poor outcomes (Li et al. 2003). Due to the non-degradability of both silicone and PTFE, the inflammatory response of the patient body often leads to the development of fibrosis around the conduit and nerve compression. As a result, a second surgery is required to remove the conduit after regeneration across the gap is achieved.

The disadvantages of non-biodegradable conduits led to the development of biodegradable nerve conduits. Biodegradable nerve conduits offer numerous advantages such as the possibility of attaching stem cells or bioactive molecules and deliver them during the degradation process and eliminate the need for conduit removal. A lot of biodegradable materials are synthetic polymers due to their flexibility. Polymers can also be modified to achieve optimal biocompatibility, degradation behavior, porosity, and mechanical strength. Aliphatic polyesters and copolyesters are the most popular choices among the biodegradable materials as nerve

conduits. A range of biodegradable materials have been used in animal studies whereas clinical applications in humans have been reported only for polyglycolic acid polymer (PGA) and polylactide-caprolactone polymer (PLCL) (Terasaka et al. 2006).

A study done by Luis *et al*., compared the effectiveness of two different biodegradable conduits with end-to-end nerve suture and autologous grafting for the reconstruction across sciatic nerve gap. The first nerve conduit tested was made of PLGA with the composition of poly(L-lactide):poly(glycolide) (90:10), the second was a commercially available nerve conduit called Neurolac® made of (DL-lactide-ε-caprolactone) copolyester (Luis et al. 2007). Compared to PLGA, Neurolac® is stiffer due to the reinforcement of the ester bonds but it degrades at a slower rate. PLGA is more flexible which is more suitable for withstanding against compressive forces during movements. PLGA with this particular composition degraded completely after 12 weeks of implantation into lactic and glycolic acids while Neurolac® took16 months and the degradation products were less acidic and disturbing to the surrounding tissue. Results from the experiment showed that the motor recovery occur fastest with the end-to-end suture followed by the autologous graft and the biodegradable conduits. However, the recovery of animals implanted with biodegradable conduits caught up at the end of 20 weeks and the extent of motor function recovery is comparable to the autologous graft group. Similar results were also seen for the nociception recovery. Luis *et al*., concluded that both PLGA and Neurolac® were equally good as substrates for nerve conduits and that the difference in the patterns of biodegradation did not influence the extent of nerve generation in this particular experiment (Luis et al. 2007).

Natural polymers offer several advantages over synthetic materials. Natural polymers are already present in nature in different forms therefore they are very likely

to have good biocompatibility, able to support the migration of cells and prevent toxic side effects. Nevertheless, most natural polymers possess poor mechanical strength, relatively fast *in vivo* degradation rate, high swelling behavior and they may contain microbes. Consequently, natural polymers are often blended with synthetic materials to improve their properties or used as fillers for nerve conduits instead. Several natural polymers have been investigated for use with nerve conduits including chitosan, collagen, polysialic acid, silk fiber, aragonite, alginate and hyaluronic acid hydrogel.

One of the most widely used natural materials used for nerve generation and repair is collagen. Collagens are the most abundant proteins in mammals and they are the major components of the extracellular matrix. The smooth microgeometry and good permeability of collagens allow diffusion nutrients and oxygen through collagen matrices. Collagens are less antigenic and are easily resorbed into the body (Cemil et al. 2009). Studies have shown that collagen filaments are able to guide regenerating axons and maintain the function of peripheral nerve function during adulthood (Alluin et al. 2009). Cemil *et al*. investigated the effectiveness of collagen biomatrix nerve conduit and omentum graft on peripheral nerve generation (Cemil et al. 2009). Results suggested that a collagen biomatrix conduit can safely and effectively support nerve generation. The omentum graft, however, did not show satisfactory results as it could not effect regeneration positively.

The human extracellular matrix (ECM) is composed of fibers and fibrils with sizes ranging from nanometers to micrometers. Thus by integrating nanostructures into nerve conduits, one can better mimic the ECM structure, design structure on a

subcellular level and possibly provide a more compatible environment for cells and the growing of axons *in vivo*.

Lin *et al.* explored the application of chitosan-gold nanocomposites for nerve conduits (Lin et al. 2008). Chitosan is a natural polysaccharide with excellent bacteriostatic (inhibits bacterial growth) and hemostatic (enhances stagnation of the blood) properties. In addition, chitosan can be degraded by enzymes to absorbable oligosaccharides. However, like most natural polymers, chitosan exhibits low mechanical strength. Gold, as a material with good biocompatibility and reasonable mechanical properties, was blended with chitosan to produce chi-Au nanocomposites. Results showed that mechanical strength increased with the amount of gold in chitosan. Cell proliferation and gene expression were also enhanced by the addition of gold nanoparticles. It was suggested that these nanoparticles alter the microstructure of chitosan resulting in the modulation of cellular responses.

Lin *et al.* also inspected the influence of micropattern on cell alignment (Lin et al. 2008). Micropatterning allows the control of cell adhesion, cell migration, cell differentiation and cell interactions (Anderson and Hinds 2011). In neurology, micropatterning is widely used as a method to investigate the interactions of ECM proteins with neurons and glia (Corey and Feldman 2003). Several techniques are available for cell micropatternining such as photolithography and soft lithography which includes microcontact printing, microfluidic patterning and stencil patterning. In the study done by Lin *et al.* (Lin et al. 2008), it was observed that most of the neural stem cells (NSC) developed multipolar branches on nonpatterned substrates, whilst developed bipolar branches on microgrooved substrates

In order to enhance nerve regeneration and better recovery for nerve injuries, especially for extended nerve gap, conditioning of the conduits have been largely

studied. Nerve conduits can be conditioned in various ways, for instance by filling of growth factors, implantation of Schwann cells, implantation of neural stem cells, introduction of vascularity, construction of inner –structure as well as combination of grafting materials. These manipulations of nerve conduits have shown positive effects towards nerve regeneration compared to the conduits alone (Meek and Coert 2002).

To improve peripheral nerve regeneration in acellular nerve grafts, Yu *et al.* investigated the release of NGF which was encapsulated in polymeric microspheres (Yu et al. 2009). Stabilized by fibrin glue, the microspheres were then loaded around nerve graft. From the histological assessment of nerve fiber counts, fiber diameter and myelin sheath thickness, autografting yielded the most favorable results followed by NGF-treated acellular grafting, fibrin-glue treatment, and lastly acellular grafting alone. Results suggested that NGF could induce neural differentiation and preservation of autonomic and sensory neurons. The Schwann cells present in autologous graft provide a favorable microenvironment through which regenerating axons grow and they also provide various important trophic factors. In this study, NGF-treated acellular grafting showed satisfactory results but they were still inferior compared to the gold standard autologous grafting.

Conclusions

This review is able to provide us with the general knowledge of almost all the common clinical procedures and ongoing researches specifically on artificial conduits, regarding peripheral nerve repair. As most nerve repairing methods are limited to only short gap injuries of only a few centimeters, artificial nerve conduits seem most potential in offering patients with the treatments they need along with the least side

effects possible. Overall, we were also able to see the problems and constraints arising in each method, which allowed us to see the setback that is preventing us from moving forward in this discipline.

After thorough research, it was apparent to us that there is still so much room left for advancements in this field of research, especially with incorporations of Bio-nanotechnology. In addition to getting insightful information, we were also able to envision the possibilities of breakthroughs concerning peripheral nerve repair.

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A ROLE OF CALCITONIN GENE-RELATED PEPTIDE ON TRANSIENT RECEPTOR POTENTIAL VANILLOID-1 EXPRESSION IN RAT TRIGEMINAL GANGLION AND PAIN SENSITIZATION

Chatchaisak, D.[1], Srikiatkhachorn, A. [2], le Grand SM.[3], Govitrapong, P.[1,4], Chetsawang, B.[1]

[1] Research Center for Neuroscience, Institute of Molecular Biosciences, Mahidol Universit, Salaya, Nakhonpathom 73170, Thailand

[2]Department of Physiology and [3]Department of Pathology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

[4]Research Center for Neuroscience and Department for Pharmacology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

Abstract

Calcitonin gene-related peptide (CGRP) plays an important role in the development of pain and migraine pathogenesis. Transient receptor potential vanilloid-1 (TRPV1) is a calcium-permeable ion channel, which is activated by a number of pain inducing stimuli. In this study, we aimed to investigate the functional role of CGRP on pain sensitization via the mediation of TRPV1 expression in rat trigeminal ganglion (TG). Following CGRP intravenous injection, an induction in the amount of TRPV1, CGRP, phosphorylation of PKC (p-PKC) and cyclic AMP responsive element-binding protein **(**CREB) were observed in rat TG. In addition, an increase in c-fos levels was determined in trigeminal nucleus caudalis (TNC) of CGRP-treated rats. The results of the present study reveal the role of CGRP in trigeminal nociceptive system. It seems that pain sensitization in migraine headache is driven by CGRP-activated and -sensitized peripheral neurons in TG, which then activate and sensitize central neurons in TNC.

Keywords: Migraine, CGRP, TRPV1, phospho-PKC, trigeminal ganglion, pain sensitization

It has been hypothesized that calcitonin gene-related peptide (CGRP) implicates in the pathogenesis of migraine attacks (Fusayasu E et al, 2007). CGRP is a neuropeptide found in the trigeminovascular system and involved in migraine pain (Johansson HW et al., 2010). A marked increase in plasma levels of CGRP is observed during migraine headache (Edvinsson L et al, 2005). In addition, plasma CGRP levels from migraineurs with and without aura have been found to be considerably higher than in healthy controls (Fusayasu E et al, 2007, Goadsby et al., 1990). It has also been reported that paediatric migraineurs have high plasma CGRP levels than non-migraine patients, and the CGRP levels is higher during migraine attack than non-attack (Fan PC et al. 2009). Migraine is believed to be a trigeminovascular disease (Goadsby et al., 2009). Migraine pain seems to be the consequence of multiple pathophysiological changes which is related to the activation of trigeminal nociceptive neurons and secondary nociceptive neurons in trigeminal nucleus caudalis (TNC). They have been demonstrated that trigeminal ganglion (TG) and TNC are likely to be sites of action of CGRP in migraine (Storer RJ et al. 2004, Jenkins DW et al. 2004). CGRP is found in 35-50% of all trigeminal neurons and over 90% of those with diameters below 15μm (Lazarov NE, 2002). CGRP receptor is a G protein-coupled receptors, which are distribution in peripheral and central nervous system (Edvinsson L and Goadsby PJ, 1995), trigeminovascular system and TNC (Lennerz JK et al, 2008). CGRP-activated CGRP receptors are associated with several effects including neurogenic inflammation (plasma extravasation and mast cell degranulation) and vasodilation of meningeal vessels (Xu X et al., 2009; Zhang Z et al., 2007; Nicoletti P et al, 2007).

Transient receptor potential vanilloid (TRPV1), originally named VR1, is a member of transient receptor potential (TRP) ion channels that responds to many direct stimuli such as thermal and chemical. It can be phosphorylated by kinase enzymes including $Ca^{2+/}$CaM-dependent kinase II (CaMKII), protein kinase A (PKA) (Vetter I et al., 2008) and protein kinase C (PKC) (Jeske NA et

al., 2009; Xu X et al., 2009). PKC induces phosphorylation in TRPV1 receptor, which leads to enhance the translocation of receptor from cytoplasm to localization on cell membrane (Xu X et al., 2009). TRPV1 is considered as a marker of nociceptive primary afferent neurons (Tominaga M and Tominaga T, 2005). TRPV1-positive neurons are mostly small and medium sizes of trigeminal neurons (Bae YC et al., 2004). TRPV1-positive neurons in TG send unmyelinated fibers to terminate at laminae I and II of trigeminal sensory nuclei caudalis (Bae YC et al, 2004), which play a pivotal role in pain sensitization.

Recent evidences have demonstrated that CGRP and TRPV1 play an important role in migraine pathogenesis and targeting for migraine therapy. In trigeminovascular system CGRP and TRPV1 are co-localization in trigeminal afferent neurons (Bae YC et al., 2004; Price TJ and Flores CM, 2007), TNC and cranial vascular. Activated TRPV1 results increase releasing of CGRP, glutamate and substance P (SP) in afferent neuron. Increasing evidences have been emphasized on the increase in the activity of peripheral nociceptors can induce change in the expression of various proteins in the trigeminal ganglionic neurons. Taken together, high plasticity of this system has been proposed to explain the change in clinical features of headache. Therefore, in the present study we aimed to investigate the modulatory roles of CGRP on activation in intracellular signaling cascades and its consequent role in induced-increase in TRPV1 levels in TG. We also investigated the functional role of CGRP on activation and sensitization of trigeminovascular neurons in trigeminal nucleus caudalis by observing an induction in c-fos levels.

Experimental procedures

Experimental animals

The present study was conducted in accordance with the NIH Guidelines on the Care and Use of Animals and the protocol was approved by the Institute of Molecular Biosciences Animal

Care and Use Committee (MB-ACUC), Mahidol University, Thailand (COA. NO. MB-ACUC 2011/ 002). Every effort was taken to minimize the number of animals used in the experiment.

Male Wistar rats weighting 250-300 g were obtained from National Laboratory Animal Centre, Mahidol University. All procedures were performed under anesthesia with intraperitoneal injection (i.p.) of 60 mg/kg sodium pentobarbiturate (Nembutal®). Control rats were intravenous injected (i.v.) with normal saline solution (NSS) into their femoral vein. CGRP-treated rats were intravenous injected (i.v.) with 600ng/kg CGRP (Sigma-Aldrich®)-dissolved in NSS into their femoral vein.

Western Immunoblotting

Animals were decapitated under anesthesia, and rapidly removed trigeminal ganglion and trigeminal nucleus caudalis. Tissues were homogenized in ice cold lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 1mM PMSF, 1mM EDTA, TritonX-100, Na deoxycholate, 0.1%SDS, PMSF, Phosphatase inhibitor (100mM NaF, 100mM Na3VO4, and 400 mM NaTartate dehydrate) and protease inhibitor. Protein assay was performed according to Lowry method. Samples were denatured and separated by SDS-polyacrylamide gel and transferred onto a PVDF membrane (Amersham Biosciences). The membranes were blocked in 3% non-fat milk in 0.1% Tween-Tris-buffered saline (TBST) for 1h at room temperature and then the membrane was washed with TBST. It was then incubated overnight at 4°C with primary antibodies against VR1 (P-19) (1:1,000, Santa cruz biotechnology), phospho-PKC (pan) (βII Ser660) (1:1,000, Cell signalling) and c-fos (1:1,000, Calbiochem). After incubation, the membrane was washed with TBST three times (5 min. each time), and then incubated for 1.5 h with HRP-conjugated secondary antibody and washed with TBST three times (5 min. each time). Chemiluminescence ECL Plus-Western Blotting detection

reagents were used to develop the immunoblots. The immunoblots were quantified by measuring the density of each protein band using densitometry analysis with Scion image program (National Institutes of Health, Bethesda, MD, USA). Re-probing of the same blot with β-actin (1:10,000, Chemicon) was performed to normalize for protein loading. The antibody against specific target protein was discarded by stripping buffer (2%SDS, 100mM 2-Mercaptoethanol, and 62.6mM Tris base, pH 6.7). The stripping buffer was heated to 50°C in water bath and then incubated with nitrocellulose membrane for 5 min with shaking. The membrane was washed with TBST three times (5 min each time) and then returned to the normal process as indicated above.

Immunohistochemistry

Animals were deeply anesthetized and perfused transcardially with ice cold phosphate buffered saline (PBS) 250ml, and fixed with 4%paraformaldehyde in 0.1M PBS pH7.4. The TG and TNC were removed and immersed in 4% paraformadehyde in 0.1M phosphate buffer. In the free-floating method, tissues were placed in 30% sucrose overnight and transverse 30-μm thick sections were cut on a cryomicrotome at -20°C. In paraffin section method, tissues were paraffin processed and transverse 3-μm thick sections were cut and deparaffinized before immunostaining.

Immunoperoxidase staining

TG paraffin sections were stained for VR-1 and CGRP immunoperoxidase staining. TNC sections were stained for c-fos immunoperoxidase staining using free-floating method. Trigeminal sections were warmed with antigen retrieval solution in microwave (citrate buffer, Dako). Endogenous peroxidase activity was blocked with Dako Peroxidase Blocking Reagent (Dako) for 5min, and non-specific staining was blocked with Antibody diluent (Dako) for 10min. Sections were incubated with primary antibodies against VR-1(1:200, Santa cruz) or CGRP (1:6,000, Sigma)

overnight at 4°C. TNC sections were blocked endogenous peroxidase activity with 1% H_2O_2 in 50% methyl alcohol for 30 min, and non-specific staining was blocked with Antibody diluent (Dako). Sections were incubated with primary antibody against c-fos (1:2,000, Santa cruz) overnight at 4°C. They were then incubated with horseradish peroxidase conjugated-anti IgG. Antigen visualization was carried out using EnVision^{TM} Detection System (Dako) and sites of peroxidase activity were visualized using 3, 3´-diaminobenzidene tetrahydrochloride (DAB: 0.005%, 30% H_2O_2 in Tris-HCl 0.05M, pH 7.2). Immunoperoxidase staining was visualized under light microscope. The staining image was saved in TIFF format (10X) and TRPV1 or CGRP positive neurons were manually counted in TG sections, and c-fos positive neurons were manually counted in TNC sections. TG neurons were classified into small, medium and large sizes based on their cellular cross- sectional area less than 400 μm^2, 400-800 μm^2 and more than 800 μm^2, respectively.

Immunofluorescent staining

TG paraffin sections were warmed in microwave and non-specific staining blocked in the same process of immunoperxidase staining. Sections were overnight incubated with a combination of antibody against VR-1(1:200) and one of the antibody against CGRP (1:6,000, Sigma) or phospho-PKC (p-PKC) (1:200, Cell signaling), respectively in Antibody diluents (Dako) at 4°C. After washed with phosphate-buffered saline, section were incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG (Dako) for 60 min at 37°C. Sections were mounted with Fluorescence Mounting Medium containing dye4', 6-diamidino-2-phenylindale (DAPI) and covered with coverslip. Stained slides were visualized and photographed with a camera under a confocal microscope (ZEISS).

Statistical analysis

Data were expressed as mean ± S.E.M. An unpaired t test was used to analyze differences between groups. Statistical analysis was performed using the scientific statistic software GraphPad Prism®. A p value of less than 0.05 was considered statistically significant.

Results

CGRP-induced increase in TRPV1 and CGRP levels in rat trigeminal ganglion.

Western immunoblotting were performed to determine the TRPV1 protein levels in rat TG. Intravenous injection of 600 ng/kg CGRP for 45 min tend to increase TRPV1 levels, whereas 60 min of CGRP injection significantly increased both 75 and 130 kDa of TRPV1 protein levels in TG of rat (Figure 1.). Up-regulation of TRPV1 in TG of CGRP-treated rats was confirmed with TRPV1 immunoperoxidase staining. The results showed that intravenous injection of 600ng/kg CGRP for 60 min significantly increased in small and medium but not large sizes of TRPV1 positive staining TG neurons (Figure 2.). The effects of CGRP treatment on the CGRP levels of TG neurons was evaluated using immunoperoxidase staining. The results showed that intravenous injection of 600ng/kg CGRP for 60 min significantly increased in small and medium but not large sizes of CGRP positive staining TG neurons (Figure 3.). The immunofluorescent staining was performed to visualize co-localization of TRPV1 and CGRP in TG neurons. The cell nucleus was stained with a nuclear fluorescence staining dye 4', 6-diamidino-2-phenylindole, which showed in blue color. The TRPV1 and CGRP immunofluorescence staining TG neurons showed in green and red colors, respectively. Confocal microscopy results from merged images indicate higher levels of co-localization of TRPV1 and CGRP in TG neurons of CGRP-treated rats (Figure 4).

CGRP-induced increase in p-PKC and CREB levels in rat trigeminal ganglion.

To determine whether up-regulation TRPV1 is dependent upon PKC and CREB, the levels of PKC phosphorylation (p-PKC) and CREB was determined in TG. Western immunoblotting analysis showed that intravenous injection of 600ng/kg CGRP for 60 min significantly increased p-PKC (Figure 5B.) and CREB (Figure 6B.) levels in TG of rats. Similarly, an increase in p-PKC (Figure 5A.) and CREB (Figure 6A.) in TRPV1 immunofluorscence staining cells was observed in TG neurons of CGRP-treated rats.

The effects of CGRP on the expression of c-fos in rat trigeminal nucleus caudalis (TNC).

In order to determine whether TG activation with CGRP can sensitize TNC neurons, the levels of c-fos expression was determined in TNC using western immunoblotting and immunohistochemistry. Intravenous injection of 600ng/kg CGRP for 60 min significantly increased c-fos levels in TNC of rats (Figure 7B). The immunohistochemical data are consistent with data obtained from immunoblotting analysis. The c-fos immunoreactive cells in the superficial laminae I and II of TNC in CGRP-treated rats were increase when compared with saline-injected control rats (Figure 7A.).

Discussion

The pathogenesis of headache in migraine is not clearly understood. However, more recent concept emphasizes the role of trigeminal nociceptive system and activation and sensitization of central trigeminovascular neurons in TNC are associated with migraine pain. Moreover, numerous studies demonstrated the pivotal role of endogenous pain mediators such as glutamate, substance P and CGRP in the sensitization of trigeminal nociceptiors. It has been reported that CGRP level is associated with the degree of pain (Edvinsson L, 2006) and CGRP level is higher in migraineur

plasma than normal. Recent evidences have indicated that TG and TNC are likely to be site of action of CGRP in migraine (Storer RJ et al., 2004 and Jenkins DW et al., 2004). CGRP is able to sensitize P2X3 receptors in nociceptive sensory neurons to increase impulse flow to brain stem trigeminal nuclei. The study in mouse trigeminal ganglion neuron cultures demonstrated that CGRP can activate Calmodulin-dependent kinase II and accompany by increase in CREB phosphorylation and P2X3 receptor expression (Simonetti M et al, 2008). In this study, we proposed the effect of CGRP on expression and sensitization of TRPV1, a non-selective cation channel and a key molecule in peripheral nociception (Tominaga M and Tominaga T, 2005) in TG, which consequently resulting in activation and sensitization of central trigeminovascular neurons in TNC. The results of the present study demonstrated that intravenous injection CGRP significantly increased TRPV1, CGRP, p-PKC and CREB levels in TG and c-fos levels in TNC of rats. It seems that CGRP-mediated signals are transduced by activation of intracellular signaling cascades, such as PKC and CREB, which then induced TRPV1 and CGRP expression in TG neurons. Results from this study support an autocrine and paracrine function for CGRP within TG (Thalakoti S el al., 2007). On such sensory neurons, up-regulation of TRPV1 and CGRP may sustain and facilitate to transmit nociceptive stimuli to brainstem trigeminal nuclei in TNC. Our immunohistochemically quantitative analysis of TRPV1 positive neurons indicated that CGRP evoked a significant increase in the number of TRPV1in CGRP-positive TG neurons, especially in the small and medium size cells. It has been reported that only small and medium TG neurons are immunopositive staining for TRPV1, which frequently co-localize with SP and CGRP in primary afferent neurons (Bae YC, 2004).

Trigeminal nucleus caudalis (TNC) is the key relay neurons for nociceptive input in head and neck. Induction of immediate early *c-fos* gene within TNC neurons is a marker of neuronal

activity within central nociceptive pathway (Mitsikistas DD and Del Rio MS, 2001). The results of the present study exhibited CGRP injection-activated c-fos expression in rat TNC especially in laminae I and II of TNC, which is an important area for the transmission of pain to higher brain structures. It was established in the present study that intravenous injection of CGRP was not only increased TRPV1 levels but also CGRP levels in TG neurons of rats. It is well accepted that CGRP is the most abundant pain mediator in TG neurons (Durham PL, 2004) and acute migraine headaches can often be relieved by CGRP receptor antagonist (Olesen J et al. 2004). Recent evidence has demonstrated that capsaicin can activate TRPV1, which lead to evoke release of CGRP from TG neurons and then culminate in excitation of trigeminal sensory neurons in brainstem slices of rats (Meng J et al. 2009). In addition, it has been reported that inhibition of CGRP receptor by CGRP receptor antagonist, olcegepant (Sixt ML et al. 2009) and BIBN4096BS (Koulchitsky S et al. 2009) can reduce spinal trigeminal neurons activation in rats. Moreover, cortical spreading depression (CSD) is an electrical phenomenon underlying the aura phase of migrane. It has been demonstrated that trigeminovascular nociception-evoked by CSD significantly increase c-fos IR cells in the superficial laminae of TNC of rats (Moskowitz MA et al., 1993).

CONCLUSION

These findings may emphasize the pivotal role of CGRP in the process of pain sensitization in migraine headache via enhancing TRPV1, a pain receptor expression in TG. It seems that migraine headache is brought about by a gain in the expression and activation of sensory nociceptors, which enhance and sustain their signal transmission to the brain. However, the molecular mechanisms underlying CGRP-evoked or –sensitized pain signal in trigeminovascular system deserves to be explored.

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Fig. 1. The effect of CGRP on TRPV1 expression in trigeminal ganglion (TG) of rats. Male Wistar rats were intravenous injected with normal saline solution (control) or 600ng/kg CGRP for 45 min and 60 min, respectively.

A. Western blot analysis of TRPV1 in TG of control and CGRP-treated rats. A specific band is seen at 130 kDa and two more around 75 kDa.

B. The density of TRPV1 protein band was quantified using Scion Image® and Graphad Prism® and the changes are presented in graph. The results are expressed as mean ± S.E.M of four independent experiments. **p < 0.01 and ***p < 0.001 compared with control, respectively.

Fig. 2. Immunohistochemical staining of TRPV1 in trigeminal ganglion (TG) of rats. Male Wistar rats were intravenous injected with normal saline solution (control) or 600ng/kg CGRP for 60 min.

A. Representative sections of TRPV1 immunostaing in TG of rats. The TRPV1 positive immunoreactive (IR) cells were shown in small and medium-sized trigeminal neurons (arrows). Scale bar = 100 μm.

B. Quantification of TRPV1 positive-IR TG neurons. The number of TRPV1 positive-IR TG neurons was determined by manual counting through whole TG section of saved-image in TIFF format. Each number represents the mean ± S.E.M. of four independent experiments. * p <0.05 and ** p < 0.01 compared with control, respectively.

Fig. 3. Immunohistochemical staining of CGRP in trigeminal ganglion (TG) of rats. Male Wistar rats were intravenous injected with normal saline solution (control) or 600ng/kg CGRP for 60 min.

A. Representative sections of CGRP immunostaing in TG of rats. The CGRP positive immunoreactive (IR) cells were shown in small and medium-sized trigeminal neurons

(arrows). Scale bar = 100 μm.

B. Quantification of CGRP positive-IR TG neurons. The number of CGRP positive-IR TG neurons was determined by manual counting through whole TG section of saved-image in TIFF format. Each number represents the mean ± S.E.M. of four independent experiments. * $p < 0.05$ and *** $p < 0.001$ compared with control, respectively.

Fig. 4. Triple immunofluoresce staining for DAPI (blue; nuclei marker)(A, E), TRPV1 (green)(B, F), CGRP (red)(C, G) and merged images (D, H) in trigeminal ganglion (TG) of rats. Male Wistar rats were intravenous injected with normal saline solution (control) or 600ng/kg CGRP for 60 min. The superimposed of triple fluorescence or merged image shows the colocalization of TRPV1 and CGRP in TG neurons (arrows). Scale bar = 10μm.

Fig. 5. The effect of CGRP on PKC phosphorylation (p-PKC) in trigeminal ganglion (TG) of rats. Male Wistar rats were intravenous injected with normal saline solution (control) or 600ng/kg CGRP for 60 min.

A. Triple immunofluoresce staining for DAPI (blue; nuclei marker)(A, E), TRPV1 (green) (B, F), p-PKC (red)(C, G) and merged images (D, H) in trigeminal ganglion (TG) of rats. The superimposed of triple fluorescence or merged image shows the colocalization of TRPV1 and p-PKC in TG neurons. Scale bar = 10μm.

B. Western blot analysis of p-PKC in TG of control-untreated and CGRP-treated rats. The density of p-PKC protein band was quantified using Scion Image® and Graphad Prism® and the changes are presented in graph. The results are expressed as mean ± S.E.M of four independent experiments. * $p < 0.05$ compared with control.

Fig. 6. The effect of CGRP on CREB levels in trigeminal ganglion (TG) of rats. Male Wistar rats were intravenous injected with normal saline solution (control) or 600ng/kg CGRP for 60 min.

A. Triple immunofluoresce staining for DAPI (blue; nuclei marker)(A, E), TRPV1 (green)(B, F), CREB (red)(C, G) and merged images (D, H) in trigeminal ganglion (TG) of rats. The superimposed of triple fluorescence or merged image shows the colocalization of TRPV1 and CREB in TG neurons. Scale bar = 10μm.

B. Western blot analysis of CREB in TG of control-untreated and CGRP-treated rats. The density of CREB protein band was quantified using Scion Image® and Graphad Prism® and the changes are presented in graph. The results are expressed as mean ± S.E.M of four independent experiments. *** $p < 0.05$ compared with control.

Fig. 7. The effect of CGRP-induced c-fos expression in trigeminal nucleus caudalis (TNC) of rats. Wistar rats were intravenous injected with normal saline solution (control) or 600ng/kg CGRP for 60 min.

A. Immunohistochemical staining of c-fos in TNC of control and CGRP-treated rats. The expansion image shows the c-fos positive immunoreactive cells in the superficial laminae I and II of TNC. Scale bar = 500 μm.

B. Western blot analysis of c-fos in TNC of control and CGRP-treated rats. The density of c-fos protein band was quantified using Scion Image® and Graphad Prism® and the changes are presented in graph. The results are expressed as mean ± S.E.M of four independent experiments. ** $p < 0.01$ compared with control.