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Research

Serotonin Influences the Endogenous Opiate Peptides in the Rat Spinal Cord to Participates in Pain Modulation

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ABSTRACT

Spinal cord is a necessary pathway that transfers the body nociceptive inputs to the brain. Endogenous opiate peptides have been proven to participate in the nociceptive process at spinal level. It has reported that serotonin (5-HT, 5-hydroxytryptamine) in spinal cord plays a role in pan modulation, which can be blocked by opiate receptor antagonists. The present study was designed to investigate the interaction between 5-HT and endogenous opiate peptides at rat spinal level effecting on pain modulation. The results showed that 1) pain stimulation increased not only leucine-enkephalin (L-Ek), β-endorphin (β-Ep) and dynorphin A_{1-13} (DynA$_{1-13}$) concentrations but also 5-HT and 5-hydorxyindoleace acid (5-HIAA, the 5-HT main metabolic product) concentrations in spinal cord significantly; 2) 5-HT could increase L-Ek, β-Ep and DynA$_{1-13}$ concentrations in spinal cord in a dose-dependent manner, whereas cypotolamine (a 5-HT receptor antagonist) decreased L-Ek, β-Ep and DynA$_{1-13}$ concentrations in spinal cord. The data suggested that 5-HT antinociceptive role might be involved in the endogenous opiate peptide system through 5-HT receptors at spinal level.

Keywords: Serotonin; Endogenous Opiate Peptide; Spinal Cord; Antinociception

1. Introduction

Serotonin (5-HT, 5-Hydroxytryptamine) is a monoamine neurotransmitter. In animals including humans, serotonin is synthesized from the amino acid L-tryptophan by a short metabolic pathway consisting of two enzymes: tryptophan hydroxylase (TPH) and amino acid decarboxylase (DDC). The TPH-mediated reaction is the rate-limiting step in the pathway. TPH has been shown to exist in two forms: TPH1, found in several tissues, and TPH2, which is a brain-specific isoform [1]. Serotonin is mainly metabolized to 5-hydorxyindoleace acid (5-HIAA), which involves first oxidation by monoamine oxidase (MAO) to the corresponding aldehyde, and then it is followed by oxidation by aldehyde dehydrogenase to 5-HIAA, the indole acetic acid derivative. Serotonin is synthesized in serotonergic neurons in central nervous system where it has various functions of the regulation of mood, appetite, sleep, memory and learning, as well as muscle contraction. Many experiments have discovered that serotonin in spinal cord plays an important role in pan modulation. Intrathecal sildenafil effectively attenuated the pain evoked by formalin injection, in which se-

rotonin receptors may be involved in the antinociceptive action of sildenafil at the spinal level [2]. The spinal serotonin system plays an important role in the mode of action of spinal cord stimulation involving the activation of descending serotonergic pathways that may inhibit spinal nociceptive processing partially via a GABAergic link [3]. A loss or decrease in the descending inhibitory serotonin system upon the spinal processing of nociceptive information appears to occur following spinal nerve injury, and this kind of decrease in the descending inhibitory serotonin system is proposed to be involved in the development of central sensitization and ultimately to the nerve injury-induced neuropathic pain [4].

Since Hughes *et al*. purified and identified L-Ek and M-Ek in 1975 [5], the endorphin had been confirmed in 1976 [6] and dynorphin in 1979 [7]. Endogenous opiate peptides include three series—enkephalin, endorphin and dynorphin [8], which have been proven to participate in the pain modulation in the spinal cord [9-13].

The spinal cord is a necessary pathway that transfers the body nociceptive inputs to the brain, in which it has been proven the relationship between serotonin and endogenous opiate peptide system relating with pain modulation. Serotonin in the spinal cord has an analgesic

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role, which can be blocked by an opiate receptor antagonist-naloxone [14]. β-Endorphin produces analgesia at spinal level via an opiate receptor-mediated interaction with spinal monoaminergic nerve terminals [15]. Selective degeneration of spinal cord serotoninergic pathway can influence the role of morphine regulates pain [16]. Spinal system participate in the development of copulatory analgesia, which is mediated by descending serotonergic fibers, although intrinsic spinal systems would involve both opiate and GABA interneurons [17]. However, there is not direct evidence that interaction between the serotonin and endogenous opiate peptides at spinal level relating with pain modulation. The communication was designed to investigate the interaction between serotonin and endogenous opiate peptide in spinal cord effecting on the pain modulation.

2. Materials and Methods

2.1. Animals

Adult male Sprague-Dawley rats weighing 180 - 220 g were used in all experiments (Animal Center of Yangzhou University, Yangzhou, Jiangsu, China). Animals were housed in a colony room under controlled temperature, humidity and a 12 hours light/dark cycle (light on at 6:00 am), with food and water available *ad libitum*. All procedures were conducted according to the guidelines of the International Association for the Study of Pain [18] and approved by the Animal Care and Use Committee of Yangzhou University.

2.2. Materials

Leucine-enkephalin (L-Ek), β-endorphin (β-Ep) and dynorphin A_{1-13} (DynA$_{1-13}$) were obtained from Peninsula Laboratories, San Carlos, CA, USA; ^{125}Iodine was from Amersham Pharmacia, Buckinghamshire, UK; Serotonin, 5-HIAA (the 5-HT main metabolizing product), cypoheptadine (5-HT receptor antagonist) and other chemical reagents were from Sigma Co., St. Louis, MO, USA.

Rabbit anti-rat L-Ek, β-Ep or DynA$_{1-13}$ serum was made by Department of Neurobiology, Second Military Medical University, Shanghai, China. The specificity of each kind of antiserum was more than 99% reactivity with its corresponding antigen and less than 1% reactivity with other similar peptides. The effective dilution of the antiserum was 1: 20,000 - 80,000 for radioimmunoassay.

2.3. Surgery

Under pentobarbital sodium (35 mg/kg, intraperitoneal injection) anesthesia, the rat was implanted a chronic intrathecal catheter (PE-10, 12 cm in length, 0.6 cm outer diameter) extending into the lumbar enlargement of the spinal cord for intrathecal injection (*i*th). All operations were carried out under aseptic conditions and the animals were allowed to recover for at least 14 days after the surgery.

2.4. Spinal Cord Administration

Ten μl of artificial cerebral spinal fluid (ACSF, containing 0.1 M NaCl, 1.0 mM KH$_2$PO$_4$, 4.0 mM KCl, 2.0 mM MgSO$_4$, 2.0 mM CaCl$_2$, 2.1 mM NaHCO$_3$ and 8.0 mM Glucose), which could dissolve the drug, was gently injected into the lumbar enlargement of the spinal cord through the chronic intrathecal catheter over 10 min.

2.5. Pain Stimulation

All animals were tested under the condition of free activity in the small cages (30 cm in diameter, 25 cm in height) from 8:00 to 10:00 am. We used the potassium iontophoresis inducing tail-flick served as pain stimulus. The small wet cotton with the potassium iontophoresis was set on the skin of the tail. The cotton was exposed to direct electrical current, and the anode led the potassium iontophoresis to permeate the skin of the tail. If the current was strong enough, the permeated potassium iontophoresis resulted in the animal feeling the pain stimulation. The intensity of current at the moment of the response was recorded as the pain threshold, which was expressed as mA (WQ-9E Pain Threshold Measurer, Shanghai, China). Through the positive electrodes producing the direct electrical current was generated from Pain Threshold Measurer to induce the potassium iontophoresis into the animal tail skin and result in acute pain. The intensity was fixed to 1.2 - 1.4 × pain threshold (0.6 - 0.7 mA) for 1 min.

2.6. Prepare of Tissue Sample

After the decapitation, the lumber spinal cords were taken out and put into the liquid nitrogen. After weighing, the tissues were homogenized in 1.0 ml of 0.1 M acetic acid at 4°C. Two hours later, the same volume of 0.1 M sodium hydroxide was mixed in the homogenate. Using the centrifugation at 10,000 g at 4°C for 20 min, the supernatants were withdrawn and stored at −80°C for assay.

2.7. Radioimmunoassay for Peptide Measurement

The L-Ek, β-Ep and DynA$_{1-13}$ concentrations were determined with specific rabbit antiserum. The peptides were labeled ^{125}Iodine using the chloramines-T method and iodinated peptides were purified by Sephadex G-50. The assay sensitivities for the L-Ek, β-Ep or DynA$_{1-13}$ were 3.0, 1.2 and 6.3 pg/tube and intra- and inter-assay coefficients of variation were less than 5.1% and 8.0%,

respectively.

2.8. High Performance Liquid Chromatograph for Neurotransmitter Measurement

Serotonin and 5-HIAA concentrations were measured by Waters Breeze high performance liquid chromatograph (HPLC) system with 2465 electrochemical detection (ECD) and 1525 HPLC pump. The hydrogen peroxide was detected electrochemically with a platinum electrode set at 2 mV (vs Ag/AgCl). The 2.1 × 150 mm 3.5 μm Waters SymmetryShield^{TM} RP18 analytical column was protected by a 2.1 × 10 mm 3.5 μm Waters Symmetry-Shield™ Sentry™ Guard column. The mobile phase was 0.01 M sodium bicarbonate containing 11% methanol, 0.58 mM sodium octyl sulfate (SOS) and 0.02 mM ethyenediaminetetraacetic acid (EDTA). The pH was adjusted to 2.9 using hydrochloric acid, and then filtered by 0.22 μm membranes. The flow rate was fixed at 0.25 ml/min. The 25-μl solution of the standard or sample was injected into the column directly.

2.9. Statistical Analysis

Data were expressed as mean ± standard error of the mean (S.E.M.), which the repeated experiment time was the animal number of each group, and were analyzed between groups by the analysis of variance (ANOVA) and χ^2 test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Pain Stimulation Increased Endogenous Opiate Peptide and Serotonin Concentrations in the Spinal Cord

Giving the animal 1min pain stimulation, L-Ek concentration in the spinal cord was increased from 8.4 ± 2.1 pg/mg (tissue weight) to 35.5 ± 5.6 pg/mg in 5 min ($P < 0.001$) and 17.6 ± 4.3 pg/mg in 10 min ($P < 0.05$) after the stimulation (**Figure 1(a)**); β-Ep concentration in the spinal cord was increased from 10.6 ± 2.7 pg/mg (tissue weight) to 48.9 ± 8.2 pg/mg in 5 min ($P < 0.001$) and 35.4 ± 6.5 pg/mg in 10 min ($P < 0.01$) after the stimulation (**Figure 1(b)**); DynA$_{1-13}$ concentration in the spinal cord was increased from 6.5 ± 1.8 pg/mg (tissue weight) to 18.7 ± 4.3 pg/mg in 5 min ($P < 0.001$) and 11.4 ± 2.1 pg/mg in 10 min ($P < 0.01$) after the stimulation **Figure 1(c)**); Serotonin concentration in the spinal cord was increased from 7.8 ± 2.0 pg/mg (tissue weight) to 42.5 ± 10.2 pg/mg in 5 min ($P < 0.001$) and 29.8 ± 7.3 pg/mg in 10 min ($P < 0.01$) after the stimulation (**Figure 1(d)**); And 5-HIAA concentration in the spinal cord was increased from 12.3 ± 3.8 pg/mg (tissue weight) to 67.8 ± 14.3 pg/mg in 5 min ($P < 0.001$) and 39.7 ± 12.4 pg/mg

in 10 min ($P < 0.01$) after the stimulation (**Figure 1(e)**). In control group, L-Ek, β-Ep, DynA$_{1-13}$, serotonin and 5-HIAA concentrations did not changed.

3.2. Serotonin Increased Endogenous Opiate Peptide Concentrations in the Spinal Cord in a Dose-Dependent Manner

Administration of 20 ng serotonin into the spinal cord increased L-Ek concentration in the spinal cord from 8.4 ± 2.1 pg/mg (tissue weight) to 33.8 ± 5.9 pg/mg in 5 min ($P < 0.001$) and 23.4 ± 4.6 pg/mg in 10 min ($P < 0.001$); Administration of 10 ng serotonin into the spinal cord increased L-Ek concentration in the spinal cord from 8.4 ± 2.1 pg/mg (tissue weight) to 18.7 ± 3.6 pg/mg in 5 min ($P < 0.01$) and 11.7 ± 3.7 pg/mg in 10 min; In control group, L-Ek concentration did not changed (**Figure 2(a)**).

Administration of 20 ng serotonin into the spinal cord increased β-Ep concentration in the spinal cord from 10.6 ± 2.7 pg/mg (tissue weight) to 47.9 ± 9.7 pg/mg in 5 min ($P < 0.001$) and 27.5 ± 7.4 pg/mg in 10 min ($P < 0.001$); Administration of 10 ng serotonin into the spinal cord increased β-Ep concentration in the spinal cord from 10.6 ± 2.7 pg/mg (tissue weight) to 26.5 ± 6.6 pg/mg in 5 min ($P < 0.001$) and 17.6 ± 5.2 pg/mg in 10 min; In control group, β-Ep concentration did not changed (**Figure 2(b)**).

Administration of 20 ng serotonin into the spinal cord increased DynA$_{1-13}$ concentration in the spinal cord from 6.5 ± 1.8 pg/mg (tissue weight) to 29.4 ± 6.7 pg/mg in 5 min ($P < 0.001$) and 18.9 ± 5.7 pg/mg in 10 min ($P < 0.001$); Administration of 10 ng serotonin into the spinal cord increased DynA$_{1-13}$ concentration in the spinal cord from 6.5 ± 1.8 pg/mg (tissue weight) to 16.7 ± 3.5 pg/mg in 5 min ($P < 0.001$) and 10.7 ± 3.9 pg/mg in 10 min; In control group, DynA$_{1-13}$ concentration did not changed (**Figure 2(c)**).

3.3. Serotonin Receptor Antagonist Decreased the Endogenous Opiate Peptide Concentrations in the Spinal Cord in a Dose-Dependent Manner

Administration of 100 ng cypotolamine (a serotonin receptor antagonist) into the spinal cord decreased L-Ek concentration in the spinal cord from 8.4 ± 2.1 pg/mg (tissue weight) to 1.8 ± 0.7 pg/mg in 5 min ($P < 0.001$), 2.9 ± 1.3 pg/mg in 10 min ($P < 0.001$) and 5.1 ± 1.3 pg/mg in 20 min ($P < 0.01$); Administration of 50 ng cypotolamine into the spinal cord decreased L-Ek concentration in the spinal cord from 8.4 ± 2.1 pg/mg (tissue weight) to 3.4 ± 1.1 pg/mg in 5 min ($P < 0.001$), 5.5 ± 1.2 pg/mg in 10 min ($P < 0.05$) and 7.7 ± 2.0 pg/mg in 20 min; In control group, L-Ek concentration did not changed (**Figure 3(a)**).
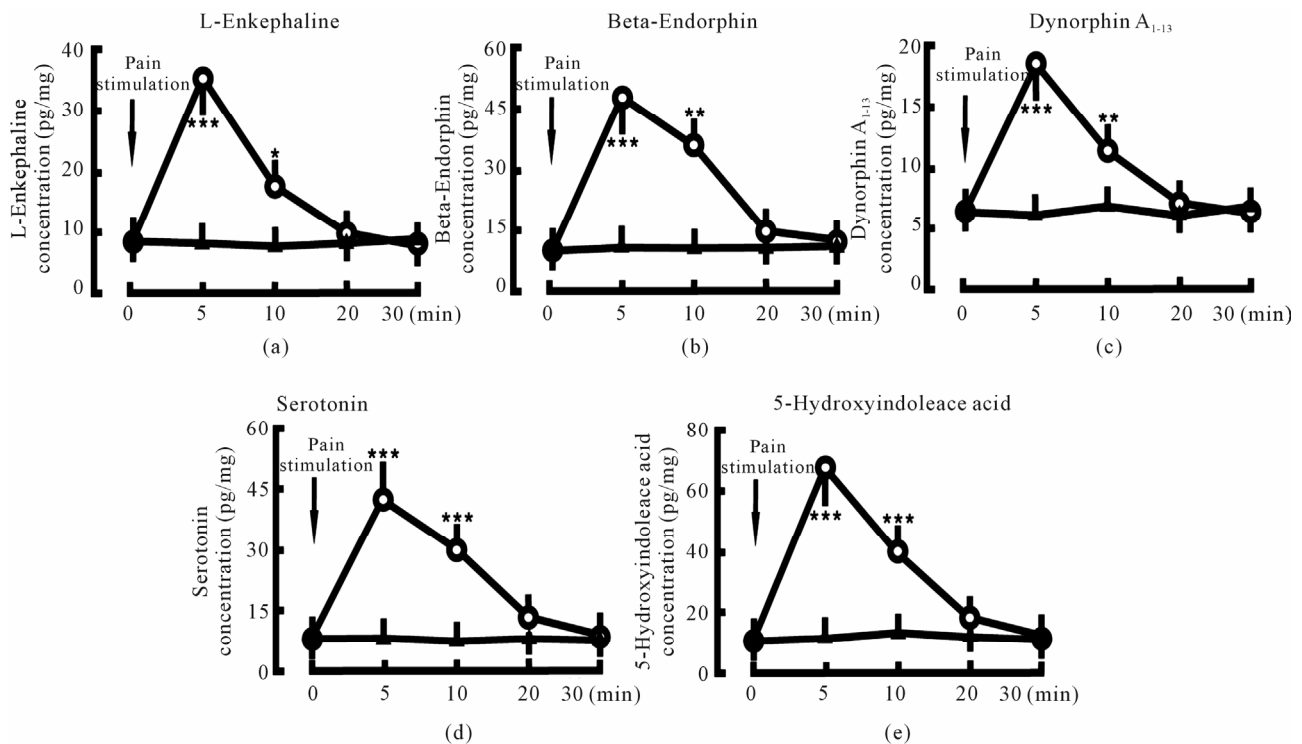
Figure 1. Effect of pain stimulation on endogenous opiate peptide and serotonin concentrations in spinal cord. Pain stimulation denotes the beginning of pain stimulation. Pain stimulation group (○, n = 8): the animal was given 1 min pain stimulation; Control group (▲, n = 8): the animal was given the sham treatment. N indicates the animal number in each group. The data are expressed as mean ± standard error mean (SEM). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ are used for the comparison of the change of L-enkephaline, β-endorphin, dynorphin A_{1-13}, serotonin or 5-hydroxyindoleace acid concentration from pain stimulation group and control group.
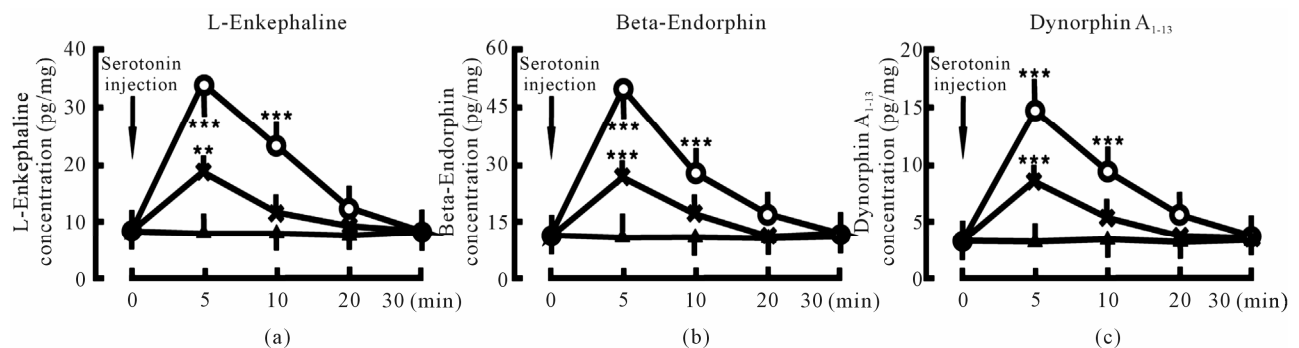

Figure 2. Effect of serotonin on endogenous opiate peptide concentration in spinal cord. Serotonin injection denotes the beginning of serotonin administration. Serotonin 20 ng group (○, n = 8): the animal was given 20 ng serotonin into the spinal cord; Serotonin 10 ng group (×, n = 8): the animal was given 10 ng serotonin into the spinal cord; Control group (▲, n = 8): the animal was not given serotonin into the spinal cord. The data are expressed as mean ± standard error mean (SEM). **$P < 0.01$ and ***$P < 0.001$ are used for the comparison of the change of L-enkephaline, β-endorphin or dynorphin A_{1-13} concentration from serotonin 20 ng group or serotonin 10 ng group and control group.

Administration of 100 ng cypotolamine into the spinal cord decreased β-Ep concentration in the spinal cord from 10.6 ± 2.7 pg/mg (tissue weight) to 3.2 ± 1.0 pg/mg in 5 min ($P < 0.001$), 5.9 ± 1.1 pg/mg in 10 min ($P < 0.001$) and 8.7 ± 2.1 pg/mg in 20min ($P < 0.01$); Administration of 50 ng cypotolamine into the spinal cord decreased β-Ep concentration in the spinal cord from

10.6 ± 2.7 pg/mg (tissue weight) to 5.6 ± 1.2 pg/mg in 5 min ($P < 0.001$), 7.3 ± 1.3 pg/mg in 10 min ($P < 0.01$) and 9.1 ± 2.2 pg/mg in 20 min; In control group, β-Ep concentration did not changed (**Figure 3(b)**).

Administration of 100 ng cypotolamine into the spinal cord decreased DynA$_{1-13}$ concentration in the spinal cord from 6.5 ± 1.8 pg/mg (tissue weight) to 1.2 ± 0.4 pg/mg
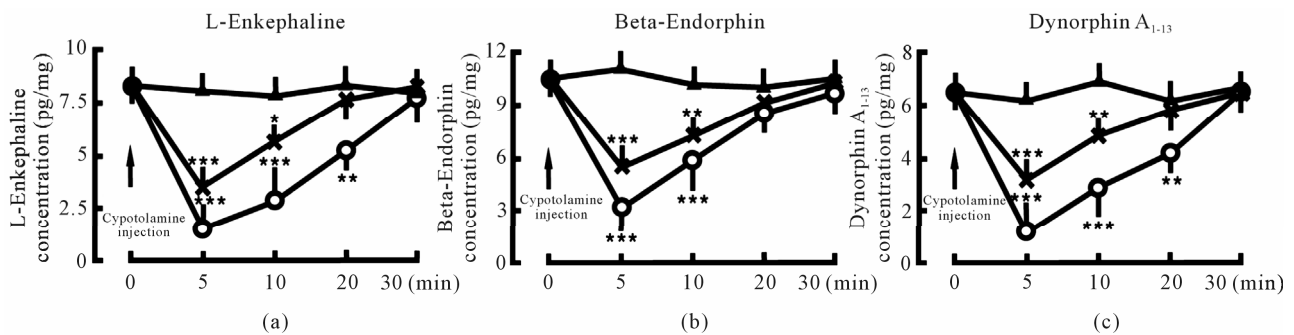
Figure 3. Effect of serotonin receptor inhibitor—cypotolamine on endogenous opiate peptide concentration in spinal cord. Cypotolamine injection denotes the beginning of cypotolamine administration. Cypotolamine 100 ng group (○, n = 8): the animal was given 100 ng cypotolamine into the spinal cord; Cypotolamine 50 ng group (×, n = 8): the animal was given 50 ng cypotolamine into the spinal cord; Control group (▲, n = 8): the animal was not given cypotolamine into the spinal cord. The data are expressed as mean ± standard error mean (SEM). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ are used for the comparison of the change of L-enkephaline, β-endorphin or dynorphin A1-13 concentration from cypotolamine 100 ng group or cypotolamine 50 ng group and control group.

in 5 min ($P < 0.001$), 2.9 ± 1.3 pg/mg in 10 min ($P < 0.001$) and 4.2 ± 1.4 pg/mg in 20 min ($P < 0.01$); Administration of 50 ng cypotolamine into the spinal cord decreased $DynA_{1\text{-}13}$ concentration in the spinal cord from 6.5 ± 1.8 pg/mg (tissue weight) to 3.1 ± 1.0 pg/mg in 5 min ($P < 0.001$), 4.8 ± 1.3 pg/mg in 10 min ($P < 0.01$) and 5.8 ± 1.4 pg/mg in 20 min; In control group, $DynA_{1\text{-}13}$ concentration did not changed (**Figure 3(c)**).

4. Discussions

Serotonin is an important neurotransmitter to regulate the pain process in the spinal cord through 5-HT receptor. 5-HT1/2A/2C and 5-HT1/2C receptors increases the descending facilitation mechanisms induced by incision in the ipsilateral paw; 5-HT2A/3 receptors contribute to descending pronociceptive pathways conveyed by lamina X spinal neurons [19]. The descending serotonergic pathways and spinal 5-HT7 receptors play a crucial role in the antinociceptive effects [20].

Endogenous opiate peptide system is involved in the serotonin effecting pain modulation. Endogenous opiate peptide may partly act as a necessary mediator for the serotonin-induced suppression on the spinal transmission of nociceptive input [21,22]. Some studies have pointed that endogenous opiate peptide is involved in serotonin-produced antinociception at the spinal level using the tail-flick assay, which effect may be mediated through different types of opiate receptors [14]. Combination of serotonin and δ-selective opiates is more effective in suppressing noxiously evoked activity than combinations with μ-selective opiates [23]. The present study showed that 1) pain stimulation increased not only L-Ek, β-Ep, $DynA_{1\text{-}13}$ concentrations but also serotonin and 5-HIAA concentrations in the spinal cord significantly; 2) serotonin increased L-Ek, β-Ep and $DynA_{1\text{-}13}$ concentrations in the spinal cord in a dose-dependent manner; 3) cypo-

tolamine, a serotonin receptor antagonist decreased L-Ek, β-Ep and $DynA_{1\text{-}13}$ concentrations in the spinal cord. The data suggested that the antinociceptive role of serotonin at spinal level was relating with the endogenous opiate peptide system through serotonin receptors. Our previous study has shown that pain stimulation can influence the endogenous opiate peptide and serotonin system in the nucleus raphe magnus [24].

Chronic nerve injury evoked hypernociception may be contributed by genetic differences of descending serotonergic inhibitory control [25]. Opiates mediates their stimulatory effects on stimulate prolactin release, at least in part, through a serotonergic mechanism in adult rats [26]. Serotonin may influence the gene expression or peptide synthesis process to act the endogenous opiate peptide system in spinal cord. However, it needs to be studied in the near further.

In conclusion, the present study makes it clear that 1) the spinal cord releases endogenous opiate peptides and serotonin during pain process; 2) serotonin enhances, whereas the serotonin receptor antagonist inhibits the spinal cord release of endogenous opiate peptides. The data indicated that the antinociceptive role of serotonin at spinal level was relating with endogenous opiate peptide system via serotonin receptors.

5. Acknowledgements

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