Acetylcholine participates in pain modulation by influencing endogenous opiate peptides in rat spinal cord

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ABSTRACT

The spinal cord is a necessary pathway that transfers the body nociceptive inputs to the brain, and endogenous opiate peptides (EOP) play an important role in pain modulation. Our previous work has proven that arginine vasopressin (AVP) antinociception in the caudate nucleus (CdN) relates with the acetylcholine (Ach) system mainly. The communication was designed to investigate the interrelations between Ach system and EOP system at the spinal level during pain process. The results showed that: 1) pain stimulation increased L-enkephalin (L-Ek), β-endorphin (β-Ep), dynorphin A_{1-13}(DynA_{1-13}), Ach and choline (Ch, an Ach metabolic product) concentrations in the spinal cord; 2) Ach increased L-Ek, β-Ep and DynA_{1-13} concentrations in the spinal cord; and 3) Atropine (M-receptor inhibitor) or hexahydric gallamine (N-receptor inhibitor) decreased L-Ek, β-Ep and DynA_{1-13} concentrations in the spinal cord. The data suggested that Ach antinociception was involved in the EOP system at the spinal level.

Keywords: Achetylcholine; Endogenous Opiate Peptide; Pain Modulation; Spinal Cord

1. INTRODUCTION

Hypothalamic paraventricular nucleus (PVN) is a complex neural structure, which has been implicated in a remarkable number of functions including control of pituitary-adrenocortical activity in response to stress [1], body fluid homeostasis [2], milk ejection reflex [3], hormone probation [4], circadian rhythm [5], food intake [6], gastrointestinal and cardiovascular functions [7], sexual activity [8], learning and memory [3]. Electrical stimulation of PVN increases pain threshold, whereas electrical cauterization of PVN decreases pain threshold;

Microinjection of L-glutamate sodium into PVN, which only excites the PVN neurons [9], increases pain threshold; Removing the pituitary, which blocks the neurohypophysis effect, does not influence the role of PVN enhancing analgesia [10,11]. PVN can regulate pain process through the central nerve system rather than the peripheral organs.

Arginine vasopressin (AVP), a nonapeptide hormone, is an important bioactive substance that is synthesized in the PVN [3]. Pain stimulation increases AVP, not oxytocin (OXT), L-enkephalin (L-Ek), β-endorphin (β-Ep) and dynorphin A_{1-13}(DynA_{1-13}) concentrations in both the PVN tissue and PVN perfuse liquid; pain stimulation decreases the number of AVP-immunoreactive neurons and enhances AVP mRNA expression in the PVN; intraventricular injection of anti-AVP serum or V_2 receptor antagonist, d(CH_2)$_5$[D-Ile$_2$,Ile$_4$, Ala-NH$_2^9$]AVP completely reversed the antinociception induced by PVN stimulation [12]. AVP is the most important bioactive substance in PVN regulating pain process [13].

The spinal cord is a necessary pathway that transfers the body nociceptive inputs to the brain. PVN stimulation increases and PVN cauterization decreases L-Ek, β-Ep, DynA_{1-13} concentrations in the spinal cord; the spinal cord pretreatment with naloxone, an opiate receptor antagonist partly reverses the analgesia induced by PVN stimulation [14]. PVN analgesia is involved in the endogenous opiate peptide (EOP) system in the spinal cord independently.

It is not clear how AVP in the PVN influences the EOP system at the spinal level. However, our previous study has pointed that AVP, which comes from the PVN, induces the caudate nucleus (CdN) releasing acetylcholine (Ach) during pain process [15,16]. Ach can influence the release of EOP in the brain including the hypothalamus [17-19]. The communication was designed to investigate the interaction between Ach system and EOP system at the spinal level during pain process.

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 [20] and approved by the Animal Care and Use Committee of Yangzhou University.

2.2 Materials

L-Ek, β-Ep and DynA$_{1-13}$ were obtained from Peninsula Laboratories, San Carlos, CA, USA; ^{125}Iodine was from Amersham Pharmacia, Buckinghamshire, UK; Ach, choline (Ch, an Ach metabolic product), atropine (M-receptor antagonist), hexahydric gallamine (N-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 (*ith*).

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 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 stimula-

tion. 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 $1.2 - 1.4 \times$ pain threshold (0.6 - 0.7 mA) for 1 min.

2.5. For 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 the drug was dissolved, was gently injected into the lumbar enlargement of the spinal cord through the chronic intrathecal catheter over 10 min.

2.6. Prepare of Tissue Sample

After the decapitation, the lumber spinal cords were taken out and put into the liquid nitrogen quickly. 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 (RIA)

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 werepurified by Sephadex G-50. The assay sensitivies 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 [21-23].

2.8. HPLC for Neurotransmitter Measurement

Ach concentration was measured by HPLC-ECD instrumentation, using a mobile phase, pH 8.5, containing 50 mM sodium phosphate buffer and 0.5% Kathon reagent (BAS) microbicide [24]. Samples were injected into a polymeric reversed phase column (BAS Ach-choline assay kit). Ach was then converted into hydrogen peroxide and betaine in a postcolumn enzyme reactor containing immobilizedacetylcholinesterase and choline oxidase (BAS). The hydrogen peroxide was detected electrochemically with a platinum electrode set at 50 mV (vs Ag/AgCl). The Ach standards was prepared weekly using ACSF and stored in –80˚C.

2.9. Statistical Analysis

Data were expressed as mean ± standard error of the mean (S.E.M.) 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 the EOP and Ach Concentrations in the Spinal Cord

Giving the animal 1 min pain stimulation, the 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)**); the 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)**); the DynA$_{1\text{-}13}$ 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)**); the Ach concentration in the spinal cord was increased from 5.4 ± 1.6 pg/mg (tissue weight) to $21.2.5 \pm 4.6$ pg/mg in 5 min ($P < 0.001$) and 12.4 ± 3.7 pg/mg in 10 min ($P < 0.001$) after the stimulation (**Figure 1(d)**); and the Ch concentration in the spinal cord was increased from 4.7 ± 1.5 pg/mg (tissue weight)

to 17.6 ± 5.3 pg/mg in 5 min ($P < 0.001$) and 9.8 ± 4.4 pg/mg in 10 min ($P < 0.01$) after the stimulation (**Figure 1(e)**). In control group, the L-Ek, β-Ep, DynA, 5-HT and 5-HIAA concentrations did not change.

3.2. Ach Increased the EOP Concentrations in the Spinal Cord

Administration of 20 ng Ach into the spinal cord increased the L-Ek concentration in the spinal cord from 8.4 ± 2.1 pg/mg (tissue weight) to 27.6 ± 5.2 pg/mg in 5 min ($P < 0.001$) and 16.7 ± 3.5 pg/mg in 10 min ($P < 0.001$); administration of 10 ng Ach into the spinal cord increased the L-Ek concentration in the spinal cord from 8.4 ± 2.1 pg/mg (tissue weight) to 15.3 ± 3.2 pg/mg in 5 min ($P < 0.01$) and $9.5.7 \pm 2.7$ pg/mg in 10 min. In control group, the L-Ek concentration did not change (**Figure 2(a)**).

Administration of 20 ng Ach into the spinal cord increased the β-Ep concentration in the spinal cord from 10.6 ± 2.7 pg/mg (tissue weight) to 31.2 ± 8.8 pg/mg in 5 min ($P < 0.001$) and 19.8 ± 6.1 pg/mg in 10 min ($P < 0.05$); administration of 10 ng Ach into the spinal cord increased the β-Ep concentration in the spinal cord from 10.6 ± 2.7 pg/mg (tissue weight) to 19.3 ± 5.3 pg/mg in 5 min ($P < 0.01$) and 13.5 ± 4.4 pg/mg in 10 min. In control group, the β-Ep concentration did not change (**Figure 2(b)**).

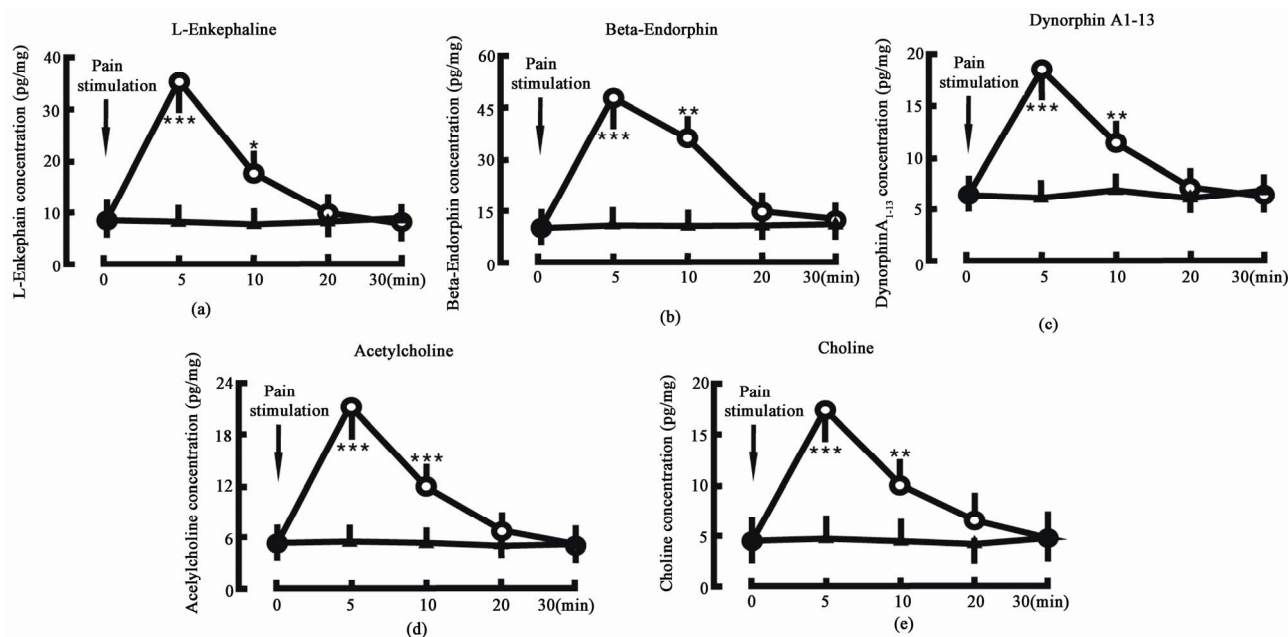Administration of 20 ng Ach into the spinal cord in-

Figure 1. Effect of pain stimulation on endogenous opiate peptide and acetylcholine concentrations in the 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-enkephalin, β-endorphin, dynorphin A$_{1\text{-}13}$, acetylcholine or choline concentration from pain stimulation group and control group.

creased the $DynA_{1-13}$ concentration in the spinal cord from 6.5 ± 1.8 pg/mg (tissue weight) to 13.4 ± 3.5 pg/mg in 5 min ($P < 0.01$) and 10.7 ± 2.9 pg/mg in 10 min ($P < 0.05$); administration of 10 ng Ach into the spinal cord increased the $DynA_{1-13}$ concentration in the spinal cord from 6.5 ± 1.8 pg/mg (tissue weight) to 8.7 ± 2.5 pg/mg in 5 min and 7.2 ± 1.9 pg/mg in 10 min. In control group, the $DynA_{1-13}$ concentration did not change (**Figure 2(c)**).

3.3. M-Receptor Inhibitor Decreased the EOP Concentrations in the Spinal Cord

Administration of 2 µg atropine into the spinal cord decreased the L-Ek concentration in the spinal cord from 8.4 ± 2.1 pg/mg (tissue weight) to 2.9 ± 0.8 pg/mg in 5 min ($P < 0.001$), 4.2 ± 1.5 pg/mg in 10 min ($P < 0.01$) and 6.2 ± 1.8 pg/mg in 20 min; administration of 1 µg atropine into the spinal cord decreased the L-Ek concentration in the spinal cord from 8.4 ± 2.1 pg/mg (tissue weight) to 5.1 ± 1.0 pg/mg in 5 min ($P < 0.05$), 6.2 ± 1.1

pg/mg in 10 min and 7.7 ± 1.9 pg/mg in 20 min. In control group, the L-Ek concentration did not change (**Figure 3(a)**).

Administration of 2 µg atropine into the spinal cord decreased the β-Ep concentration in the spinal cord from 10.6 ± 2.7 pg/mg (tissue weight) to 4.8 ± 0.9 pg/mg in 5 min ($P < 0.001$), 6.1 ± 1.2 pg/mg in 10 min ($P < 0.05$) and 8.2 ± 2.2 pg/mg in 20 min; administration of 1 µg atropine into the spinal cord decreased the β-Ep concentration in the spinal cord from 10.6 ± 2.7 pg/mg (tissue weight) to 7.1 ± 1.5 pg/mg in 5 min ($P < 0.05$), 8.6 ± 1.9 pg/mg in 10 min and 9.5 ± 2.4 pg/mg in 20 min. In control group, the β-Ep concentration did not change (**Figure 3(b)**).

Administration of 2 µg atropine into the spinal cord decreased the $DynA_{1-13}$ concentration in the spinal cord from 6.5 ± 1.8 pg/mg (tissue weight) to 2.8 ± 0.7 pg/mg in 5 min ($P < 0.01$), 3.7 ± 1.1 pg/mg in 10 min ($P < 0.05$) and 5.9 ± 1.5 pg/mg in 20 min; administration of 1 µg
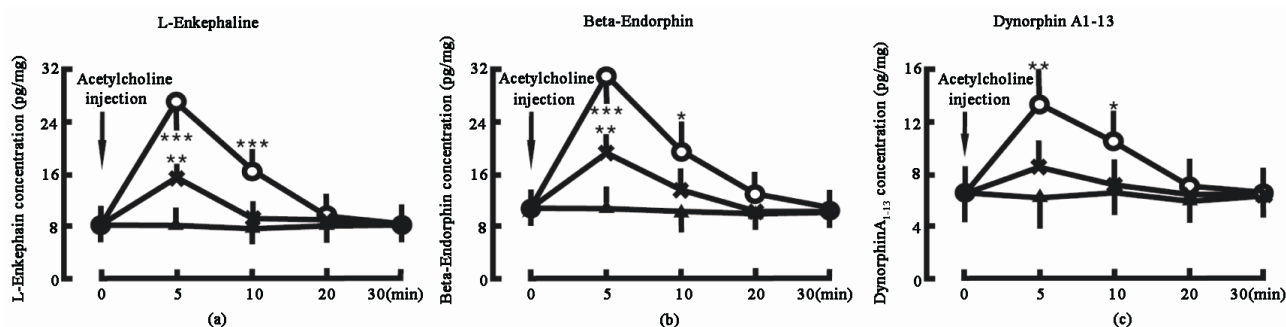

Figure 2. Effect of acetylcholine on the endogenous opiate peptide concentrations in the spinal cord. Acetylcholine injection denotes the beginning of acetylcholine administration. Acetylcholine 20 ng group (○, n = 8): the animal was given 20 ng acetylcholine into the spinal cord; Acetylcholine 10 ng group (X, n = 8): the animal was given 10 ng acetylcholine into the spinal cord; Control group (▲, n = 8): the animal was not given any acetylcholine 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- enkephalin, β-endorphin or dynorphin A1-13 concentration from acetylcholine 20 ng group or acetylcholine 10 ng group and control group.
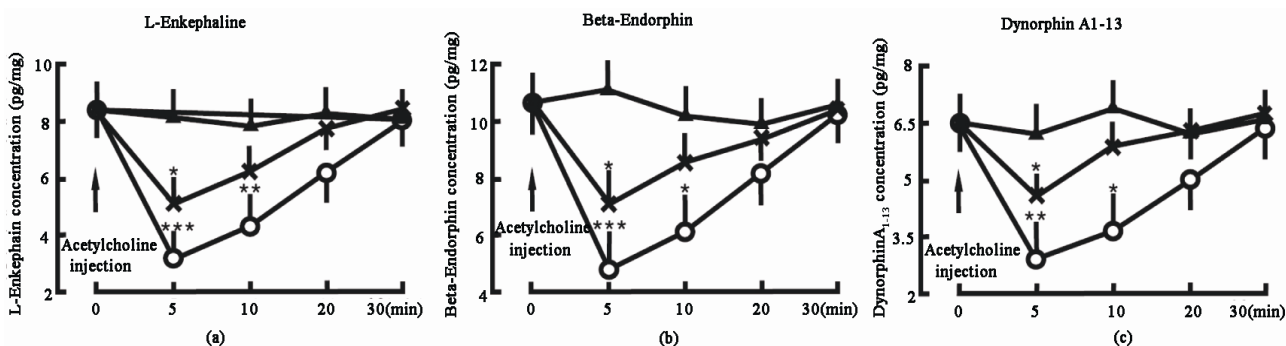

Figure 3. Effect of M-receptor inhibitor—atropine on the endogenous opiate peptide concentrations in the spinal cord. Atropine injection denotes the beginning of atropine administration. Atropine 2 µg group (○, n = 8): the animal was given 2 µg atropine into the spinal cord; Atropine 1 µg group (X, n = 8): the animal was given 1 µg atropine into the spinal cord; Control group (▲, n = 8): the animal was not given any atropine 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 hange of L-enkephalin, β-endorphin or dynorphin A_{1-13} concentration from atropine 2 µg group or atropine 1 µg group and control group.

atropine into the spinal cord decreased the DynA$_{1-13}$ concentration in the spinal cord from 6.5 ± 1.8 pg/mg (tissue weight) to 4.6 ± 1.0 pg/mg in 5 min ($P < 0.05$), 5.9 ± 1.6 pg/mg in 10 min and 6.3 ± 1.8 pg/mg in 20 min. In control group, the DynA$_{1-13}$ concentration did not change (**Figure 3(c)**).

3.4. N-Receptor Inhibitor Decreased the EOP Concentrations in the Spinal Cord

Administration of 2 μg hexahydric gallamine into the spinal cord decreased the L-Ek concentration in the spinal cord from 8.4 ± 2.1 pg/mg (tissue weight) to 4.6 ± 1.4 pg/mg in 5 min ($P < 0.01$), 5.8 ± 1.8 pg/mg in 10 min and 7.3 ± 2.0 pg/mg in 20min; administration of 1 μg hexahydric gallamine into the spinal cord decreased the L-Ek concentration in the spinal cord from 8.4 ± 2.1 pg/mg (tissue weight) to 5.8 ± 1.3 pg/mg in 5 min, 6.8 ± 1.8 pg/mg in 10 min and 7.9 ± 2.1 pg/mg in 20 min. In control group, the L-Ek concentration did not change (**Figure 4(a)**).

Administration of 2 μg hexahydric gallamine into the spinal cord decreased the β-Ep concentration in the spinal cord from 10.6 ± 2.7 pg/mg (tissue weight) to 5.8 ± 1.4 pg/mg in 5 min ($P < 0.001$), 7.2 ± 1.3 pg/mg in 10 min ($P < 0.01$) and 8.8 ± 2.2 pg/mg in 20 min; administration of 1 μg hexahydric gallamine into the spinal cord decreased the β-Ep concentration in the spinal cord from 10.6 ± 2.7 pg/mg (tissue weight) to 7.3 ± 1.6 pg/mg in 5 min ($P < 0.05$), 8.9 ± 1.9 pg/mg in 10 min and 9.5 ± 2.3 pg/mg in 20 min. In control group, the β-Ep concentration did not change (**Figure 4(b)**).

Administration of 2 μg hexahydric gallamine into the spinal cord decreased the DynA$_{1-13}$ concentration in the spinal cord from 6.5 ± 1.8 pg/mg (tissue weight) to 3.5 ± 0.7 pg/mg in 5 min ($P < 0.01$), 4.2 ± 1.1 pg/mg in 10 min and 5.8 ± 1.4 pg/mg in 20 min; administration of 1 μg

hexahydric gallamine into the spinal cord decreased the DynA$_{1-13}$ concentration in the spinal cord from 6.5 ± 1.8 pg/mg (tissue weight) to 4.7 ± 0.8 pg/mg in 5 min, 5.5 ± 1.1 pg/mg in 10 min and 6.8 ±1.2 pg/mg in 20 min. In control group, the DynA$_{1-13}$ concentration did not change (**Figure 4(c)**).

4. DISCUSSION

PVN contains two types of functional cells, the large-sized cells and small-sized cells. The large-sized cells, which are called magnocellular cells that synthesize either AVP or OXT and their respective neurophysins, areneuroendocrine cells, which project to the capillaries in the posterior pituitary lobe, where they secrete their contents as hormones into the systemic circulation. The small-sized cells, which are called parvocellular cells that synthesize these neuropeptides serve one of a number of specialized roles in the brain: some of these cells secrete releasing hormones into the portal circulation of the anterior pituitary gland; others activate, inhibit, or modulate activity in other neurons in the brain; and still other synapse on blood vessels in the brain, where they may influence vascular dynamics [3]. PVN has an analgesic role in the body nocieprion [10,11], which may be through EOP system in the spinal cord [14].

AVP has been identified as an important factor governing analgesia [6,25]. AVP intraventricular injection increases pain threshold and anti-AVP serum administration decreases pain threshold, whereas intrathecal or intravenous injection of AVP or anti-AVP serum does not influence pain threshold [26]. Our previous work has pointed that AVP is the most important bioactive substance in PVN antinociception [12,27,28]. AVP antinociception in PVN is through many brain areas including CdN [15]. Pain stimulation changes AVP concentration in CdN [28]; CdN treatment with AVP enhances analge-
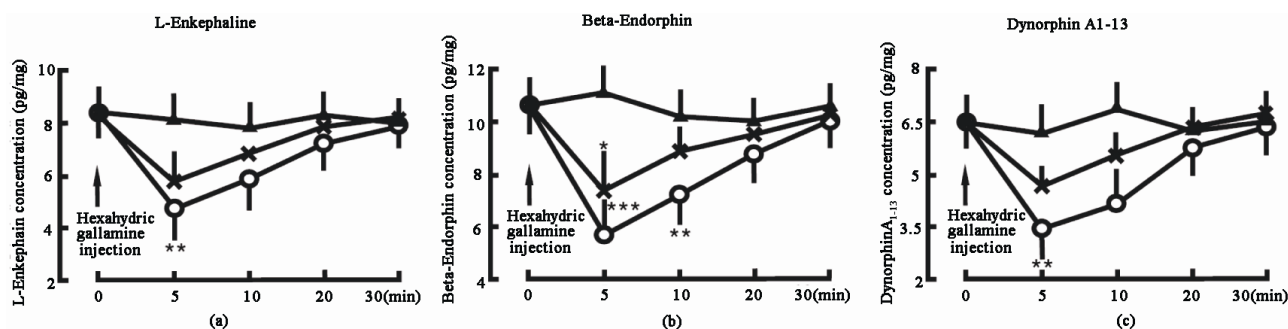

Figure 4. Effect of N-receptor inhibitor——hexahydric gallamine on the endogenous opiate peptide concentrations in the spinal cord. Hexahydric gallamine injection denotes the beginning of hexahydric gallamine administration. Hexahydric gallamine 2 μg group (○, n = 8): the animal was given 2 μg hexahydric gallamine into the spinal cord; Hexahydric gallamine 1 μg group (X, n = 8): the animal was given 1 μg hexahydric gallamine into the spinal cord; Control group (▲, n = 8): the animal was not given any hexahydric gallamine 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-enkephalin, β-endorphin or dynorphin A$_{1-13}$ concentration from hexahydric gallamine 2 μg group or hexahydric gallamine 1 μg group and control group.

sia, and CdN treatment with AVP receptor antagonist weakens analgesia [16]. Pain stimulation increases both AVP and Ach concentrations in the CdN perfusion liquid; AVP induces CdN releasing Ach, while AVP receptor antagonist including $d(CH_2)_5Tyr(Me)AVP$ (V_1 receptor antagonist) and $d(CH_2)_5[D-Ile_2, Ile_4, Ala-NH_2^9]AVP$ (V_2 receptor antagonist) inhibits this effect [15]. AVP analgesic effect in the CdN is involved in Ach system.

Acetylcholine (ACh) is a neurotransmitter in both the peripheral nervous system and central nervous system in many organisms including humans. Acetylcholine is one of many neurotransmitters in the autonomic nervous system and the only neurotransmitter used in the motor division of the somatic nervous system.

Many experiments have reported that Ach in the spinal cord plays an important role in pan modulation [29,30]. Intrathecal injection (ith) of clonidine dose-dependently reduced tonic pain behaviors [31]. Subcutaneous injection of formalin into a paw of mice causes the licking and biting, which were inhibited by intrathecal injection of the muscarinic antagonist atropine or the M_3 receptor antagonist 4-diphenylacetoxy-N-methylpiperidine methiodide [32].

Since Hughes *et al*. purified and identified L-Ek and M-Ek in 1975 [33], the endorphin had been confirmed in 1976 [34] and dynorphin in 1979 [35]. Endogenous opiate peptides include three series—enkephalin, endorphin and dynorphin [36], which have been proven to participate in the pain modulation in the spinal cord [37-40].

It has been proven that the interaction between Ach and endogenous opiate peptides in the brain relates with pain modulation [41]. The μ- and δ-opioids in the hypothalamic paraventricular nucleus (PVN) contribute to the control of ACh release [18]. The opioid peptide dynorphin A_{1-8} dynorphin B, methionine-enkephalin (M-Ek) or leucine-enkephalin (L-Ek) increased Ach release, which effect was reversed by the opiate antagonist naloxone [42]. The spinal cord is a necessary pathway that transfers the body nociceptive inputs to the brain. EOP system includes three series—enkephaline, endorphin and dynorphin, all of which are involved in analgesia [43]. PVN stimulation increases and PVN cauterization decreases the EOP concentrations in the spinal cord; administration of naloxone, an opiate receptor antagonist in the spinal cord partly attenuates the analgesia induced by PVN stimulation [14]. The date suggested that PVN regulating pain process may be through the EOP system at the spinal level. Ach system in the spinal cord is involved in the pain relieving effect [30]. Many studies have proven that there is interaction between Ach system and EOP system in central nerve system [17-19], which effects on the nociceptive suppression at the spinal level [44]. EOP induces Ach releasing evoked by K^+ or glutamic acid [45], and Ach can cause the spinal cord releasing the neuropeptides [29]. Our present study showed that: 1) Pain stimulation increased L-Ek, β-Ep, $DynA_{1-13}$, Ach and Ch concentrations in the spinal cord; 2) Ach increased L-Ek, β-Ep and $DynA_{1-13}$ concentrations in the spinal cord; and 3) Atropine (M-receptor inhibitor) or hexahydric gallamine (N-receptor inhibitor) decreased L-Ek, β-Ep and $DynA_{1-13}$ concentrations in the spinal cord. The data indicated that Ach antinociception at the spinal level was involved in the EOP system.

However, we have not investigated how Ach effects on the EOP system and where Ach comes from in the spinal cord during pain modulation.

5. ACKNOWLEDGEMENTS

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