Formalin pain increases the concentration of serotonin and its 5-hydroxyindoleacetic acid metabolite in the CA1 region of hippocampus

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ABSTRACT

Background and the purpose of the study: The hippocampal formation is involved in nociception. Prenatal serotonin depletion results in a significant decrease in the concentration of nociceptive sensitivity during the second phase of behavioral response in the formalin test.

Methods: A microdialysis probe was inserted via a guide cannula into the right CA1 region of the hippocampus. Extracellular serotonin (5HT) and its 5- hydroxyindoleacetic acid (5HIAA) metabolite overflow were collected every 10 min during the formalin test and measured by HPLC with electrochemichal detector.

Results: Compared to the sham group, formalin injection in the hind paw of the rat significantly increased 5HT after 10, 30, 40, and 50 min and increased 5HIAA after 10, 30, 40, 50, and 60 min collection time periods in hippocampal dialysate. (n=6 for each group at each sampling time). In the formalin treated rats serotonin and 5HIAA concentrations increased in the biphasic pattern in concert with the first and second phases of formalin pain.

Conclusion: The hippocampal formation might be involved in the processing of nociceptive information and serotonin-related mechanisms in the hippocampus may play a role in the biphasic behavioral responses to formalin noxious stimulation.

Keywords: Formalin test; Hippocampus; Serotonin; 5- hydroxyl-indole-acetic-acid; HPLC - ECD

INTRODUCTION

Physiological, pharmacological and behavioral findings suggest that the hippocampal formation is involved in nociception (1-5). Some hippocampal formation neurons respond exclusively to noxious stimuli (6-8). Microinjection of lidocaine into dorsal hippocampal formation has been shown to attenuate the nociceptive behavior to the unconditioned hind paw resulting from injection of the formalin (3) and partial hippocampectomy has been reported to alleviate chronic pain (9). Furthermore, electrical stimulation of the hippocampal formation which evokes painful sensations in human (10), and blockade of neural transmission along the major afferent (3), or efferent (11) hippocampal pathways has been shown to reduce pain behaviors. Finally, peripheral noxious stimulation alters the induction of Fos (1,12,13) and Early Grows Response1(Egr1) (1,14,15) in the hippocampal formation.

Serotonin (5-hydroxtryptamine, 5-HT) is described to exert either algesic or analgesic effects depending on the site of action and the receptor subtype which it acts on. 5-HT application in peripheral tissues produces pain in human (16) and nociceptive behaviors (17,18) and hyperalgesia in rodents (19). 5-HT also participates in the nociception induced by formalin or carrageenan (20).

The CA1 region of hippocampus receives collateral serotonergic projections from median raphe nuclei (21, 22) that have a powerful influence on hippocampal activity (23) and are excited during noxious stimulation (24). 3,4-Methylenedioxymethamphetamine (MDMA), a selective serotonint depletor in the cortex, hippocampus, striatum, brain stem, and cervical portion of the spinal cord, enhances analgesic effect of morphine in tail immersion test (25). It is also reported that prenatal serotonin depletion in rats results in a significant decrease in the concentration of nociceptive sensitivity during the second phase of behavioral response in the formalin test (26).

Based on the above information, it appears that a relation exist between hippocampal extracellular serotonin concentration and nociception. This study was, therefore, designed to investigate changes in extracellular serotonin and 5HIAA in the CA1 region of hippocampus during formalin test. In order to

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support this hypothesis, intra- CA1 dialysates was collected and quantified for 5HT and 5HIAA during a persistent nociception.

MATERIAL AND METHODS

Animals

Male albino rats of Wistar strain, each weighing 250-280 g, were used in this study. They were housed four per cage in a temperature and light-controlled room (21 ºC, light/ dark cycle 12hrs/12hrs, and light on 06:00) with water and food *ad libitum*. The experiments followed the ethical guidelines of the International Association for the Study of Pain (27) which was approved by the Pasteur Institute of Iran ethical committee for animal experimentation.

Surgery

Each rat after administration of a mixture of ketamine and xylazin (60 and 12 mg/kg respectively, i.p.) for surgical anesthesia, was placed in a stereotaxic apparatus, and a guide cannula was implanted above the right CA1 region of hippocampus (ML -2.2 and AP -3.8 mm from bregma, DV 2.6 mm from outer skull surface) according to the atlas of Paxinos & Watson 2005 (28). The guide cannula was fixed to the skull using stainless steel screws and dental acrylic. Animals were housed separately in the postoperative period and allowed 7 days for recovery prior to further experimentation.

Formalin test

Seven days after surgery, each rat was placed in a transparent acrylic cage and allowed to move freely for 15–20 min to habituate. A mirror was placed under the cage to allow an unobstructed view of the animal's paws by the behavioral observer. Each rat was restrained and received a 50-µl subcutaneous injection of 5% buffered formalin acetate (Sigma) into the left hind-paw and placed in the observation box for another 60 min. The pain response to the formalin test consisted of an initial display of nociceptive behaviors that subsides after approximately 5 min and re-appears 10-15 min later and then slowly diminishes following sequent 40-60 min (28). Rats in this experiment were observed for 60 min following formalin injection and each 15 sec their pain behavior were continuously rated by a 4- point scale (28,29).

Scores of each time point (five minute intervals) were calculated by the equation $(0 \times T0 + 1 \times T1 + 2 \times T2 + 3 \times T3) / 300 = \text{score}$, where T0-T3 designates the number of times that rats obtained scores of 0-3, respectively (28). By this way, pain behaviors are expressed during each period of five minutes intervals during the initial acute phase (0-5 min) or the second, tonic phase (20-60 min). (28)

Microdialysis procedure

In vivo microdialysis sampling was carried out by a microdialysis probe (MAB 4.15.1.Cu; Microbiotech, Stockholm, Sweden) that was lowered into the guide cannula approximately 15 min prior to microdialysis sessions. Each rat was placed into transparent acrylic cage for formalin test and microdialysis processing. The microdialysis inflow and outflow tubing were connected to a fluid swivel (Eicom, Japan). Artificial cerebrospinal fluid (aCSF) consisting of (mM) NaCl 114, KCl 3, CaCl2 1, MgSO4 2, NaH2PO4 1.25, NaHCO3 26, NaOH 1, glucose 10, at a pH of 7.4 (29), pumped at a flow rate of 5 µl/ min through the probe. The lag time between dialysates flowing through the probe and sample collection was 10 min. This time was used as a delay time and the data were corrected accordingly. Dialysate samples (50 µl) were collected at 10 min intervals and immediately stored at -70 ºC until analysis. When all samples were collected, they assayed for 5HT and 5HIAA via high performance- liquid chromatography with electrochemical detector (HPLC-ECD). Microdialysis samples were collected throughout the pre- and post- formalin injection period for 30 and 60 min respectively.

HPLC-ECD analysis of 5HT and 5HIAA in dialysate samples

Aliquots of the dialysate (40 µl) were injected into a HPLC column (Teknokroma, 120 ODSA, 150× 4.6 mm) that was coupled to an electrochemical detector (Pharmacia LKB, type-2143 RPE, USA). A glassy carbon electrode was set at a potential of +750 mV relative to Ag/AgCl reference. Mobile phase consisted of sodium phosphate (8.4g), 1-octane-sulfonic acid (360 mg) , (EDTA) 30 mg and 20% of methanol per liter of water (final pH= 3.5) and was pumped (Waters 510, USA) at a rate of 1.0 ml/ min (29). Chromatographic data were acquired on-line and exported to a software system (Autochro 2000, USA) for peak amplification, integration, and analysis. Standards of 5HT and 5HIAA were run daily before dialysate samples. A monoamine standard mix containing 5HT and 5HIAA was injected before and after the experiment to insure validity of the retention times of constituents. Peak heights of unknowns were compared to peak heights of standards and the lower limit of assay sensitivity was 5 fmol/ 40 µl sample (Fig.1). (30)

Histology

At the end of each experiment, rats were euthanized with overdose of ether; brains were immediately removed and stored in 10% formalin for at least 48 hrs. The placement of microdialysis probe tips within the specific area of interest was verified from 10 µm coronal sections, and only rats with correct placement were include in data analysis. Only six rats out of seven were used for data selection. Figure 2 shows representative photomicrographs of microdialysis site.

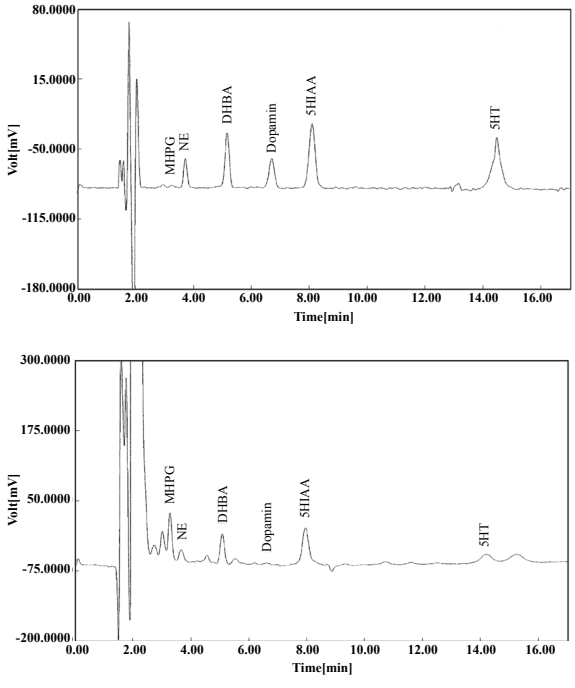

Figure 1. The HPLC– ECD peaks of serotonin and 5HIAA in the standard (top) and dialysate samples (bottom) are shown.

Statistical analyses

The data were analyzed by ANOVA and also paired and unpaired t-test. All results have been shown as mean percent from baseline± S.E.M. In all statistical comparisons, $P < 0.05$ considered as significant.

RESULTS

Formalin test

In the test group, formalin injection into the dorsal surface of the left hind paw led to a two-phase pain response. In the early phase, the rats had a 0.16± 0.008 nociceptive score and in the late phase, it had 0.13± 0.005 score (Fig.3). In the sham group, after injection of saline, no change could be detected. The changes observed in the test group were significantly different from those observed in sham group.

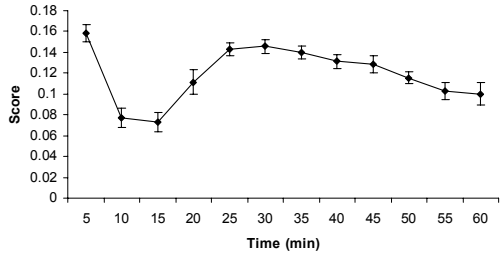

Figure 3. Average pain scores after formalin administration. Each point is the mean ± SEM of the time spent in nociceptive behavior (n=8).

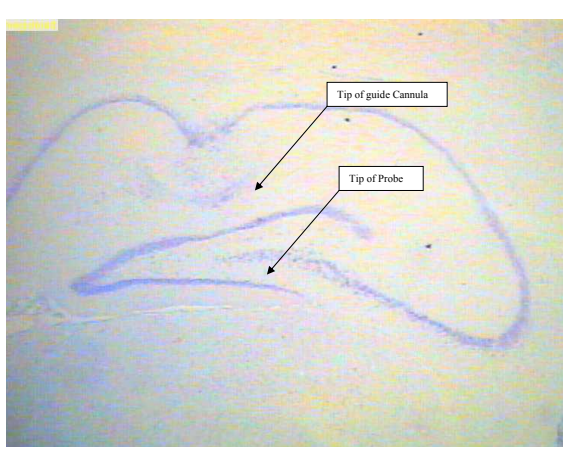

Figure 2. Nissl-stained coronal brain section from cannulated and dialysated rats.Cannula and dialysation position in CA1 is shown.

Microdialysis

Serotonin (5HT)

The basal concentration of serotonin in the CA1 region of hippocampus was 25.03± 5.8 (mean ± SEM) fmol/40µl sample (n =6). which was calculated by the equation: [(Area in each interval – Basal concentration) / Basal concentration] ×100 = Mean percent from baseline.

In the sham group, the injection of saline had no significant effect on the concentration of extracellular serotonin in the CA1 region of hippocampus. In the formalin group, however, the concentration of serotonin increased significantly at 10 ($P< 0.002$), 30 ($P< 0.046$), 40 ($P< 0.036$), and 50 ($P< 0.05$) min intervals after formalin injection. These increases were significantly different from the basal concentration and from that measured in sham group (Fig. 4).

With respect to time intervals there was only a significant difference in the concentration of serotonin between 10 and 20 min intervals ($P< 0.020$) in the formalin group.

5HIAA

The basal concentration of 5HIAA in the CA1 region of hippocampus was 313.8± 31.2 (mean ± SEM) fmol/40µl sample (n=6). Using the above equation,

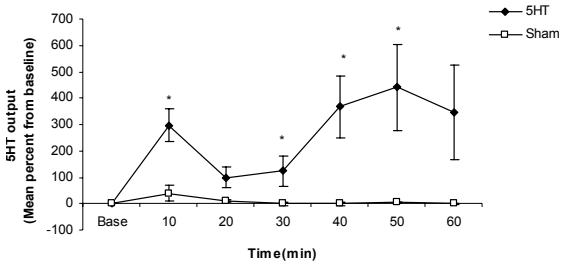

Figure 4. Time- course of the basal and formalin- evoked release serotonin concentration in the extracellular fluid. Each point is the mean percent of baseline± SEM (* $p< 0.05$ vs baseline; n=6).

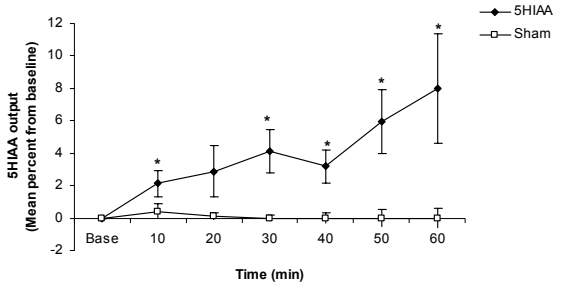

Figure 5. Time- course of the basal and formalin- evoked 5HIAA concentration in the extracellular fluid. Each point is the mean percent of baseline± SEM (* p< 0.05 vs baseline; n=6).

the amount as mean percent from baseline was calculated. In the sham group, injection of saline had no significant effect on the concentration of extracellular 5HIAA in the CA1 region. However in the formalin group, the concentration increased significantly at 10 (P< 0.045), 30 (P< 0.027), 40 (P< 0.027), 50 (P< 0.028), and 60 (P< 0.026) min intervals after injection of formalin. These increases were significantly different from the basal concentration and from that measured in sham group (Fig. 5).

A compareison of different time intervals showed there was a significant differences in the concentration of serotonin between 10 with 60 (P< 0.035) and also 20 with 60 (P< 0.035) min intervals.

DISCUSSION

The present experiment showed the biphasic increase of extracellular serotonin and its 5HIAA metabolite in the CA1 region of hippocampus during noxious stimulation in the formalin test.

Some evidences suggest that the hippocampal formation is involved in nociception (2,5,31) and in the biphasic response to formalin (32). The pyramidal cells and interneurons in the dorsal hippocampal CA1 response to persistent noxious activation (33, 34) and formalin injection induce a decrease in spontaneous activity of the CA1 pyramidal cells (33). In addition, this area receives collateral projections from median raphe nuclei (22). It has further been demonstrated that the serotonergic system has a powerful influence on hippocampal electrical activity (23).

Results of this study showed a significant difference between formalin and saline groups in the concentration of extracellular serotonin and 5HIAA which appears to be related to pain and not the short duration of noxious stimulation (e.g. injection of saline).

The measure biphasic increases of serotonin and its 5HIAA metabolite in the CA1 region of hippocampus during the first and second phases of formalin pain, indicates that the CA1 region of hippocampus might be critically involved in the perception of acute and tonic pain and/or in the biphasic behavior responses to noxious stimulation (32).

It has been shown that serotonin receptors are present on GABA interneurons (35, 36) and pyramidal cells (37, 38). Therefore, the noxious stimulation-induced increase of serotonin and 5HIAA might result from pyramidal cells and interneurons responses in the CA1 region of the hippocampus. The serotonergic input might decrease the activity of the pyramidal cells and increase the inhibitory GABAergic tonus impinging upon these cells. This is supported by previous finding that subcutaneous formalin induced depression of pyramidal cell synaptic excitability and increase firing rate of GABAergic interneurons in the CA1 region of hippocampus (31, 35, 37, 38).

It was also observed that extracellular concentrations of serotonin and 5HIAA undergo biphasic changes. The first peak occurs 10 min and the second peak occurs 30-50 min after formalin injection. Therefore, our data is in agreement with reported nociception behaviour response to formalin injection; including an early phase nociceptive drive which decreases towards sham within 10 min following formalin injection followed by a later (late phase) increase in nociceptive drive (8). The functional meaning of the serotonin and 5HIAA increase in the CA1 might be related to pain nociception by hippocampal activation during noxious stimulation. It was also shown that $5HT_{2A/2C}$ receptors antagonist microinjection in the CA1 region of hippocampus decreased pain behaviour (5). Results of this study show an increase of serotonin and its metabolite 5HIAA. It is suggested that the serotonin and 5HIAA of the CA1 region of hippocampus increases as part of a nociception mechanism.

In conclusion, formalin injection in the hind paw increases extracellular serotonin and its metabolite 5HIAA in the CA1 region of hippocampus suggesting that these monoamines are involved in the CA1 response to noxious stimulation.

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