L-type calcium channel blockers enhance 5-HTP-induced antinociception in mice

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ABSTRACT

AIM: To investigate the involvement of L-type Ca^{2+} channels in antinociceptive action induced by the 5-HT precursor, 5-hydroxytryptophan (5-HTP). **METHODS:** Female Kunming mice were treated with either 5-HTP (20-80 mg/kg, ip) alone, or the combination of 5-HTP and fluoxetine (2-8 mg/kg, ip), pargyline (15-60 mg/kg, ip), nimodipine (2.5-10 mg/kg, ip), nifedipine (2.5-10 mg/kg, ip), verapamil (2.5-10 mg/kg, ip), $CaCl_2$ (5-20 mmol/L, icv), or EGTA (0.5-3 mmol/L, icv) prior to the hot-plate test (55 ºC, hind-paw licking latency). In addition, locomotor activity in mice treated with 5-HTP alone was measured using an ambulometer with five activity boxes. **RESULTS:** Ip injection of 5-HTP alone had no influence on the spontaneous locomotor activity, whereas dose-dependently increased the latency to licking hind-paw in the hot-plate test in mice. The inhibitory effects of 5-HTP on nociceptive response were significantly enhanced by fluoxetine in the mouse hot-plate test. At a sub-effective dose, pargyline could cause a leftward shift in the dose-response curve of 5-HTP-induced antinociception. Co-administration with 5-HTP and nimodipine, nifedipine, or verapamil obviously potentiated the antinociceptive effects elicited by 5-HTP. Interestingly, 5-HTP-induced antinociception was antagonized by $CaCl_2$ and enhanced by EGTA injected icv in the mouse hot-plate test. **CONCLUSION:** These findings suggest that systemic administration of 5-HTP may yield the antinociceptive effects, which are related to Ca^{2+} influx from extracellular fluid through L-type Ca^{2+} channels.

INTRODUCTION

Although the numerous studies have shown the importance of the peripheral and central serotonin (5-hydroxytryptamine, 5-HT) in modulating nociceptive transmission, several modes of its actions require clarification. 5-HT is generally described as pronociceptive substance in peripheral tissues, but as antinociceptive substance in the central nervous systems (CNS), including spinal and supraspinal levels. However, there exist several lines of evidence which are in conflict with the view of a generalized antinociceptive action of 5-HT in spinal and/or supraspinal levels. Depending on the nature of the noxious stimulus, the dose and route administered, 5-HT may either facilitate or inhibit nociceptive transmission[1,2]. Recently, the use of selective agonists and antagonists of 5-HT receptor subtypes has brought to light a complex picture in which nociception is mediated through these receptors in spinal and supraspinal levels, even agonists selective for several 5-HT receptor subtypes can produce pro- or antinociception[2,3]. Systemic administration with 5-hydroxytryptophan (5-HTP), a precursor of 5-HT, can

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increase extracellular 5-HT levels in both peripheral tissues and CNS[4-7]. One goal of this study was to determine how systemic administration of 5-HTP affected thermal nociception in the mouse hot-plate test.

Calcium ion (Ca^{2+}) is known to act as a second messenger in the modulation of cellular responses. It has been reported that Ca^{2+} plays an important role in the regulation of pain sensitivity[8,9]. Recently, our data have demonstrated that Ca^{2+} influx from extracellular fluid and release of Ca^{2+} from Ca^{2+}/caffeine-sensitive microsomal pools may be involved in buspirone-induced antinociception[10]. The main route of extracellular Ca^{2+} influx to the cells is voltage dependent Ca^{2+} channels (VDCCs)[11]. Although the electrophysiological characteristics, pharmacological sensitivities, and physiological roles of four types of VDCC, including L-, N-, T- and P-type, are different, Ca^{2+} conductance in these types of Ca^{2+} channels is inhibited by activation of various 5-HT receptor subtypes[12, 13]. However, the effects of Ca^{2+} influx through Ca^{2+} channels from extracellular fluid on antinociception induced by 5-HTP have not been documented. The present study was undertaken to investigate the hypothesis that Ca^{2+} channels, in particular L-voltage-gated Ca^{2+} channels, might be involved in antinociception induced by 5-HTP administered systemically in mice.

MATERIALS AND METHODS

Animals Female Kunming mice (Grade II, Certificate No 99001) weighing 18-24 g were purchased from the Department of Laboratory Animal Science, Peking University Health Science Center. The animals were housed in groups of 5-6 in clear plastic cages with free access to water and food in a room that was kept at a constant temperature (22±1 ºC) and on a 12-h/12-h light/dark cycle (lights on at 08:00 h). Mice were acclimatized to laboratory conditions for 2-3 d before the test. Experiments were performed between 09:00 and 17:00, and each animal was tested only once. All experiments were conducted according to the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No 80-23, revised 1996). The experimental procedures were approved by the local Committee on Animal Care and Use.

Drugs and chemicals The drugs and chemicals used included 5-hydroxy-*L*-tryptophan (5-HTP), pargyline HCl, fluoxetine HCl (Changzhou SIYAO Pharmaceuticals Co, Ltd, China), nimodipine (Tianjin Central Pharmaceutical Manufactory, China), nifedipine, verapamil, $CaCl_2$ (Tianjin Dengzhong Chemical Factory, China) and (β-aminoethyl-ether)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA). Unless otherwise noted, drugs were purchased from Sigma Chemical Co (St Louis, MO, USA). All the drugs and chemicals were dissolved in 0.9 % saline with the exception of nifedipine and nimodipine, which were dissolved in one drop of ethanol and diluted with saline. Saline was used as vehicle control for all the experiments. All the drugs and chemicals were injected ip in a volume of 10 mL/kg, except $CaCl_2$ and EGTA administered icv.

The intracerebroventricular (icv) injection The icv injection was carried out as described previously[14]. Drugs or vehicle were injected in a volume of 10 μL into the mouse lateral ventricle. The injection sites were verified by an icv injection of 10 μL of 1 % methylene blue. The distribution of the dye in the cerebral ventricles and spinal cord was examined. The dye was found in cerebral ventricles, the ventral surface of the brain, and the upper cervical portion of the spinal cord with injection accuracy greater than 95 %.

Assessment of antinociception The hot plate (Medical Electronic Apparatus Factory of Zhejiang, China) consisted of a circular, pure copper slab (diameter: 22 cm), which was maintained at a temperature of 55.0±0.5 ºC and surrounded by a clear plastic cylinder (height: 16 cm). Mice were placed individually on the center of the hot plate and the latency (s) to the licking of the hind paws was recorded with a stopwatch. An arbitrary cutoff time of 60 s was adopted, after which the animals were immediately removed from the hot plate. In the (rare) event that a mouse jumped prior to licking its paw, data were disregarded.

Locomotor activity Locomotor activity was measured by an ambulometer with five activity boxes (JZZ98, Institute of Materia Medica, Chinese Academy of Medical Sciences, China). Each activity chamber of 26 cm×14 cm×15 cm (length×width×height) consisted of white opaque perspex walls, a transparent perspex lid and a floor consisting of 25 parallel copper bars spaced at 1-cm intervals. The odd bars were earthed and the even bars were active and connected with a few micro-amper energy source. The paws of the mice connected or disconnected the active bars producing random configurations that were converted into pulses. The pulses, which were proportional to the spontaneous locomotor activity of the mice, were recorded as

the cumulative total counts of motor activity for a selected minute period.

Procedures Animals were examined with the hot-plate test 30 min after ip injections of 5-HTP (20, 40, 60, and 80 mg/kg), verapamil (2.5, 5, and 10 mg/kg), pargyline (15, 30, and 60 mg/kg). To investigate the effects of fluoxetine, pargyline, nimodipine, nifedipine, verapamil, $CaCl_2$ and EGTA on 5-HTP antinociception, mice were treated ip or icv with these drugs 10 min before 5-HTP, which was injected 20 min prior to the hot-plate test. In addition, to evaluate the side effects of 5-HTP injected ip alone on the spontaneous locomotor activity in mice, locomotion was measured 30 min post-treatment of it at the same doses as mentioned above.

Statistical analysis All data were analyzed by one-way analysis of variance (ANOVA) followed by Independent-Sample *t*-test with the exception of those of the effects of combining pargyline with 5-HTP, which were analyzed by two-way ANOVA. The results were expressed as mean±SD. $P<0.05$ was considered as significant.

RESULTS

Effects of fluoxetine and pargyline on 5-HTP-induced antinociception Animals treated with 5-HTP (20-80 mg/kg) did not show the modification of the spontaneous locomotor activity. Given alone, however, 5-HTP yielded obvious antinociceptive effects that were similar to those reported previously[10]. 5-HTP at the same doses induced dose-dependent increases in the pain threshold in the mouse hot-plate test (Fig 1).

As shown in Fig 2, fluoxetine (2-8 mg/kg), a selective serotonin re-uptake inhibitor (SSRI), significantly prolonged 5-HTP-induced licking latency in mice. In the previous studies from our laboratory[10], 2.5-10 mg/kg of fluoxetine had no antinociception in the mouse hot-plate test. When administered alone, pargyline (15-60 mg/kg), a monoamine oxidase inhibitor (MAOI), showed no analgesic properties in mice (Fig 3A). Co-administration ip with pargyline (15 mg/kg) and 5-HTP potentiated 5-HTP-induced antinociception. A dose of pargyline that was unable to elicit any antinociception could cause a leftward shift in the 5-HTP dose-response curve (Fig 3B).

Nimodipine, nifedipine, and verapamil potentiated 5-HTP-induced antinociception To investigate whether L-type Ca^{2+} channels are involved in the

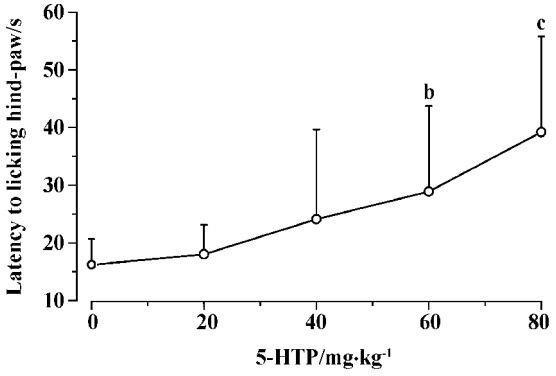

Fig 1. Antinociceptive effects of ip injection of 5-HTP in the hot-plate test in mice. *n*=12-13. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* saline control (0 mg/kg group).

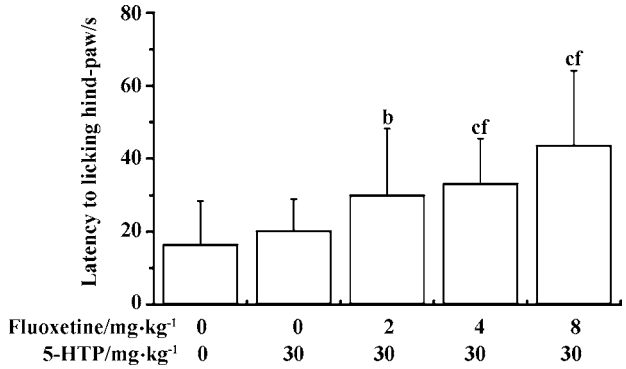

Fig 2. Fluoxetine potentiated 5-HTP-induced antinociception in the mouse hot-plate test. *n*=12. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* saline+saline group. $^fP<0.01$ *vs* saline+5-HTP (30 mg/kg) group.

antinociception induced by 5-HTP systemically administered, the effects of nimodipine, nifedipine or verapamil ip treatment on the inhibition of nociceptive response elicited by 5-HTP were examined. Although verapamil (2.5-10 mg/kg) failed to modulate latency to licking hind-paw in the mouse hot-plate test (data not shown), it did significantly enhance the antinociception elicited by ip injection of 5-HTP at the sub-effective dose of 30 mg/kg (Fig 4C). As we had observed in earlier studies[10], nimodipine (2.5-10 mg/kg) and nifedipine (2.5-10 mg/kg) appeared not to affect latency to licking hind-paw in the mouse hot-plate test. As shown in Fig 4A and Fig 4B, in the presence of nimodipine or nifedipine, a sub-effective dose 30 mg/kg of 5-HTP systemically administered produced significantly antinociceptive effect. Latency to licking hind-paw of mice in hot-plate test appeared to be enhanced with increasing doses of nimodipine (2.5-10 mg/kg) or

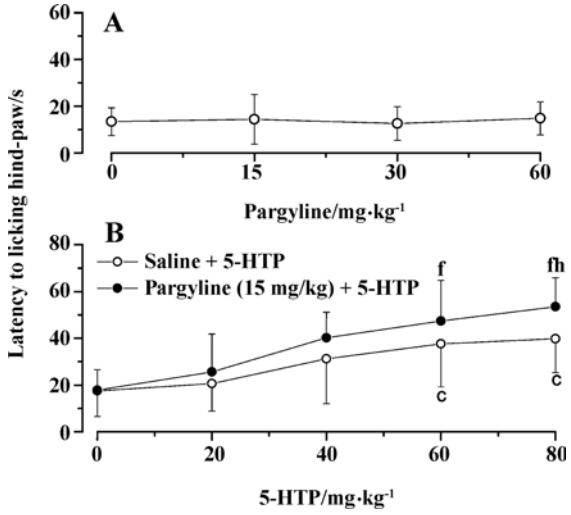

Fig 3. Effect of pargyline alone (A) or plus 5-HTP (B) on latency to licking hind-paw in the hot-plate test in mice. *n*=10-14. Mean±SD. $^{c}P<0.01$ *vs* saline+saline group. $^{f}P<0.01$ *vs* pargyline (15 mg/kg)+saline group. $^{h}P<0.05$ *vs* corresponding 5-HTP-treated alone (saline+5-HTP) group.

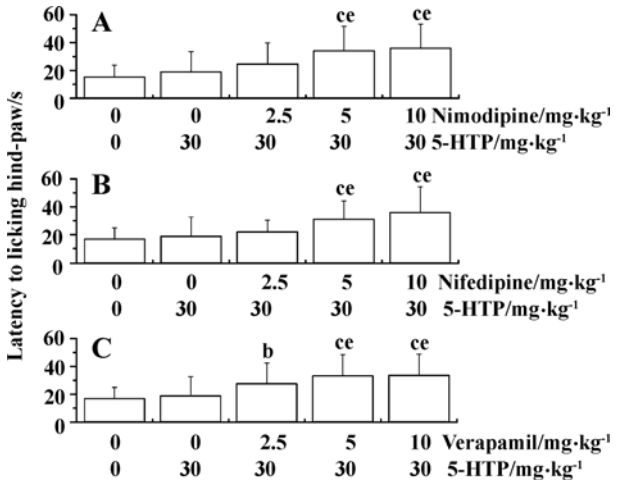

Fig 4. Effects of nimodipine (A), nifedipine (B) or verapamil (C) on 5-HTP-induced antinociception in the hot-plate test in mice. *n*=12. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* saline+saline group. $^{e}P<0.05$ *vs* saline+5-HTP (30 mg/kg) group.

nifedipine (2.5-10 mg/kg).

Effects of Ca^{2+} and EGTA administered icv on 5-HTP-induced antinociception The experiments were designed to determine the hypothesis that directly increasing or decreasing concentration of calcium in CNS would affect pharmacological profile of antinociception injected ip with 5-HTP. The previous experiments in our laboratory indicated that $CaCl_2$ (2.5-20 mmol/L) administered icv did not change latency to licking hind-paw of mice in hot plate test[10]. However, administration with $CaCl_2$ (5, 10, and 20 mmol/L, icv 10 μL) significantly and dose-dependently attenuated latency to licking hind-paw induced by the ip injection of 5-HTP at 70 mg/kg, which obviously produced antinociceptive action in the mouse hot-plate test (Fig 5A). Conversely, although EGTA, a selective Ca^{2+} chelator, did not influence the latency at 3 mmol/L injected icv alone, co-administration with EGTA (0.5, 1.5 and 3 mmol/L, icv 10 μL) and 5-HTP (30 mg/kg, ip) increased the licking latency in a dose-dependent manner (Fig 5B).

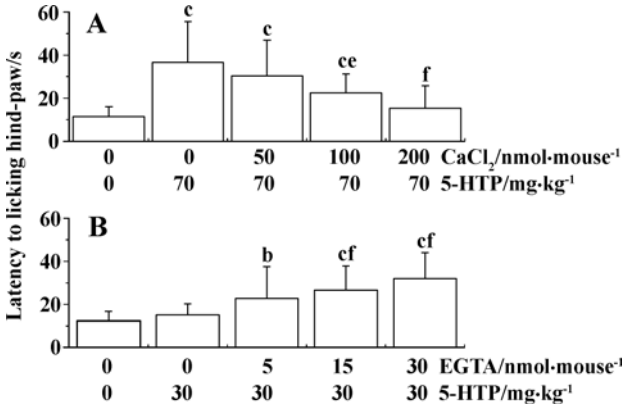

Fig 5. Effects of Ca^{2+} (A), or EGTA (B) on 5-HTP-induced antinociception in the hot-plate test in mice. *n*=10-12. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* sterilized H_2O+saline group. $^{e}P<0.05$, $^{f}P<0.01$ *vs* sterilized H_2O+5-HTP group.

DISCUSSION

The present study demonstrated that systemic (ip) application of 5-HTP induced obvious and dose-dependent antinociceptive effect in the mouse hot-plate test, which was in line with the results reported previously on antinociception elicited by ip injection of 5-HTP[10]. Fluoxetine, a selective serotonin re-uptake inhibitor, and pargyline, a monoamine oxidase inhibitor, both increase extracellular levels of 5-HT in CNS[15-17]. The present study indicates that fluoxetine and pargyline could potentiate the antinociceptive action of 5-HTP. These data confirm and extend that 5-HTP exerts antinociceptive effects mediated by central serotonergic system. In view of certain drugs antinociception upon motor behavior in the mouse hot-plate test, the relationship between antinociceptive and motor properties is a critical factor in assessing the effects of drugs on nociceptive transmission[18]. Serotonin has been shown to influence a broad range of physiological systems, such as cardiovascular regulation, respiration and thermoregulation, and a variety of behavioral functions, including circadian rhythm entrainment, sleep-wake cycle,

appetite, aggression, sexual behavior, sensorimotor reactivity, pain sensitivity, and learning. In general, serotonin is considered as an inhibitor of behavioral responding in modulating motor behavior[19]. In this study, we investigated the effects of 5-HTP (20-80 mg/kg, ip) on the spontaneous locomotor activity in mice. Our data indicated that 5-HTP administered systemically did not affect motor activity in mice at the same doses of 5-HTP-induced antinociception, which provided strong evidence that the antinociceptive action of 5-HTP could not be attributed to a mechanism underlying non-specific suppression in CNS.

Serotonin distributes widely in the spinal cord, brain, and even the peripheral tissues. 5-HTP administered systemically can pass the blood brain barrier into the brain and is converted to 5-HT in the brain, the spinal cord, and the periphery[4-7]. There are differences concerning the effects of 5-HT on nociceptive transmission in the peripheral, spinal, and supraspinal levels. 5-HT in the peripheral tissues can stimulate the nerve terminals to facilitate the nociceptive transmission, whereas increasing 5-HT levels in the brain can produce the antinociceptive action. In the spinal cord, there are conflicting findings regarding the involvement of 5-HT in the modulation of nociception. Intrathecal administration of 5-HT has been reported to facilitate or to inhibit the transmission of the nociceptive impulse[2,3]. In the present study, ip injection of 5-HTP may affect 5-HT levels in spinal, supraspinal centers, as well as peripheral tissues. Our data indicated the inhibitory effects of 5-HTP on nociceptive response in the mouse hot-plate test, which was dependent upon the total effects of 5-HT on the nociceptive transmission pathway at peripheral, spinal, and supraspinal levels.

There are more than 14 molecularly identified 5-HT receptor subtypes, related by amino acid sequence, pharmacology and intracellular mechanisms[20]. Diversity of 5-HT receptors provides the organism with flexibility in the response to 5-HT and facilitates adaptability during physiological and environmental challenge[21]. A similar degree of differences exists regarding the possible role played by various 5-HT receptor subtypes in nociceptive transmission. In our recent study[10], systemic (ip) administration of buspirone, a partial 5-HT_{1A} receptor agonist, produces a dose-dependent antinociceptive effect. However, it has been reported that activation of the 5-HT_2 receptors may facilitate the nociceptive transmission[22]. In the case of 5-HT_3 receptors, some studies have shown that intrathecal injection of 5-HT_3 receptor agonist has a pro-nociceptive effect[23,24], whereas Xu *et al*[25] has failed to demonstrate 5-HT_3 receptor-mediated control of nociception. It has long been known that the involvement of 5-HT in the modulation of nociception is the non-selective actions of 5-HT at multiple receptors. In all probability, systemic administration (ip) of 5-HTP that can increase the 5-HT levels in the spinal cord and the brain results in the inhibition of nociceptive response in the mouse hot-plate test. These alterations may be attributed to the integration of activation of various 5-HT receptor subtypes, which produces antinociceptive effects induced by 5-HTP administered systemically.

Numerous studies have demonstrated that Ca^{2+} channel currents may be potently inhibited by 5-HT and there is the functional coupling of 5-HT receptors such as 5-HT_{1A} to various types of Ca^{2+} channels, including N-, P/Q-, T-, or L-type, in neurons[10,12,13]. The intracellular mechanisms underlying 5-HTP-induced antinociception are unclear. The cellular actions of serotonin have been hypothesized to be associated with the Ca^{2+} levels inside the neurons. In the experiments, nimodipine (2.5-10 mg/kg), nifedipine (2.5-10 mg/kg), and verapamil (2.5-10 mg/kg), at the doses that had no effects in the mouse hot-plate test, could potentiate the antinociceptive effects induced by 5-HTP administered ip. Our data firstly showed that L-type Ca^{2+} channels might be involved in 5-HTP-induced antinociception. One possibility was that ip injection of 5-HTP might increase 5-HT levels outside neurons in CNS, and then the combination of serotonin and L-type Ca^{2+} channel blockers would further decrease the neuronal Ca^{2+} levels and thus enhance 5-HTP-induced antinociception.

The most important results in our study were that Ca^{2+} (5-20 mmol/L) injected icv dose-dependently reversed antinociception by ip administration of 5-HTP (70 mg/kg) in the mouse hot-plate test, whereas 5-HTP antinociception was potentiated when EGTA (0.5-30 mmol/L, icv) and 5-HTP (30 mg/kg, ip) were given parentally in mice. Nociception has been hypothesized to be related to the Ca^{2+} levels inside the neurons. It has been reported that the antinociceptive action of buspirone, a partial 5-HT_{A1} agonist, is antagonized by the decrease and potentiated by the increase in the neuronal Ca^{2+} [10]. Therefore, we hypothesized that there might be interaction between 5-HTP-induced antinociception and cytosolic Ca^{2+} in the CNS. Several lines of evidence indicate that, in contrast to the effects produced by the icv injection of Ca^{2+} chelators, Ca^{2+} injected icv may

directly increase synaptosomal Ca^{2+} levels and act within neurons[26,27]. Raising the extracellular Ca^{2+} levels in lateral ventricles of the brain and consequent increment of Ca^{2+} inside neurons may completely block 5-HTP-induced antinociception. Similar to L-type Ca^{2+} blockers, egtazic acid, when administered icv, may reduce intracellular Ca^{2+} availability, resulting in potentiating antinociceptive effects injected ip with 5-HTP. It has been reported that the regulation of intracellular Ca^{2+} levels is related to Ca^{2+}-induced Ca^{2+} release from Ca^{2+} microsomal pools[28]. Thus, it is likely that the reversal of Ca^{2+} injected icv on 5-HTP-induced antinociception, in part, may be due to the amplification of cytosolic Ca^{2+} levels as a result of Ca^{2+}-induced Ca^{2+} release from Ca^{2+} microsomal pools. Certainly, the effects of the release from intracellular Ca^{2+} pools on 5-HTP-induced antinociception need further investigation.

In summary, we have shown that systemic administration of 5-HTP may produce significant antinociceptive effects in the mouse hot-plate test, which is related to central serotonergic system. Of all in the present study, the most important findings indicate that there is positively synergistic interaction on 5-HTP-induced antinociception between 5-HTP and L-type Ca^{2+} channel blockers, including nimodipine, nifedipine, and verapamil. It is very interesting that the antinociceptive action of 5-HTP may be antagonized by central administration of Ca^{2+} and potentiated by the selective Ca^{2+} chelator egtazic acid. Therefore, our results firstly provide the strong evidence showing that 5-HTP-induced antinociception may be associated with Ca^{2+} influx from extracellular fluid through L-type Ca^{2+} channels.

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