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Research report

N-Methyl-D-aspartate receptor antagonist D-AP5 prevents pertussis toxin-induced alterations in rat spinal cords by inhibiting increase in concentrations of spinal CSF excitatory amino acids and downregulation of glutamate transporters

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ABSTRACT

Recently, we found that intrathecal (i.t.) pertussis toxin (PTX) injection produces thermal hyperalgesia and is associated with increasing concentrations of excitatory amino acids (EAAs) in spinal cerebrospinal fluid (CSF) dialysates; a reduction in the antinociceptive effects of morphine and glutamate transporters (GTs) was also observed. The reduction in the morphine-induced analgesic effects is directly related to increased extracellular EAA levels, which are maintained by GTs at physiological levels. In this study, we aimed to examine the role of GT isoforms in thermal hyperalgesia, determine the EAA concentrations in CSF dialysates, and elucidate the role of *N*-methyl-D-aspartate (NMDA) receptors in PTX-induced reduction in the antinociceptive effects of morphine. Two i.t. catheters and one microdialysis probe were inserted into male Wistar rats: one catheter was used for PTX (1 μg) and morphine (10 μg) injection and the other was connected to an osmotic pump for NMDA receptor antagonist D-2-amino-5-phosphonopentanoic acid (D-AP5; 2 μg/h for 4 days) continuous infusion. The microdialysis probe was used to collect CSF dialysates for EAA measurements by high-performance liquid chromatography. Intrathecal morphine failed to produce antinociceptive effects in PTX-treated rats, and D-AP5 coinfusion prevented the PTX-induced reduction in the antinociceptive effect and associated downregulation of the GTs. We conclude that NMDA receptor suppression inhibits EAA excitation and reduces the morphine-induced antinociception in PTX-treated rats.

1. Introduction

Pertussis toxin (PTX) treatment results in adenosine diphosphate (ADP) ribosylation of the α-subunit of inhibitory guanine nucleotide-binding regulatory proteins (G_i/G_o), thus disrupting inhibitory G-protein-mediated signal-transduction [5,17]. PTX-sensitive G-protein-coupled receptors, including opioid, α_2-adrenergic, gamma-aminobutyric acid (GABA), and A_1-adenosine receptors, are involved in antinociceptive signaling. Womer et al. [47] and McCormack et al. [26] suggested that intrathecal (i.t.) injection of PTX may be used as a model for studying the central mechanisms of neuropathic pain. Numerous studies have also suggested that the development of the neuropathic pain syndrome may involve increased release of excitatory amino acids (EAAs) and the subsequent activation of EAA receptors in the spinal cord [11,29,50].

Similar to the phenomena of morphine tolerance, i.t. PTX-induced neuropathic pain syndromes are also resistant to treatment with opioids [49]. In our previous studies, we found that i.t. injection of PTX attenuates the antinociceptive effects of opioids and produces thermal hyperalgesia; a concomitant increase was observed in the EAA levels in spinal cerebrospinal fluid (CSF) dialysates [44,46,49]. Moreover, increased expression of protein kinase C-gamma (PKCγ) in the dorsal horn of the lumbar spinal cord was also observed; this effect was significantly blocked by *N*-methyl-D-aspartate (NMDA) receptor antagonists [46]. In the same study,

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we also found that pretreatment with a PKC inhibitor markedly inhibits morphine-induced spinal EAA release in PTX-treated rats. NMDA receptor antagonists inhibit morphine tolerance in rats [22,39,48]. The EAA signaling cascade plays an important role in reducing the morphine-induced antinociceptive effects. However, the mechanism by which NMDA receptors cause PTX-induced thermal hyperalgesia and reduce morphine's antinociceptive potency is not clear.

The EAA glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) and plays an important role in regulating morphine-induced antinociceptive effects and nociceptive sensitization. Under normal neurophysiological conditions, the extracellular concentration of glutamate in the synapse needs to be maintained at an appropriate level (<1 μM) for ensuring a high signal-to-noise ratio during synaptic signaling and neuron protection [8]. Therefore, under neuropathological conditions, large-scale glutamate removal is essential for maintaining functional communication between neurons and preventing the glutamate concentration from reaching toxic levels. Five distinct eukaryotic high-affinity glutamate transporters (GTs) have been cloned, three of which have been identified in the mammalian CNS: Glu-Asp transporter (GLAST), Glu transporter-1 (GLT-1), and neuronal transporter excitatory amino acid carrier 1 (EAAT1) [4,13,20,30–32]. Mao et al. [23] and Sung et al. [37] have demonstrated that downregulation of spinal GTs (GLAST, GLT-1, and EAAC1) plays an important role in extracellular glutamate homeostasis in morphine-tolerant rats and neuropathic rats, respectively. Many neuropathological disorders are associated with a change in the localization and/or expression of GTs [27,35]. We found increased EAA levels in the spinal CSF of PTX-treated rats accompanied with thermal hyperalgesia and a reduction in the morphine-induced analgesic effects [44,45]. Moreover, Tsai et al. [40] and Lin et al. [21] also demonstrated that i.t. PTX induces downregulation of GT protein expression in rat spinal cord. In the present study, we attempted to prove the hypothesis that NMDA receptor antagonists inhibit PTX-induced GT downregulation, which in turn prevents the reduction in morphine-induced antinociceptive effects.

2. Materials and methods

2.1. Intrathecal catheter and microdialysis probe implantation

Male Wistar rats (400–420 g) were used in this study. As described previously [45], under isoflurane (2%) anesthesia, two i.t. catheters (PE_5 tube: 9 cm, 0.008-in. inner diameter, 0.014-in. outer diameter; Spectranetics, Colorado Springs, CO, USA) and a microdialysis probe were simultaneously inserted via the atlantooccipital membrane into the i.t. space to the level of the lumbar enlargement of the spinal cord, and externalized and fixed to the cranial aspect of the head [45]. The rats were then returned to their home cages for a 4-day recovery period; each rat was housed individually and maintained on a 12-h light/dark cycle with food and water freely available. Rats were excluded from the study if they showed evidence of gross neurological injury or the presence of fresh blood in the CSF. The use of animals conformed to the Guiding Principles in the Care and Use of Animals of the American Physiology Society and was approved by the National Sun Yat-sen University and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

2.2. Construction of the microdialysis probe

The microdialysis probe was constructed as described previously [24,45], using two 7-cm PE_5 tubes (0.008-in. inner diameter, 0.014-in. outer diameter) and a 4-cm cuprophan hollow fiber (300 μm outer diameter, 200 μm inner diameter, 50 kDa molecular weight cut-off; DM-22, Eicom Co., Kyoto, Japan). To make the probe sufficiently firm for implantation, a Nichrome-Formavar wire (0.0026 in.; A-M system, Everret, Inc., WA, USA) was passed through a polycarbonate tube (194 μm outer diameter, 102 μm inner diameter; 0.7 cm in length) and the cuprophan hollow fiber (active dialysis region), and connected to a PE_5 catheter with epoxy glue. The fiber was then bent in the midsection of the cuprophan hollow fiber, forming a "U"-shaped loop. The two ends of the dialysis fiber, consisting of silastic tubes, were sealed with silicon sealant. The dead space of the dialysis probe was 8 μL. During in vitro measurements, the recovery rate of the dialysis probe was 39.5 ± 4.32% (mean ± S.D.) at an infusion rate of 5 μL/min.

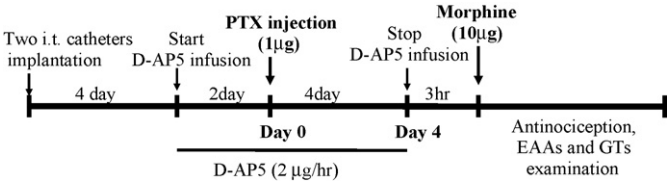

Fig. 1. Time course of the study. Two intrathecal catheters and one microdialysis probe were implanted in the rats. After a 4-day recovery, one catheter was used to infuse D-AP5 (2 μg/h) or saline (1 μL/h); 48 h later (day 0), PTX (2 μg) or saline was injected via the other catheter. On day 4 after PTX injection, morphine was intrathecally injected 3 h after stopping D-AP5 infusion.

2.3. Intrathecal PTX injection and antinociceptive test

Four days after i.t. catheterization, one catheter was connected to a mini-osmotic pump (model 2001; Alzet, Palo Alto, CA, USA) and saline or D-AP5 (2 μg/μL) were continuously infused for the rest of the experimental period at a rate of 1 μL/h. Forty-eight hours later, saline or PTX (2 μg, Calbiochem-Novabiochem International, San Diego, CA, USA) was injected via the other catheter, followed by a flush with 10 μL of saline. The dose of D-AP5 (2 μg/μL) was used similar to our previous study [46]. On day 4 (after PTX injection), the rats were placed in plastic restrainers for morphine injection and antinociceptive examination. Intrathecal morphine (10 μg) was injected 3 h after the discontinuation of i.t. D-AP5 or saline infusion by the other catheter. The antinociceptive test, i.e., tail-flick latency, was measured by the hot water immersion test (52 ± 0.5 °C) at 30, 60, 90, and 180 min after i.t. morphine injection. The cut-off time was set at 10 s to prevent tissue injury. Each tail-flick latency was an average of three measurements over a 6-min testing period. The experimental protocol is summarized in Fig. 1.

2.4. CSF sampling and excitatory amino acid measurement

After the 4-day recovery period, one end of the externalized microdialysis probe was connected to a syringe pump (KD Scientific, MA, USA) for cerebrospinal fluid (CSF) sampling in plastic restrainers. The dialysis system was perfused with artificial CSF, consisting of 151.1 mM Na^+, 2.6 mM K^+, 122.7 mM Cl^-, 21.0 mM HCO_3^-, 0.9 mM Mg^{2+}, 1.3 mM Ca^{2+}, 2.5 mM $HPO4^{2-}$, and 3.5 mM dextrose, bubbled with 5% CO_2 in 95% O_2 to adjust the pH to 7.3. CSF samples were collected using a standard procedure of a 30-min washout period, followed by a 30-min sample collection period at a flow rate of 5 μL/min. On day 4 (after PTX or saline injection), 2.5 h after discontinuation of i.t. D-AP5 infusion, basal CSF dialysate was collected. After basal CSF dialysate collection, rats were administered an i.t. injection of morphine (10 μg), and CSF dialysates were continuously collected in polypropylene tubes on ice every 30 min for 180 min and stored at −80 °C until assayed. Concentrations of EAAs were analyzed by high-performance liquid chromatography (Agilent 1200 HPLC System, Palo Alto, CA, USA) using a fluorescence detector (Ex = 340 nm, Em = 450 nm) as described in the Agilent method. In brief, amino acids were assayed by precolumn derivatization with *o*-phthalaldehyde/*t*-butylthiol (OPA) reagent by a robotic autosampler. Derivatization was performed by adding 0.5 μL of OPA reagent (10 mg/mL, Fluka Chemical Co., Buchs, Switzerland) to 0.5 μL of sample with 2.5 μL borate buffer (0.4N, pH 10.2, Fluka Chemical Co. Buchs, Switzerland), shaking the mixture, then allowing it to react for 1 min. Then, 3.5 μL of the derivatized sample was injected onto a reverse-phase Agilent Zorbax Eclipse-AAA column (4.6 mm × 150 mm, 3.5 μm) and eluted at a flow rate of 2 mL/min at 40 °C. A linear gradient from 100% eluent A [40 mM Na_2HPO_4 adjusted to pH 7.8 with NaOH] to 100% eluent B [acetonitrile/methanol/water (45/45/10 v/v/v)] was used to separate the amino acids. The separation was obtained at a flow rate of 2 mL/min with a gradient program that allowed for 1.9 min at 0% B followed by a 16.3-min step that raised eluent B to 53%. Then, washing at 100% B and equilibration at 0% B was performed in a total analysis time of 26 min. All solvents were vacuum-filtered through a 0.22 μm membrane (Millipore) and degassed by sonication before use. External standard solutions containing 0, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M standard amino acids were run before and after each sample group. The percentage change relative to the basal EAA release was calculated as the area under the curve (AUC) from 0 to 180 min after morphine injection, which was derived from the original data by the trapezoidal method [33].

2.5. Spinal cord preparation and Western blot analysis

After the experiment, the rats were rapidly decapitated and the dorsal portion of the enlarged portion of the lumbar spinal cord was removed and stored at −80 °C until it was used for Western blotting. Western blotting of spinal GTs protein expression was performed as in our previous study [45]. The dorsal portions of the spinal cord were homogenized in an ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2% Triton X-100, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin), and then centrifuged at 68,000 rpm (TXL-100, BEKMAN) for 30 min at 4 °C. The supernatant was decanted from the pellet and retained for Western blot analysis. Protein concentrations were determined by the DC protein Assay kit (Bio-Rad, Hercules, CA, USA). An equal volume of sample buffer (2% SDS, 10% glycerol, 0.1% bromophenol