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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\section*{A B S T R A C T}

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 \textmu g) and morphine (10 \textmu g) injection and the other was connected to an osmotic pump for NMDA receptor antagonist D-2-amino-5-phosphonopentanoic acid (D-AP5; 2 \textmu g/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 failure 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.

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\section*{1. Introduction}

Pertussis toxin (PTX) treatment results in adenosine diphosphate (ADP) ribosylation of the \textalpha; subunit of inhibitory guanine nucleotide-binding regulatory proteins (G\textalpha;/G\textbeta;), thus disrupting inhibitory G-protein-mediated signal-transduction [5,17]. PTX-sensitive G-protein-coupled receptors, including opioid, \textalpha;2- adrenergic, gamma-amino butyric acid (GABA), and A1-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 (PKC\textgamma;) 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,
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) [14,13,20,30–32]. Mao et al. [23] and Sung et al. [37] have demonstrated that downregulation of spinal GTs (GLAST, GLT-1, and EAAT1) 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 (PE1, 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 l.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 Physiologv 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 sufferance.

2.2. Construction of the microdialysis probe

The microdialysis probe was constructed as described previously [24,45], using two 7-cm PE tubes (0.008-in. inner diameter, 0.014-in. outer diameter) and a 4-cm cuprophan hollow fiber (360 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-Formvar wire (0.0026 in.; A-System, Everett, Inc., WA, USA) was passed through a polycarbonate tube (194 μm outer diameter, 120 μm inner diameter; 0.7 cm in length) and the cuprophan hollow fiber (active dialysis region), and connected to a PE catheter with epoxy glue. The fiber was then bent in the midsection of the cuprophan hollow fiber, forming a “U”-shaped loop. 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/μL) 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) was 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 (52.5 ± 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–, 210 mM HCO3–, 0.9 mM Mg2+, 1.3 mM Ca2+, 2.5 mM HPO42–, and 3.5 mM dextrose, bubbled with 5% CO2 in 95% O2 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/d- butyrylthiol (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-AA column (4.5 μm = 150 mm, 35 μm) and eluted at a flow rate of 2 mL/min at 40 °C. A linear gradient from 100% eluent A [40 mM NaHPO4 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, 10−6, 10−7, 10−8, and 10−9 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 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 GT 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, 25 mM Trition 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
blue, 2% 2-mercaptoethanol, and 50 mM Tris–HCl, pH 7.2) was added to the sample (100 µg), which was then loaded onto a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and electrophoresis was performed at 150 V for 60 min. The proteins were transferred to a polyvinylene difluoride membrane (Immobilon-P, Millipore, 0.45 µM pore size) at 135 mA overnight at 4°C in transfer buffer (50 mM Tris–HCl, 380 mM glycine, 1% SDS, and 20% methanol). Then, the membrane was blocked for 50 min at room temperature with 5% non-fat dry milk (TTBS) and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (1:1000 dilution; Chemicon, Temecula, CA, USA), EAAC1 (EAT3, 1:1000 dilution; BD Transduction Laboratories, San Jose, CA, USA). It was then washed three times in TTBS for 10 min, blocked with 5% non-fat dry milk/TTBS, and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (dilution 1:2000). The GLAST and GLT-1 antibody, at a band at 65 kDa; the GLT-1 antibody, at a band at ~70 kDa; and the EAAC1 antibody, at a band at ~70 kDa. Immunoblotting was performed using appropriate antibodies followed by secondary horseradish-peroxidase-conjugated antibody. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL kit; Millipore, Bedford, MA, USA). Images were visualized with the UVP BioChem Imaging System, and relative quantification by densitometry was performed with LabWorks 4.0 software (UVP, Upland, CA). β-Actin (A-5441, Sigma) was used as an internal control for protein loading, and data are expressed as a ratio of the protein of interest to β-actin.

3. Statistical analysis

All data are presented as the mean ± S.E.M. For statistical analysis, all data were analyzed by one-way analysis of variance (ANOVA), followed by Student–Newman–Keul’s post-hoc test for multiple comparisons. For the immunoreactivity data, the intensity of each test band was expressed as the relative optical density (ROD) calculated from the average control optical density value for all the control X-ray films. A significant difference was defined as a P value <0.05.

4. Results

The mean tail-flick latency of naïve rats was approximately 2.3 ± 0.25 s in the hot water immersion test (52 ± 0.5°C). No significant differences in the basal tail-flick latency (before drug injection) were observed among the experimental groups (Table 1). The time course of the study is shown in Fig. 1. On day 4, i.t. PTX (1 µg) injection resulted in a slight, but not significant, decrease in the nociceptive threshold. In other words, the PTX + Δ-AP5 and Δ-AP5 treatment produced a significant antinociceptive effect on day 4, in which the tail-flick latency returned to baseline after Δ-AP5 infusion was stopped for 3 h (Table 1). Intrathecally morphine (10 µg) injection produced a significant antinociceptive effect in the control group but not in the PTX (1 µg) treatment group. The PTX-induced reduction in morphine analgesia was inhibited by i.t. Δ-AP5 (2 µg/h) infusion for 6 days (2 days before and 4 days after PTX treatment; Fig. 2).

As in our previous study, acute i.t. morphine (10 µg) injection produced a significant increase in aspartate (Fig. 3A and B) and glutamate (Fig. 3C and D) concentrations in the CSF (CSF) dialysates of the PTX-treated rats; in contrast, Δ-AP5 (2 µg/h) infusion inhibited the morphine-induced increase in the amino acid concentrations (Fig. 3). Western blot analysis of EAAC1, GLAST, and GLT-1 per-formed 4 days after the i.t. PTX injection showed a significant downregulation of the GTs in the dorsal part of the lumbar spinal cord of the PTX-treated rats (Fig. 4). Downregulation of GLAST and EAAC1 was prevented by i.t. Δ-AP5 (2 µg/h) infusion for 6 days (2 days before and 4 days after PTX injection; Fig. 4). Intrathecal Δ-AP5 infusion alone had no effect on the expression of GLAST, GLT-1, or EAAC1 in the naïve rat spinal cord.

5. Discussion

In this study, we demonstrated that reduction in the antinociceptive effects of morphine is associated with downregulation of GTs and increase in EAA levels in spinal CSF dialysates of PTX-treated rats. Intrathecal PTX induced the downregulation of GLT-1, GLAST, and EAAC1 in the rat spinal cord; these effects were almost completely inhibited by Δ-AP5 infusion before PTX treatment. Our previous study showed that i.t. Δ-AP5 injection significantly inhibits PTX-induced thermal hyperalgesia [44]. The present findings support the observation that GT downregulation retards synaptic EAA reuptake, resulting in EAA accumulation and subsequent thermal hyperalgesia due to reduction in morphine-induced antinociceptive effects in PTX-treated rats.

Both neuropathic pain and morphine tolerance show common features of nociceptive sensitization and morphine resistance [3,18,19]. Numerous studies have indicated that spinal EAAs play
Fig. 3. Effect of d-AP5 on the morphine-evoked increase in the levels of spinal aspartate (A and B) and glutamate (C and D) in PTX-treated rats. On day 4 after intrathecal injection of either PTX (2 μg) or saline, a morphine challenge (10 μg, intrathecal) was administered 3 h after discontinuing d-AP5 infusion. The basal spinal CSF dialysate was obtained just before the morphine challenge and was expressed as 100%. Four other dialysate samples were collected at 30, 60, 90, and 180 min after the morphine challenge. The area under the curve (AUC; 0–180 min) obtained using the data from A and C was expressed as the AUC (percent change from baseline × min). The data are presented as mean ± standard error of the mean for the number of rats indicated in A and C. # P < 0.05 compared with the PTX group; * P < 0.05 compared with the control group.

Fig. 4. d-AP5 prevents PTX-induced GT downregulation. Western blotting of GLT-1, GLAST, and EAAC1 from the dorsal lumbosacral segment of rat spinal cords in the control, PTX, PTX + d-AP5, and d-AP5 groups (A). Densitometric quantification of the western blotting results for GLT-1 (B), GLAST (C), and EAAC1 (D). The optical density of each protein band was quantified by densitometry and the relative optical density was calculated using the average value of the samples from six rats. The GT/β-actin density ratio of the control band was considered as 100%. Values are expressed as mean ± standard error of the mean. * P < 0.05 compared with the control group; # P < 0.05 compared with the PTX group.
an important role in the development of morphine tolerance and neuropathic pain [7,18,22,25]. Many previous studies have also indicated that the development of neuropathic pain involves increased EAA release and subsequent activation of EAA receptors in the dorsal horn of the spinal cord [11,29,50]. Intrathecal injection of EAA receptor agonists induces allodynia and hyperalgesia, similar to the symptoms seen in patients with neuropathic pain [1,51]. Moreover, our previous studies demonstrated that intrathecal PTX injection not only attenuates the antinociceptive effects of opioid-receptor agonists but also produces thermal hyperalgesia [44,49]. In the present study, 4 days after PTX injection, an increase in the glutamate and aspartate levels was observed in the CSF dialysate following the morphine challenge, with decreased antinociceptive effects of morphine; moreover, these effects could be prevented by d-AP5 infusion.

Durcan and Morgan [12] and Huston et al. [16] have demonstrated that PTX not only increases glutamate release but also enhances NMDA receptor sensitivity. EAA receptors, especially NMDA receptors, can be activated by extracellular EAA. PTX increases the release of EAAAs from neuronal (cerebellar granule neurons and dorsal root ganglion) and non-neuronal cells (chromaffin cells) by modulation of Ca2+ channel activity [9,10,16,24]. Activation of Ca2+ channels results in increased intracellular Ca2+, in turn increasing Ca2+-dependent glutamate exocytosis [34]. The increasing intracellular Ca2+ level, due to NMDA receptor activation, can also lead to the production of second messengers such as IP3 and DAG, which stimulate PKC [2,42]. Urban et al. [41] found that PKC-mediated NMDA receptor phosphorylation enhances NMDA receptor activity, subsequently increasing EAA release and producing a positive feedback to the nociceptive sensitization. Our previous study indicated that i.t. PTX-induced thermal hyperalgesia is accompanied by PKC activation in the rat spinal cord and that both these effects are inhibited by d-AP5 [44]. Moreover, PKC activation inhibits GT activity and protein expression [14,15,36,38,43]. Papp et al. [28] also indicated that NMDA receptor hypofunction may have beneficial therapeutic effects on neurological disorders characterized by impaired NMDA receptor-mediated transmission. Therefore, we suggest that d-AP5 may prevent the PTX-induced NMDA–PKC signal-transduction cascade, which might play a critical role in neuropathological changes and GT downregulation in PTX-treated rats.

PTX is believed to cause ADP ribosylation of the α-subunit of G-protein (Gαi5/Gi6) and disrupts Gαi5/Gi6–protein–coupled receptor signal-transduction. According to Crain and Shen’s theory [6,7], opioid receptors can be activated via either the inhibitory Gαi5/Gi6 protein or stimulatory Gs–proteins. Intrathecal administration of PTX may disrupt the balance between the actions of the opioid-receptor-coupled inhibitory and excitatory G-proteins, by which the stimulatory Gs–protein signal-transduction pathway becomes prominent. Thus, in PTX-treated rats, morphine administration activates the Gs-coupled opioid-receptor signal-transduction pathway, resulting in increased EAA release and thus causing loss of morphine-induced analgesic effects. Moreover, in our previous and present observations, NMDA receptor antagonists not only prevented the reduction in morphine-induced analgesia but also blocked the morphine-evoked increase in spinal CSF EAAAs in morphine-tolerant rats [45] and PTX-treated rats. We suggest that this morphine–evoked increase in the levels of spinal EAAAs may activate NMDA receptors, enhancing nociceptive transmission and thus attenuating the analgesic effects of morphine. Therefore, activation of the glutamatergic receptor system by PTX treatment plays an important role in the reduction of these analgesic effects. The proposed role of NMDA receptors in PTX–induced alternations of the EAA concentrations induced by morphine is depicted in Fig. 5.

In conclusion, except the disruption of inhibitory G-protein-coupled opioid-receptor signal-transduction, i.e. PTX treatment induces an increase in the spinal EAA concentrations, probably via the downregulation of GTs (GLAST, EAAC1, and GLT-1). Intrathecal PTX injection also induces a reduction in the analgesic effects of morphine. d-AP5 infusion not only attenuates the reduction in morphine-induced antinociceptive effects but also blocks morphine-evoked EAA accumulation and GT downregulation on PTX treatment. Our present study provides evidence that NMDA receptor suppression restores the antinociceptive effects of morphine by inhibiting the release of EAAs and downregulation of GTs in PTX-treated rats.

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