DEXAMETHASONE MODULATES THE DEVELOPMENT OF MORPHINE TOLERANCE AND EXPRESSION OF GLUTAMATE TRANSPORTERS IN RATS

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Abstract—We recently demonstrated an increase in spinal cerebrospinal fluid (CSF) excitatory amino acids (EAA)s in morphine-tolerant rats after morphine challenge. The present study examined whether co-infusion of the glucocorticoid dexamethasone (DEX) co-infusion inhibited morphine tolerance and the morphine challenge-induced EAA increase after long-term morphine infusion. Intrathecal (i.t.) catheters and one microdialysis probe were implanted to male Wistar rats. Rats were divided into four groups: i.t. morphine (15 μg/h), saline (1 μl/h), DEX (2 μg/h), or DEX (2 μg/h) plus morphine (15 μg/h) infusion for 5 days. Tail-flick responses were examined before drug infusion and daily after the start of infusion for 5 days. Moreover, on day 5 after morphine challenge (50 μg, i.t.), CSF EAA s was also measured. Rat spinal cords were removed on day 5, and prepared for Western blot analysis of different glutamate transporters (GTs). The AD50 (analgesic dose) on day 5 was 1.33 μg in saline-infused rats, 83.8 μg in morphine-tolerant rats, and 10.15 μg in DEX plus morphine co-infused rats. Single DEX (2 μg, i.t.) injection did not enhance morphine's antinociceptive effect in either naive or morphine-tolerant rats. No difference in CSF EAA level was observed in all groups between baseline (before drug infusion) and on day 5 after tolerance developed. Surprisingly, on day 5, after morphine challenge, an increase in glutamate and aspartate (284 ± 47% and 201 ± 18% of basal) concentration was observed, and morphine lost its antinociceptive effect (maximum percent effect, MPE=41±12%), whereas DEX/morphine co-infusion inhibited morphine-evoked EAA increase with a MPE=97±2%. DEX co-infusion prevented the down-regulation of glial glutamate transporters (GLAST (Glut-Asp transporter) and GLT-1 (Glut transporter-1)), but not the neuronal GT EAAC1 (excitatory amino acid carrier). Ureapulation of GLT-1 was also observed (204 ± 20% of basal). DEX co-infusion inhibits the morphine-challenge induced EAA increase and prevents the loss of morphine’s antinociceptive effect after long-term morphine infusion. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: excitatory amino acids, microdialysis, morphine tolerance, NMDA receptor, glutamate transporters.

Opioids are the therapeutically mainstay in clinical pain management; however, long-term administration leads to tolerance. In clinical practice, tolerance is still a concern issue, though it is not always observed during pain management. Colpaert et al. (1980) demonstrated that noxious mechanical stimuli blocked the development of opioids tolerance. Similar finding was reported by Vaccarino et al. (1993); they also failed to observe morphine tolerance rats in the presence of formalin-induced pain. Adrenocorticotropin hormone (ACTH) had been found to prevent morphine tolerance (Hendrie, 1998). Furthermore, Vaccarino and Couret (1995) suggested that blockade of morphine tolerance by the presence of formalin-induced pain might via activation of the hypothalamic–pituitary–adrenal (HPA) axis. They also found that the formalin-induced pain prevented the antinociceptive tolerance of morphine in Fischer rats (with normal HPA axis response), but not Lewis rats that lack a stress-activated HPA axis. Budziszewska et al. (1995) further demonstrated that repeated morphine administration down-regulated glucocorticoid, but not mineralocorticoid, receptors in the rat hippocampus. By using corticosterone synthesis inhibitor metyrapone, Vaccarino and colleagues (1997) further demonstrated that glucocorticoid prevented the antinociceptive tolerance of morphine in formalin-pain animal model.

The actions of opioids are mediated and modulated by a complex group of receptors. We previously demonstrated that, in addition to opioid receptor uncoupling and down-regulation (Wong et al., 1992a,b), N-methyl-D-aspartate acid (NMDA) receptors are also involved in the development of opioid tolerance (Trujillo and Akil, 1991; Wong et al., 1996; Hsu and Wong, 2000). In a rat spinal model, we found that both competitive and non-competitive NMDA receptor antagonists inhibit morphine tolerance and prevent the reduction of μ-opioid receptor high-affinity sites in continuously morphine infused rats (Wong et al., 1996). Furthermore, in morphine-tolerant rats, we observed an increase in [3H] MK-801 binding affinity (Wong et al., 2000a). In clinical cancer pain patients receiving long-term intrathecal (i.t.) morphine for pain relief, we observed an increase in the levels of glutamate and aspartate in the cerebrospinal fluid (CSF) which was accompanied by a

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Abbreviations: AD50, analgesic dose for 50%; CSF, cerebrospinal fluid; DEX, dexamethasone; EAAs, excitatory amino acids; EAAC1, excitatory amino acid carrier; GLAST, Glu-Asp transporter; GLT-1, Glu transporter-1; GTs, glutamate transporters; HPA, hypothalamic–pituitary–adrenal; i.t., intrathecal; MPE, maximum percent effect; NMDA, N-methyl-D-aspartate acid; OPA, o-phthalaldehyde–l-butythiol; TTBS, 0.1% Tween 20 in 20 mM Tris-HCl, 137 mM NaCl, pH 7.4.
loss of the analgesic effect of morphine (Wong et al., 2002). Similarly, we also recently demonstrated an increase in CSF excitatory amino acid (EAA) levels of morphine-tolerant rats after morphine challenge (Wen et al., 2004). Thus, activation of EAA, particularly the NMDA receptors by the increased EAA in synapse is implicated as one of the mechanisms of opioid tolerance.

As there are no enzymes that extracellularly metabolizing glutamate, it must be removed from the synaptic cleft by cellular uptake system. Glutamate transporters (GTs) are responsible for maintaining the extracellular glutamate concentration at physiological level, thus preventing neuronal excitotoxicity and neuroplasticity (Danbolt, 2001). Five GTs have been cloned which consist of two neuronal transporters, excitatory amino acid carrier (EAAC1) and excitatory amino acid transporter (EAAT4), two glial transporters, Glu-Asp transporter (GLAST) and Glu transporter-1 (GLT-1), and retinal transporter, EAAT5 (Danbolt, 2001 for review). Mao et al. (2002) demonstrated that morphine tolerance is accompanied by downregulation of GTs in dorsal horn of the spinal cord, while Nakagawa et al. (2001) found that increasing of GT activity attenuates morphine tolerance. Dexamethasone (DEX) was reported to enhance glutamate uptake in rat cerebral cortex synaptosomes and neuroblastoma SH-N-SH cells (Zhu et al., 1998). Furthermore, Rauen and Wiessner (2000) demonstrated that cortisol induces upregulation of GLAST in glial cells. These findings suggested that modulation of GT function by glucocorticoids may play an important role in morphine tolerance development. In the present study, we examined the cellular mechanisms of glucocorticoid DEX in modulating morphine tolerance development and GTs expression in the spinal cord.

**EXPERIMENTAL PROCEDURES**

**Implantation of i.t. catheters and microdialysis probe**

Male Wistar rats (400–450 g) were used. As in our previous study (Wen et al., 2004), one i.t. catheter and a microdialysis probe were inserted via the atlantooccipital membrane into the i.t. space at the level of the lumbar enlargement of the spinal cord and externalized and fixed to the cranial aspect of the head. 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. All experiments conformed to the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiology Society and were approved by the National Defense Medical Center Animal Care and Use Committee. Efforts were made to minimize the number of animals used.

**Construction of the microdialysis probe**

The microdialysis probe was constructed as described previously (Marsala et al., 1995; Wen et al., 2003a), using two 7 cm PE5 tubes (0.008 inch inner diameter, 0.014 inch outer diameter) and a 4 cm cuprophan hollow fiber (300 mm outer diameter, 200 mm inner diameter, 50 kDa molecular weight cutoff; DM-22, Eicom Co., Kyoto, Japan). To make the probe firm enough for implantation, a Nichrome-Formvar wire (0.0026 inch; A-M systems, Everett, WA, USA) was passed through a polycarbonate tube (194 mm outer diameter, 102 mm inner diameter; 0.7 cm in length) and the cuprophan hollow fiber (active dialysis region) and connected to a PE5 catheter with epoxy glue. The Nichrome-Formvar wire was then bent in the middle section 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 40% at an infusion rate of 5 μl/min. Using this technique, it was possible to measure levels of CSF amino acids for up to 12 days after implantation.

**Antinociception test and tolerance induction**

Tail flick latency of the hot water immersion test (52 ± 0.5 °C) was used to measure the antinociceptive effect. At this temperature, the mean tail-flick latency was approximately 2.2 ± 0.3 s in naive rats. An automatic cutoff was set at 10 s to prevent tissue injury. The rats were placed in plastic restrainer for drug injection and antinociception test. Morphine tolerance was induced by continuous morphine infusion (15 μg/h, i.t.) for 5 days, while the effect of DEX on the development of morphine tolerance was examined by DEX (2 μg/h, i.t.) co-infusion with morphine (15 μg/h, i.t.). Rats receiving saline or DEX (2 μg/h, i.t.) infusion alone were used as controls. Tail-flick responses were examined before drug infusion and daily after the start of infusion for 5 days. All drug infusions were at the rate of 1 μl/h via a mini-osmotic pump (model 2001; Alzet, Palo Alto, CA, USA) implanted in the interscapular region. The tail-flick dose-response curves for morphine were also measured before and after morphine tolerance developed. According to our previous study (Wen et al., 2004), on day 5, morphine challenge (50 μg) was performed 3 h after discontinuation of i.t. drugs infusion. Each tail-flick latency was an average of three measurements over a 6-min testing period. The latency was converted into the maximum percent effect (MPE) using the equation:

\[
\text{MPE (\%) = \frac{\text{Test response time} - \text{Basal response time}}{\text{Cut-off time} - \text{Basal response time}} \times 100\%}
\]

**Effect of single DEX injection on morphine’s antinociception**

The acute effect of DEX on morphine’s antinociception was examined both in naive and tolerant rats. In naive rats (n=6), morphine 0.5 μg was given, while in tolerant rats (n=6), 50 μg of morphine was injected. In naive rats, one i.t. catheter was implanted for drugs injection, while in tolerant rats, two i.t. catheters were implanted, one for tolerance induction (15 μg/μl, for 5 days), and the other one for DEX and morphine injections. The DEX (2 μg, i.t.) was injected 2 h before morphine injection.

**CSF sample collection and measurement of amino acids**

After recovery, rats were transferred to a free-moving animal system and one end of the externalized microdialysis probe was connected to a syringe pump (CMA-100) for the collection of CSF samples. The dialysis system was perfused with artificial CSF (aCSF), consisting of 151.1 mM Na+, 2.6 mM K+, 122.7 mM Cl−, 21.0 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 final pH to 7.3. All CSF sample collections followed 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. Thirty microliters of dialysate was collected before pump implantation, then daily for 5 days after implantation. On day 5, at 3 h after discontinuation of i.t. drug infusion, two consecutive CSF samples were collected before morphine challenge (basal level), and another 4 samples at 30, 60, 90 and 180 min after challenge. The dialysates
were collected in polypropylene tubes on ice, and then frozen at −80 °C until assayed. Amino acid levels were analyzed by high performance liquid chromatography (Agilent 1100 series, Agilent Technologies, Wilmington, DE, USA) using a fluorescence detec-

Fig. 1. The effect of DEX on morphine tolerance development. (A) The effect of DEX on morphine tolerance was examined by i.t. co-infusing DEX (2 μg/h) with morphine (15 μg/h) for 5 days. Morphine tolerance was induced by continued morphine infusion (15 μg/h, i.t.). The antinociceptive effect of continuous DEX (2 μg/h) infusion was also examined. Saline infusion (1 μl/h, i.t.) was used as control. (B) On day 5, the antinociceptive effect of morphine was examined in rats received either morphine or morphine plus DEX infusion. The mini-osmotic pump was disconnected from each rat and a single morphine challenge (50 μg, i.t.) was given 3 h later, at which the tail-flick latency returned to less than 3 s. The tail-flick latency was then examined every 30 min for 180 min. Each data point was expressed as mean±S.E.M. * P<0.05 (compare with morphine-infused group).
The method of amino acids analysis was according to our previous studies (Wen et al., 2003a, 2004). In brief, amino acids were assayed by precolumn derivatization with o-phthalaldehyde/t-butylthiol (OPA) reagent and iodoacetamide/methanol scavenger. Derivatization was performed by adding 4 μl of OPA reagent to 40 μl of sample, shaking the mixture, then allowing it to react for 2 min. Four milliliters of reagent B (185 mg of iodoacetamide/ml of methanol) was added and the mixture allowed to react for another 2 min. The derivatized sample was then injected onto a C18 reversed phase column and eluted at a flow rate of 0.45 ml/min. A linear gradient from 100% eluent A [0.1 M sodium acetate buffer, pH 6.8/acetonitrile (80:20)] to 100% eluent B [acetonitrile/double-distilled water (80:20)] was used to separate the amino acids. 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, 10^5 M standard amino acids, were run before and after each sample group.

Western blot analysis of GTs

Rats were implanted with only one i.t. catheter. Three days after catheterization, the catheter was connected to an osmotic pump for either saline (1 μl/h), or morphine, or DEX (2 μg/h) alone, or morphine (15 μg/h) plus DEX (2 μg/h) infusion. On day 5, all rats were anesthetized with isoflurane and rapidly decapitated, then the dorsal portion of the lumbar spinal cord enlargements was removed and stored at −80 °C until use. 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 r.p.m. (TXL-100, Beckman, Fullerton, CA, USA) 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) modified from the method of Lowry et al. (1951). Western blotting was performed as our previous study (Wen et al., 2003b). In brief, 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, which was then loaded onto a 10% SDS-polyacrylamide gel and electrophoresis performed at 150 V for 60 min. The proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, 0.45 μm pore size) at 125 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 in 0.1% Tween 20 in 20 mM Tris–HCl, 137 mM NaCl, pH 7.4 (TTBS), and incubated for 180 min at room temperature with antibody against GLAST (EAT1, 1:1000 dilution; Chemicon), EAAC1 (EAT3, 1:1000 dilution; Chemicon), or GLT-1 (EAT2, 1:1000 dilution; Santa Cruz, CA, USA). The GLAST antibody recognized a band at ~65 kDa, GLT-1 antibody recognized a band at ~70 kDa, and EAAC1 antibody recognized a band at ~70 kDa in spinal cord homogenates. The selection of antibodies for GLAST, GLT-1 and EAAC1 was according to Mao et al. (2002). It was then washed three times in TTBS for 10 min, blocked with 5% non-fat dry milk/TTBS, then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (dilution 1:2000). The blots were then visualized in ECL solution (NEN) for 30 s and finally exposed to X-ray film (Kodak X-OMAT LS, Kodak, Rochester, NY, USA). The membranes were re-probed with a monoclonal mouse anti-β-actin antibody (1:2500, Sigma, St. Louis, MO, USA) as the loading control.
Data and statistical analysis

The data are presented as the mean±S.E.M. Latencies less than the baseline or higher than the cutoff time were assigned MPE values of 0% or 100%, respectively. For statistical analysis, the area under the curve (AUC) for the plot of %MPE versus time was calculated by the trapezoidal method (Rowland and Tozer, 1995) between the intervals of 30 and 180 min and computerized by SigmaPlot 7.0. Morphine’s antinociceptive dose-response latency was calculated by the computer-assisted linear regression (SigmaPlot 7.0). The analgesic dose for 50% (AD50) was defined as the dose of morphine that induced a 50% of MPE, it was converted by tail-flick latencies and calculated from linear regression equation of morphine’s dose response curves. AD50 and 95% confidence intervals (CI) were calculated using a nonparametric Wilcoxon test. For immunoreactivity data, the intensity of each test band was expressed as the relative optical density (ROD) calculated with respect to the average optical density for the corresponding control band. For statistical analysis, all data were analyzed by one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls post hoc test for multiple comparisons. A significant difference was defined as a P value of <0.05.

RESULTS

The effect of DEX on morphine’s antinociceptive effect

As our previous studies, morphine infusion (15 µg/h, i.t.) induced a maximal antinociceptive effect at day 1 and tolerance was developed at day 3 and a maximal antinociceptive tolerance 5 days after continued morphine infusion, and co-infusion DEX (2 µg/h, i.t.) with morphine attenuated morphine’s antinociceptive tolerance (Fig. 1A). DEX infusion alone did not produce any antinociceptive effect by the hot-water immersion test at 52 °C. On day 5, morphine challenge (50 µg, i.t.) had a significant antinociceptive effect in the morphine plus DEX infusion group, but was only partially effective in morphine-tolerant rats (Fig. 1B). The acute bolus injection of DEX (2 µg, i.t.) did not affect morphine’s antinociceptive effect either in naïve or tolerant rats (Fig. 2A, B). The dose-response curve of

![Graph](image)

**Fig. 3.** Morphine’s dose response curves of rats with various drugs infusion. Rats received either saline infusion (1 µl/h), or morphine (15 µg/h), or morphine (15 µg/h) + DEX (2 µg) i.t. infusion for 5 days, and the dose-response curves were examined by the tail-flick test at 52 °C. The dose-response effect was expressed as the MPE (% of MPE). Each data point (mean±S.E.M.) was the average of at six rats.
morphine revealed a significant rightward shift (63-fold) by the chronic morphine infusion when compared with the morphine naïve rats; the AD50 were 1.33 and 83.84 μg of morphine naïve and tolerant rats, respectively (Table 1). Moreover, we found that DEX co-infusion with morphine made a leftward shift of the dose-response curve of tolerance in tolerant rats, the AD50 was 10.15 μg (Table 1, Fig. 3).

The effect of DEX on the CSF EAAs concentration during morphine’s antinociceptive tolerance induction and after morphine challenge

Table 2 shows the basal concentration (prior to i.t. various drugs injection) of aspartate and glutamate in all treatment groups. For the saline, morphine and morphine plus DEX groups, the baseline concentration of aspartate and glutamate had no difference among three groups (Table 2). Chronic morphine infusion did not induce significant change in the concentration of aspartate and glutamate during the 5-day infusion when compared with the basal. Similarly, saline infusion and DEX co-infusion with morphine also did not affect the aspartate and glutamate level in CSF dialysates (Table 2). On day 5 in morphine tolerant rats, morphine challenge (50 μg, i.t.) induced a significant increasing of glutamate (284 ± 47%) and aspartate (201 ± 18%) concentration in the spinal CSF dialysates while with DEX co-infusion inhibited this phenomena (Fig. 4A, B).

The effect of morphine tolerance and DEX co-infusion on GTs protein expression

Similar to Mao et al.’s study (2002), Western blotting of GTs revealed down-regulation of all three GTs, GLAST, GLT-1 and EAAC1 protein expression in morphine tolerant rat spinal cords. Furthermore, in our present study, we found that DEX co-infusion not only prevented the down-regulation of GLAST, but even up-regulated the GLT-1 expression (Fig. 5A, B). The reduction of the neuronal GT EAAC1 protein expression did not reversed by the DEX co-infusion (Fig. 5C).

**DISCUSSION**

The present study showed that DEX, co-administration with morphine, attenuated not only morphine’s antinociceptive tolerance but also the increasing of EAAs concentration in the CSF dialysates by the morphine challenge in chronic morphine-infused rats. Acute DEX injection (2 μg, i.t.) neither produced any antinociceptive nor enhanced morphine’s analgesic effect in naive and morphine tolerant rats. The AD50 shift was 63- and nine-fold in morphine tolerant and DEX co-infused rats, respectively. Acute morphine challenge (50 μg, i.t.), on day 5, did not produce significant antinociceptive effect in morphine tolerant rats, however, DEX co-infusion maintained an antinociceptive effect by the morphine challenge. In dorsal part of the spinal cord lumbar enlargements, significant down-regulation of GTs, GLAST, GLT-1 and EAAC1, protein expression was observed, and DEX co-infusion prevented the down-regulation of GLAST expression, but not EAAC1. Moreover, an up-regulation of GLT-1 protein expression was observed by the DEX co-infusion with morphine. From the present results, we further confirm our previous study (Wen et al., 2004) that an increasing of the CSF EAAs concentration plays an important role in morphine tolerance, and down-regulation of GTs may be responsible for this increasing of CSF EAAs concentration by the morphine challenge. Inhibiting the increasing of EAAs concentration in the CSF, via modulating the GTs expression, may preserve the antinociceptive effect of morphine.

Similar to our previous report, like cyclooxygenases inhibitors (Wong et al., 2000b), the present study demonstrated that DEX co-infusion attenuated morphine’s antinociceptive tolerance. However, in our recent clinical observations, we found an increasing of CSF EAAs, but not the prostaglandins, when losing morphine’s analgesic effect (Wong et al., 2002), and therefore, the anti-inflammatory effect of DEX on attenuating the morphine tolerance is unlikely. Trujillo and Akil (1991) first demonstrated that NMDA receptor antagonist MK-801 inhibited morphine tolerance and dependence. Subsequently, serial evidence implied that activation of EAAs receptors, in particular the NMDA receptors, is involved in morphine tolerance development (Tanganelli et al., 1991; Higgins et al., 1992; Wong

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**Table 2. Basal concentration of glutamate and aspartate prior to drugs’ infusion on day 1**

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Saline (n=6)</th>
<th>Morphine (n=7)</th>
<th>Morphine + DEX (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>0.62±0.37</td>
<td>0.56±0.15</td>
<td>0.51±0.11</td>
</tr>
<tr>
<td>Glu</td>
<td>4.93±1.08</td>
<td>5.12±2.54</td>
<td>5.39±1.13</td>
</tr>
</tbody>
</table>

Mean resting concentration of aspartate (Asp) and glutamate (Glu) in lumbar dialysates of rats from each experimental group (pmol/μl), measured on day 1 prior to various drug delivery, and there was no difference among three groups at the basal concentration of glutamate and aspartate. Saline: saline (1 μl/h, i.t.); Morphine: morphine (15 μg/h, i.t.) infusion for 5 days; Morphine + DEX: morphine (15 μg/h, i.t.) + DEX (2 μg/h, i.t.) infusion for 5 days.

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**Table 3. Effect of various drugs i.t. infusion on the spinal CSF EAAs concentration**

<table>
<thead>
<tr>
<th>Amino acid treatment</th>
<th>Saline Baseline</th>
<th>Saline Day 5</th>
<th>Morphine Baseline</th>
<th>Morphine Day 5</th>
<th>Morphine + DEX Baseline</th>
<th>Morphine + DEX Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>100</td>
<td>114±10</td>
<td>100</td>
<td>128±18</td>
<td>100</td>
<td>105±17</td>
</tr>
<tr>
<td>Glu</td>
<td>100</td>
<td>114±9</td>
<td>100</td>
<td>138±21</td>
<td>100</td>
<td>111±12</td>
</tr>
</tbody>
</table>

The concentrations of aspartate (Asp) and glutamate (Glu) were expressed as percentage of day 1. Rats received either saline (1 μl/h), or morphine (15 μg/h), or morphine (15 μg/h) plus DEX (2 μg/h) i.t. infusion for 5 days. No statistical differences were observed. Day 1, as 100%, represents the basal level before drugs’ infusion, and day 5 was the concentrations before morphine challenge.
**Glutamate**

- Saline ($n = 6$)
- Morphine ($n = 7$)
- Morphine plus Dex 5 day ($n = 7$)

**Aspartate**

- Saline ($n = 6$)
- Morphine ($n = 7$)
- Morphine plus Dex 5 day ($n = 7$)

**Fig. 4.** The effect of morphine challenge on the spinal glutamate (A) and aspartate (B) concentrations in the CSF dialysates after various drugs treatment in rats. On day 5, morphine challenge (50 μg, i.t.) was performed 3 h after discontinuation of the i.t. drugs infusion, two consecutive CSF dialysates (30 min each), just before morphine challenge, were obtained as the basal concentrations expressed as 100%. Another four dialysates were collected at 30, 60, 90 and 180 min after morphine challenge. Morphine challenge significantly increased glutamate and aspartate concentration in morphine tolerant rats. Co-administration of DEX completely prevented the morphine-evoked glutamate and aspartate release. (*$P<0.05$ compared with saline infusion group; #$P<0.05$ compared with morphine infusion group.)
Recently, we were able to demonstrate a correlation between morphine’s analgesic effect and CSF EAAs concentration in terminal cancer pain patients (Wong et al., 2002). At the effective analgesic dose of morphine, a lower concentration of EAAs was observed in the CSF. However, an increasing of EAAs level in the CSF was observed when morphine loss of its analgesic effect at the same effective dose of morphine after long-term i.t. administration. Furthermore, double the effective dose of morphine neither provided a satisfactory pain relief nor a reduction of CSF EAAs concentration (Wong et al., 2002). This result suggests that the loss of morphine’s analgesic effect is directly related to the increasing of EAAs in the CSF. Amount of studies demonstrated that long-term opioids administration activated NMDA receptors (Chen and Huang, 1991; Larcher et al., 1998). Activation of NMDA receptors can be via different mechanisms, increasing of presynaptic EAAs release, post-synaptic receptors activation and hinder the EAAs reuptake by GTs. Previous studies (Jhamandas et al., 1996; Wen et al., 2004) failed to show a significant increasing of EAAs in the spinal CSF dialysates during morphine tolerance induction. Similar to our previous report (Wen et al., 2004), in present study, a bolus injection of morphine (50 μg, i.t.), on day 5 in morphine tolerant rats, did not produce significant antinociceptive effect. It was accompanied by an increasing of EAAs concentration in spinal CSF dialysates, and co-infusion of glucocorticoid agonist DEX not only attenuated tolerance development and preserved morphine’s antinociceptive effect, but blocked morphine-induced spinal EAAs release as well. Therefore, we suggest that this increasing of spinal CSF EAAs concentration is responsible for the reduction of morphine’s antinociceptive effect in morphine tolerant rats.

To ensure a high signal-to-noise ratio during synaptic signaling and to protect neurons, the extracellular concentration of EAAs in the synapse needs to be maintained low (<1 μM), and GTs, particularly the glial GTs, play an important role in maintaining the synaptic EAAs concentration (Danbolt, 2001). In the present study, similar to Mao et al.’s findings (2002), both the neuronal (EAAC1) and glial (GLAST) GTs were down-regulated, and we further demonstrated that DEX co-infusion prevented the down-regulation of glial GTs GLAST and GLT-1, but not the neuronal GT EAAC1. Rauen and Wiessner (2000) had demonstrated that glucocorticoid hormone cortisol up-regulated GLAST gene and protein expression, they suggested that transcriptional regulation of glial proteins may impact on transmitter clearance. Gene deletion, gene knockdown, and pharmacological studies had also demonstrated that GLT-1 played a more important role than GLAST in the clearance of extracellular glutamate (for review see Robinson, 1998). Knockdown of spinal GLT-1 decreased the threshold of nociceptive processing (Niederberger et al., 2003). Co-administration of GT activator MS-153 with chronic morphine treatment significantly attenuated morphine tolerance development (Nakagawa et al., 2001). Moreover, Ozawa et al. (2001) also observed a significant reduction of the GLT-1 mRNA expression in morphine dependent rats, and the expression of GLT-1 protein was reduced as well in cortical cells (Thorlin et al., 1998). Development of morphine tolerance was demonstrated accompanied by PKC activation (Mao et al., 1994, 1995; Granados-Soto et al., 2000), and activation of protein kinase C was found to inhibit GLT-1 activity in Y-79 human retinoblastoma (Ganel and Crosson, 1998). Taken together, these studies suggest that the glial GTs, in particular the GLT-1 play an important role in the development of morphine tolerance, at which failure of clearance of EAAs maybe a mechanism for the tolerance development and reduction of morphine’s antinociceptive effect. Similarly, in the present study, we also found a reduction of GLT-1 expression in morphine tolerant rat spinal cord, and DEX not only prevented this down-regulation of GLT-1 but further enhanced its protein expression, which in turn, prevented the reduction of morphine’s antinociceptive effect by the morphine challenge in chronic morphine-infused rats. Glial cells, known as housekeepers for synapses in the CNS, recently, they are thought to be communicators between pre- and post-synaptic neurons (for review Araque et al., 1999). They dynamically control neuronal functions both in physiological and pathological conditions, for instance the inflammatory and neuropathic pains. Glial cells had been suggested to be involved in the development of morphine tolerance (Song and Zhao, 2001). Therefore, the effect of DEX on inhibiting the increasing of EAAs and preserved morphine’s antinociceptive effect, by the morphine challenge on day 5, in chronic morphine-infused rats may be via inhibiting downregulation of the glial GTs, which in turn maintains the synaptic concentration of EAAs at a physiological low level. The absence of increasing of EAAs concentration during morphine tolerance induction in the present study and previous studies (Jhamandas et al., 1996; Wen et al., 2004) might be due to a progressive, in a lower extent, down-regulation of GT expression of time during morphine tolerance, and on day 5 to the maximal effect. In the present study, this increase in the EAAs concentration after morphine challenge might be due to tolerance of morphine’s inhibitory effect which unmasks the excitatory effect of morphine (Crain and Shen, 1998), it resulted in a large amount of EAAs release from presynaptic nerve terminals, accompanied with the down-regulated GTs, it further limited the reuptake of the GT protein expression of the dorsal lumbosacral segment of the rat spinal cords after various drug treatment. Control: saline (1 μl/h) infusion; Morphine: morphine (15 μg/h) infusion; Morphine + DEX: morphine (15 μg/h) plus DEX (2 μg/h). All infusions were performed intrathecally for 5 days. The corresponding (a) GLAST (b) GLT-1 and (c) EAAC1 protein bands on the Western blot are shown above each column. The optical density of each protein band was quantified by densitometry and the relative optical density was calculated using the average value for samples from six rats and taking the density of the control band as 100%. GLAST, GLT-1 and EAAC1 levels were significantly down-regulated 5 days after morphine infusion. DEX infusion (2 μg/h, i.t.) prevented the morphine-induced down-regulation of GLAST expression. Morphine plus DEX infusion significantly increases GLT-1 expression. Each group was six rats (a and c, P<0.05 compared with control; b, P<0.05 compared with morphine group).
morphine-evoked released EAAs. From the present results and our previous report (Wen et al., 2004), we suggest that this elevation of synaptic EAAs is responsible for, at lease in part, the loss of morphine’s antinociceptive effect in tolerant rats.

In conclusion, in addition to the conformational change of opioid receptors, activation of the NMDA receptors, by the increasing of synaptic EAAs concentration, may play another mechanism for the loss of morphine’s antinociceptive effect. Down-regulation of GTs GLAST, GLT-1 and EAAC1, particularly the glial GLT-1, might be responsible, at least in part, for the increasing of synaptic EAAs concentration. The mechanism of DEX on inhibiting the above phenomenon needs further investigation.

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