Research Report

Attenuation of morphine tolerance by intrathecal gabapentin is associated with suppression of morphine-evoked excitatory amino acid release in the rat spinal cord

Jui-An Lin, Meei-Shyuan Lee, Ching-Tang Wu, Chun-Chang Yeh, Shinn-Long Lin, Zhi-Hong Wen, Chih-Shung Wong

*Department of Anesthesiology, Tri-Service General Hospital, National Defense Medical Center, #325, Chenggung Road, Section 2, Neihu 114, Taipei, Taiwan

bSchool of Public Health, National Defense Medical Center, #161, Minchuan E. Road, Section 6, Neihu 114, Taipei, Taiwan

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Abstract

This study was designed to investigate the effect of acute and chronic intrathecal (i.t.) injection of gabapentin (GBP) on the antinociceptive effect of morphine and tolerance development using a tail-flick latency test. Levels of excitatory amino acids (EAA) in i.t. CSF dialysates were also measured by high performance liquid chromatography. Male Wistar rats were implanted with either one or two i.t. catheters for drug injection or pump infusion and with a microdialysis probe for CSF dialysate collection. The effect of acute GBP (10 μg i.t.) injection on the morphine dose response was examined in both naïve rats and rats made tolerant by continuous infusion of morphine (15 μg/h i.t.) for 5 days. At such a low dose (10 μg i.t.), GBP did not enhance morphine’s antinociception in naïve rats. In morphine-tolerant rats, however, acute GBP (10 μg i.t.) injection potentiated morphine’s antinociception and yielded a 14.6-fold shift in morphine’s dose–response curve. When GBP (10 μg/h i.t.) was co-infused with morphine (15 μg/h i.t.) to examine its effect on the development of morphine tolerance, GBP attenuated the development of morphine tolerance. The effect of GBP and morphine on CSF glutamate and aspartate levels was examined in naïve rats, and the effect of morphine challenge on CSF glutamate and aspartate levels was examined in tolerant rats. Acute injection of GBP (10 μg i.t.), morphine (50 μg i.t.), or GBP (10 μg i.t.) followed by morphine (50 μg i.t.) 30 min later had no significant effect on CSF EAA concentration in naïve rats; however, in tolerant rats, morphine challenge (50 μg i.t.) increased aspartate and glutamate levels to 221 ± 22% and 296 ± 43%, respectively, of those before morphine challenge, and this phenomenon was inhibited by GBP co-infusion. Our results show that GBP, at a dose without enhanced effect on morphine’s antinociception in naïve rats, not only potentiates morphine’s antinociceptive effect in morphine-tolerant rats but also attenuates the development of morphine tolerance. The mechanism of the effect of GBP on morphine tolerance might be via suppression of the EAA concentration in spinal CSF dialysate.

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1. Introduction

Long-term use of opioids results in opioid tolerance, which reduces their analgesic efficacy and is related to neuroadaptive changes and persistent neural adaptation [18,25–27]. We previously demonstrated that opioid receptor uncoupling and downregulation are involved in the development of opioid tolerance [38,39]. In addition to the conformational change induced in opioid receptors after long-term exposure to opioids, activation of the central glutamnergic receptors, in particular N-methyl-D-aspartate (NMDA) receptors, also plays an important role in the development of opioid tolerance [18,20,21,23]. In a rat spinal model, we

* Corresponding author.
E-mail address: w82556@ndmctsgh.edu.tw (C.-S. Wong).
found that both competitive and non-competitive NMDA receptor antagonists inhibit the development of morphine tolerance and prevent the reduction in the number of μ-opioid receptor high-affinity sites seen in rats continuously infused with morphine [40]. Furthermore, in morphine-tolerant rats, we observed an increase in the [3H]MK-801 binding affinity of NMDA receptors [41]. Mao et al. [20] found that prolonged exposure to morphine activates NMDA receptors and down-regulates spinal glutamate transporters (GTs). On the basis of these findings, a high synaptic glutamate concentration and NMDA activation have been suggested to be involved in the generation of opioid tolerance.

Gabapentin (GBP), used as an anticonvulsant and for neuropathic pain management, has been shown to inhibit allodynia and/or hyperalgesia in various animal models [5,12,32,33]. The neuroplastic changes seen in opioid tolerance and in neuropathic pain have much in common [23]. Drugs that modulate opioid tolerance have antihyperalgesic and/or antiallodynic effects [19]. Recently, GBP was shown to inhibit the development of morphine tolerance when given either systemically and intrathecally [8,9]. However, the interpretation of the results may be confounded by an enhancing effect of GBP on morphine-mediated antinociception which has been reported in studies on rats [30] and humans [4].

In clinical cancer pain patients, we observed an increase in CSF glutamate and aspartate levels which is accompanied by a loss of the analgesic effect of morphine after long-term intrathecal (i.t.) morphine administration [42]. Similarly, following morphine challenge, we found an increase in CSF excitatory amino acid (EAA) levels in morphine-tolerant rats [37]. Thus, activation of EAA receptors, particularly NMDA receptors, by increased levels of EAs in the synapse has been implicated as one of the mechanisms of opioid tolerance. Moreover, Patel et al. [29] found that GBP presynaptically inhibits excitatory transmission in the hyperalgesic spinal cord. The present study was designed to test the hypothesis that GBP attenuates morphine tolerance by inhibition of EAA release in the spinal cord and to try to clarify the difference between the antinociception enhancement and modulation of morphine tolerance by GBP.

2. Materials and methods

2.1. Animal model: implantation of i.t. catheters and the mini-osmotic pump

For morphine dose–response curves, male Wistar rats (400–450 g) were implanted with two i.t. catheters under chloral hydrate anesthesia (400 mg/kg i.p.). The catheters, inserted into the i.t. space through the cisternal membrane and down to the rostral edge of the lumbar enlargement, were externalized on the top of the head and used for drug injection and mini-osmotic pump infusion. After operation, the rats were returned to their home cages for a 3-day recovery period. Rats were excluded from the study if they showed evidence of gross neurological injury or the presence of blood in the CSF. A mini-osmotic pump (model 2001; Alzet, Palo Alto, CA, USA) with a pump rate of 1 μl/h was filled with morphine or other test drugs and attached to an i.t. catheter. Drugs were administered in a 10-μl of normal saline and flushed by another 10 μl of normal saline. The pump was implanted subcutaneously between the scapulae under isoflurane anesthesia. The use of animals in this study conformed to the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiological Society and by the National Defense Medical Center Animal Care and Use Committee.

2.2. Construction of the i.t. microdialysis probe

The i.t. microdialysis probe was constructed as previously described [22] and inserted into the lumbar enlargement. Each end of 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]) was connected via a polycarbonate tube (194 μm outer diameter, 102 μm inner diameter; 0.7 cm in length) to a 7-cm PE5 tube (0.008 in. inner diameter, 0.014 in. outer diameter). To make the probe firm enough for implantation, a Nichrome–Formavar wire (0.0026 in.; A-M system, Everett, WA, USA) was passed through the polycarbonate tubes and cuprophan hollow fiber (active dialysis region) and fixed inside the PE5 tubes with epoxy glue. The probe was then bent in the middle part of the cuprophan hollow fiber, forming a “U” shaped loop. The two ends of the microdialysis probe, consisting of silastic tubes, were sealed with silicon sealant. The dead space of the dialysis probe was 8 μl. During the in vitro measurements, the recovery rate of the dialysis probe was 40% at an infusion rate of 5 μl/min.

2.3. Antinociception test and tolerance induction

Tail-flick latency in the hot water immersion test (52 ± 0.1 °C) was used to measure the antinociceptive effect and the development of tolerance in naïve rats or rats made tolerant to morphine by i.t. infusion for 5 days with morphine (15 μg/h). In experiments studying the effect of acute injection of GBP on the antinociceptive effect of morphine, naïve or tolerant animals were given a single injection of GBP (10 μg i.t.) or left untreated, then injected 30 min later with different doses of morphine and the dose response measured using the tail-flick latency. In experiments studying the effect of chronic GBP treatment on development of tolerance to morphine, rats were infused for 5 days with morphine alone (15 μg/h i.t.), GBP alone (10 μg/h i.t.) or both, and the tail-flick test was performed on day 0 (before infusion) and days 1, 3 and 5 after pump implantation. Normal saline infusion (1 μl/h i.t.) was used as control. Latency was expressed as the average of three measurements taken at 5-min intervals and an automatic cut-
off was set at 10 s to prevent tissue injury. For bolus drug injection and the antinociception test, the rats were placed in a plastic restrainer and the tail-flick latency was measured before and 30 min after drug injection. All drug infusions were via a mini-osmotic pump (model 2001; Alzet, Palo Alto, CA, USA).

We chose 10 µg of GBP as the acute injection dose and a rate of 10 µg/h for infusion, far lower than the other dose used in a tolerance study in the literature (300 µg i.t.) [30]. An acute injection of 10 µg of GBP or continuous infusion of 10 µg/h for 5 days had no antinociceptive effect. Tail-flick latencies were converted into the maximum percent effect (MPE) using the equation:

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

2.4. CSF sample collection and measurement of EAA concentration

For the microdialysis study, a different set of rats were used from those used in the above behavioral study. Naïve rats were implanted with one i.t. catheter for drug injection and a dialysis probe for CSF dialysate collection; rats for chronic infusion were implanted with an additional i.t. catheter for mini-osmotic pump drug infusion. After a 3-day recovery period, one end of the dialysis probe was connected to a syringe pump (CMA-100) (inflow) and the other to another PE-10 tube (outflow) for sample collection. The dialysis system was perfused with artificial cerebrospinal fluid (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 sampling involved 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. Naïve rats were injected with normal saline (20 µl i.t.), GBP (10 µg i.t.) or morphine (50 µg i.t.) to examine if the drugs had an effect on glutamate and aspartate levels in the spinal CSF dialysates; two CSF dialysate samples were collected before drug injection and another two immediately after drug injection. Another set of naïve rats were injected sequentially with GBP (10 µg i.t.) followed by morphine (50 µg i.t.) 30 min later and two CSF dialysate samples were collected before and after the drugs injection. Rats for chronic experiments were infused for 5 days with either morphine (15 µg/h) or morphine (15 µg/h) plus GBP (10 µg/h). Two hours after stopping drug infusion, two consecutive CSF dialysate samples were collected, then the animals were injected with morphine (50 µg i.t.) at 3 h after discontinuation of drug infusion and another two samples collected. All CSF dialysate samples were collected in polypropylene tubes on ice and frozen at −80 °C until analysis.

Levels of amino acids were analyzed by high performance liquid chromatography (HPLC) using a fluorescence detector (Gilson model 121, set at 428 nm) as described previously [36]. In brief, amino acids were assayed by precolumn derivatization with o-phthalaldehyde/-butylthiol (OPA) reagent and iodoacetamide/methanol scavenger. Derivatization was performed by adding 4 ml of OPA reagent to 40 ml 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 and 10⁻⁵ M standard amino acids, were run before and after each sample group.

2.5. Statistical analysis

All data were expressed as the mean ± SD. We used linear regression model to predict AD₅₀ and generate corresponding 95% confidence interval (CI). We used generalized estimating equations with an identity link function to model the change in tail-flick latency among the four groups over time by comparison with the baseline level assuming an exchangeable correlation structure between the repeated measures [17]. Areas under the tail-flick latency curves (AUC) over 5 days among group were calculated and compared by one-way ANOVA. Scheffe’s test was used for post hoc comparison between groups. The effects of EAA changes in different treatment groups, i.e., the significances of interaction terms, were assessed by ANOVA.

3. Results

3.1. Effect of GBP on the antinociceptive effect of morphine and tolerance development

As shown in Fig. 1 and Table 1, the antinociceptive dose–response curves for morphine showed that the AD₅₀ for morphine was 1.34 µg in naïve rats and 79.0 µg in morphine-tolerant rats and that a single injection of GBP (10 µg i.t.) 30 min before morphine injection enhanced the antinociceptive effect of morphine in tolerant rats, the AD₅₀ being 5.42 µg, giving a 14.6-fold shift in the dose–response curve (P < 0.05). The 95% CI for AD₅₀ of naïve rats with and without acute injection of GBP (10 µg i.t.) were overlapped and the result revealed an insignificant shift in the dose–response curve. As in our previous study [40], the antinociceptive effect of morphine was maximal on day 1,
then decreased significantly on day 3 of continuous morphine infusion (Fig. 2). Continuous GBP infusion alone did not have a significant antinociceptive effect compared to the normal saline infusion group \( (P > 0.05) \), but co-infusion of GBP and morphine attenuated the development of morphine tolerance when compared to the morphine-infused group \( (P < 0.05) \) (Fig. 2).

3.2. Effect of bolus injection of GBP or morphine on i.t. CSF EAA levels in naïve and morphine-tolerant rats

Acute i.t. injection of naïve rats with normal saline (20 µl), GBP (10 µg), morphine (50 µg) or GBP (10 µg) followed by morphine (50 µg) 30 min later did not produce significant change in the CSF EAA levels (Table 2). Infusion of GBP alone for 5 days did not affect CSF aspartate and glutamate levels (data not shown). As in our previous study [37], in rats made tolerant by infusion with morphine for 5 days, a slight increase in aspartate and glutamate levels was seen on day 5 compared to day 0, but the difference was not statistically significant (data not shown); however, when these rats were injected on day 5 with morphine (50 µg i.t.), a significant increase in glutamate (296 ± 43% of basal levels) and aspartate (221 ± 22% of basal levels) in the CSF dialysates was seen (Table 3). This effect was not seen when rats co-infused with GBP and morphine were injected with morphine on day 5 (Table 3).

4. Discussion

In the present study, acute injection of GBP (10 µg i.t.) neither produce antinociception nor significantly potentiated the antinociceptive effect of morphine in naïve rats. The \( AD_{50} \) for the antinociceptive effect of morphine was shifted 4.19- and 14.6-fold, respectively, in naïve and tolerant rats by GBP. This 4.19-fold enhancement of GBP on morphine’s antinociception in naïve rats was insignificant due to overlapping of 95% CI for \( AD_{50} \) with and without acute injection of GBP in naïve rats. In tolerant rats, however, acute GBP (10 µg i.t.) injection enhanced morphine’s antinociception and yielded a 14.6-fold shift in morphine’s dose–response curve (Table 1). Moreover, co-infusion of GBP with morphine attenuated morphine tolerance development during the 5-day infusion period (Fig. 1). These phenomena might be explained by the antihyperalgesic effect of GBP in morphine-tolerant rats [28]. Hyperalgesia and morphine tolerance, two seemingly unrelated phenom-
ena, may be connected by common neural substrates that act at spinal EAA receptors and its downstream cellular mechanisms. Hyperalgesia has been reported in morphine-tolerant animals [19]. However, significant hyperalgesia was not observed in our present study which is insensitive by the 52 °C hot water tail-flick test.

GBP is well tolerated, with few serious adverse effects in clinical use [7,35]; however, mild to moderate adverse effects, such as somnolence (15.2%), dizziness (10.9%), asthenia (6.0%) and convulsions (0.9%), have been reported [24]. The enhancing effect of this minimal sub-antinociceptive dose of GBP (10 μg i.t) on morphine-induced antinociception in morphine-tolerant rats favors its clinical application, as few side effects are seen in patients even at doses of 300–600 mg/day orally. Therefore, GBP in combination with morphine may be useful in patients who need long-term opioids, in particular neuropathic pains, for pain relief.

Repeated exposure to opioids may induce neuronal plastic changes via interactions with NMDA receptors [19,23]. Trujillo and Akil [34] first demonstrated that the NMDA receptor antagonist, MK-801, inhibits morphine tolerance and dependence. Moreover, EAAs activate glutamate receptors, in particular NMDA receptors, and facilitate spinal sensory transmission, which contributes to the hypersensitivity and neuroplastic changes of dorsal horn neurons in chronic pain states [3,10,16]. One possible common link between morphine tolerance and GBP-induced analgesia is the modulation of glutamate receptors (NMDA, AMPA and kainate). Although the central mechanism of action of GBP has not been clearly established, several hypotheses have been suggested. GBP may inhibit Ca2+ influx into glutamatergic terminals by acting presynaptically on the α2β subunit of P/Q-type voltage-gated calcium channels, subsequently attenuating the release of the EAAs, glutamate and aspartate [6]. GBP decreases

<table>
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<th>Table 2</th>
<th>Effect of acute drug injection on spinal CSF excitatory amino acid levels in naïve rats</th>
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<td>Amino acid</td>
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The effects of an acute i.t. injection of normal saline (20 µl), gabapentin (10 µg) or morphine (50 µg) or gabapentin (10 µg) followed by morphine (50 µg) 30 min later on aspartate (Asp) and glutamate (Glu) levels in the i.t. CSF dialysates were examined. Each CSF dialysate sample was collected for 30 min. Two consecutive basal CSF samples were obtained just before drug injection and the mean concentration was used as the 100% (“before”). Another two consecutive CSF samples were collected after drug injection and the mean concentrations, presented as a percentage of the “before” levels, are shown as “after”. All data are the mean ± SD for the indicated number of animals. No statistical difference was found by three-way ANOVA with one-way repeated.
glutamate currents in the superficial lamina of the rat spinal cord [31], and the antinociceptive effect of GBP is antagonized by NMDA and AMPA in the rat formalin model [43]. Kaneko et al. [15] found that GBP, possibly acting indirectly on NMDA receptors, attenuates formalin-evoked nociception and related Fos-like protein expression in rats. Furthermore, Chen et al. [1] demonstrated a synergistic action between a non-NMDA receptor (AMPA/kainate) antagonist and GBP on allodynia in spinal nerve-ligated rats. The present results confirm that an increase in CSF EAA concentration plays an important role in morphine tolerance, and that GBP, by inhibiting the increase in EAA level caused by morphine challenge after tolerance development, which potentiated the antinociceptive effect of morphine.

In our clinical study, we found that the loss of the analgesic effect of morphine in terminal cancer patients is related to an increase in CSF EAA levels [42]. As in the study by Jhamandas et al. [14] and our recent study [37], the present results failed to demonstrate an increase in CSF EAA levels during induction of morphine tolerance. Similarly, as in our previous study [37], we observed an increase in EAA levels in morphine-tolerant rats, but not in rats co-infused with GBP and morphine, when the rats were challenged with morphine on day 5. In naïve rats, acute GBP (10 μg i.t.) or morphine (50 μg i.t.) followed by morphine (50 μg i.t.) injection 30 min later did not suppress the basal CSF EAA concentration (Table 2). In morphine-tolerant rats, morphine (50 μg i.t.) challenge resulted in a significant increase in EAA concentration in morphine-infused rats, but not in morphine + GBP co-infused rats (Table 3). The 14.6-fold increase in the AD50 of morphine’s antinociception in tolerant rats might be explained by the suppression of spinal EAA concentration by GBP treatment (Table 1). We therefore suggest that the mechanism of attenuation of morphine tolerance by GBP might be involved in the suppression of EAA concentration in the spinal CSF. Furthermore, similar to the results of Ibuki et al. [13] who found that naloxone injection evokes EAA release in morphine-infused rats, our present study showed that morphine challenge in morphine-tolerant rats also induced EAA release; however, the morphine withdrawal (discontinuation of morphine infusion for 3 h) associated EAA release [11] can be ruled out by the low basal EAA levels before morphine challenge. This increase in EAA levels after morphine challenge might be due to tolerance to the inhibitory effect of morphine which unmask the excitatory effect of morphine [2] and results in a large EAA release from presynaptic nerve terminals by morphine challenge. In our unpublished work, we have found that the GT, which is responsible for the reuptake of EAA in the synapse, is down-regulated in morphine-tolerant rats and thus cannot prevent EAA accumulation. The present results and those in our recent report [37] suggest that the increase in synaptic EAA levels is responsible, at least in part, for the antinociceptive tolerance of morphine and GBP may attenuate morphine tolerance by inhibiting this pathway.

In conclusion, at such a low dose (10 μg i.t.) of GBP without enhanced effect on morphine’s antinociception in naïve rats, it not only potentiates morphine’s antinociception in morphine-tolerant rats, but also attenuates morphine tolerance development. The mechanism of the effect of GBP on morphine tolerance may be via suppression of the EAA concentration in spinal CSF dialysate.

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