

# Magnesium Chloride and Ruthenium Red Attenuate the Antiallodynic Effect of Intrathecal Gabapentin in a Rat Model of Postoperative Pain

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**Background:** Gabapentin, a  $\gamma$ -aminobutyric acid analog anticonvulsant, has been shown to possess antinociceptive effects in animal models and clinical trials. An endogenous binding site of [ $^3$ H]gabapentin has been revealed to be the  $\alpha_2\delta$  subunit of voltage-dependent  $\text{Ca}^{2+}$  channels. Magnesium chloride, ruthenium red, and spermine have been shown to modulate [ $^3$ H]gabapentin binding to this binding site *in vitro*. In this study, the authors examined whether intrathecal magnesium chloride, ruthenium red, or spermine could affect the antiallodynic effect of intrathecal gabapentin in a rat model of postoperative pain.

**Methods:** Under isoflurane anesthesia, male Sprague-Dawley rats received an incision over the plantar surface of the right hind paw to produce punctate mechanical allodynia. Withdrawal thresholds to von Frey filament stimulation near the incision site were measured before incision, 2 h after incision, and every 30 min after intrathecal coadministration of gabapentin with normal saline or different doses of magnesium chloride, ruthenium red, or spermine for 2 h.

**Results:** Intrathecal gabapentin (30, 100, 200  $\mu\text{g}$ ) dose-dependently reduced incision-induced allodynia. Hexahydrated magnesium chloride (5, 10, 20  $\mu\text{g}$ ) and ruthenium red (0.2, 2, 20 ng) noncompetitively inhibited the antiallodynic effect of gabapentin. Spermine at doses not inducing motor weakness (30, 60  $\mu\text{g}$ ) did not affect the antiallodynic effect of gabapentin. The antiallodynic effect of intrathecal morphine (1.5  $\mu\text{g}$ ) was not affected by hexahydrated magnesium chloride (20  $\mu\text{g}$ ), ruthenium red (20 ng), or spermine (60  $\mu\text{g}$ ).

**Conclusions:** These results provide behavioral evidence to support that the  $\alpha_2\delta$  subunit of  $\text{Ca}^{2+}$  channels may be involved in the antiallodynic action of intrathecal gabapentin in the postoperative pain model.

GABAPENTIN, a novel anticonvulsant, has been shown to possess antinociceptive effects in animal pain models and clinical trials.<sup>1-4</sup> Several clinical indications have also been implied, including anxiety,<sup>5</sup> bipolar depression,<sup>6</sup> and hot flashes.<sup>7</sup> The mechanisms of action of gabapentin have

been actively explored, and different mechanisms may be involved in different actions of gabapentin.<sup>8</sup>

Even though designed as a lipophilic analog of  $\gamma$ -aminobutyric acid (GABA) to penetrate the blood-brain barrier, gabapentin shows little activity at  $\text{GABA}_A$  or  $\text{GABA}_B$  receptors in initial binding assays<sup>9</sup> but was recently claimed to be a selective agonist at the heterodimeric  $\text{GABA}_{B1a-B2}$  receptors.<sup>10-11</sup> In healthy volunteers and epileptic patients, brain GABA concentration is increased after oral intake of gabapentin.<sup>12,13</sup> This may be due to the ability of gabapentin to increase the activity of glutamic acid decarboxylase<sup>14</sup> or promote nonvesicular release of GABA.<sup>15</sup> Despite the fact that D-serine, an agonist at the glycine binding site of *N*-methyl-D-aspartate (NMDA) receptors, could reverse certain actions of gabapentin *in vitro* and *in vivo*, gabapentin seems not to interact directly with the glycine-NMDA receptor complex.<sup>9,16</sup> On the other hand, Gu and Huang<sup>17</sup> recently found that gabapentin could enhance NMDA receptor-mediated currents of spinal GABAergic neurons by increasing the glycine affinity of NMDA receptors. In the rat spinal dorsal horn, gabapentin has been shown to affect glutamatergic excitatory neurotransmission.<sup>18,19</sup> Adenosine 5'-triphosphate-sensitive  $\text{K}^+$  channels have also been reported to be involved in the action of gabapentin.<sup>20</sup>

Among various proposed mechanisms of gabapentin actions, a high-affinity binding site of [ $^3$ H]gabapentin in rat brain homogenate reported by Suman-Chauhan *et al.*<sup>9</sup> is a unique finding. They tested a wide range of neurotransmitter receptor ligands and found that only magnesium chloride and spermine, two of many tested NMDA receptor ligands, inhibit [ $^3$ H]gabapentin binding in rat cerebral cortical membranes.<sup>9</sup> Gee *et al.*<sup>21</sup> later identified this [ $^3$ H]gabapentin binding site to be the  $\alpha_2\delta$  subunit of voltage-dependent  $\text{Ca}^{2+}$  channels. Subsequently, Dissanayake *et al.*<sup>16</sup> demonstrated that  $\text{Mg}^{2+}$  and spermine could displace [ $^3$ H]gabapentin binding to this  $\alpha_2\delta$  subunit in detergent-solubilized porcine cerebral cortical membranes. Moreover, among various binding ligands of voltage- and ligand-gated  $\text{Ca}^{2+}$  channels, Taylor *et al.*<sup>22</sup> found only magnesium chloride, ruthenium red, and spermine modulate [ $^3$ H]gabapentin binding in mouse cerebral cortex in an allosteric manner.

Several gabapentin analogs with high binding affinity to the  $\alpha_2\delta$  subunit of  $\text{Ca}^{2+}$  channels also possess anticonvulsant and antinociceptive effects.<sup>23,24</sup> However, the functional consequence of gabapentin binding to the  $\alpha_2\delta$  subunit of  $\text{Ca}^{2+}$  channels is not well established.<sup>25</sup> We have previously demonstrated that intrathecal gaba-

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gabapentin possesses an antiallodynic effect in the Brennan<sup>26</sup> postoperative pain model,<sup>2</sup> a rat model of incisional pain with a time course similar to the duration of clinical postoperative pain. The system L transporter, which is responsible for the entry of gabapentin into cells,<sup>27</sup> seems not to be involved in the antiallodynic effect of intrathecal gabapentin in this model.<sup>2</sup> In the current study, we further examined whether magnesium chloride, ruthenium red, or spermine could affect the antiallodynic effect of intrathecal gabapentin in the same model to explore whether the  $\alpha_2\delta$  subunit of Ca<sup>2+</sup> channels is involved in the antiallodynic action of intrathecal gabapentin.

## Materials and Methods

This study was approved by the Animal Care and Use Committee of Mackay Memorial Hospital (Taipei, Taiwan).

### *Intrathecal Catheterization*

Male Sprague-Dawley rats (weight, 250–300 g) housed under a 12-h light–dark cycle with food and water *ad libitum* were used in all experiments. For intrathecal drug administration, rats received intrathecal catheterization under isoflurane mask anesthesia.<sup>28</sup> Intrathecal catheters (PE-5 polyethylene tubing) were advanced 8.5 cm caudally through an incision in the cisternal membrane and secured to the musculature at the incision site. Rats showing any neurologic dysfunction after catheterization were euthanized. To confirm proper position of the catheter, lidocaine (2%, 10  $\mu$ l) was injected intrathecally to induce hind limb paralysis 1 day after catheterization. Rats displaying normal grooming, ambulation, and weight gain after catheterization were used for the behavioral test.

### *Postoperative Allodynia Induction*

Postoperative allodynia was induced 5 days after intrathecal catheterization according to the method described by Brennan *et al.*<sup>26</sup> Under isoflurane mask anesthesia, rats received a 1-cm longitudinal incision over the plantar surface of right hind paw, and the plantaris muscle was incised longitudinally as previously described.<sup>2</sup>

### *Behavioral Testing*

Behavioral studies were performed on the first day after paw incision surgery. Von Frey filament (Stoelting Co., Wood Dale, IL) testing was performed over the area near the incision wound to determine the punctate mechanical withdrawal threshold. The 50% likelihood withdrawal threshold was calculated using the Dixon “up-and-down” method.<sup>29</sup> Withdrawal thresholds were determined before incision (preincision threshold), 2 h after paw incision (postincision threshold), and every 30 min after intrathecal injection for 2 h (postdrug threshold). The antiallo-

dynic effects induced by test drugs were expressed as percent of maximal possible effect (%maximal possible effect): %maximal possible effect = 100  $\times$  (postdrug threshold – postincision threshold)/(preincision threshold – postincision threshold). Only rats showing marked allodynia after paw incision were used for drug evaluation. The person performing the test was blinded to the drugs administered.

### *Experimental Treatments*

All drugs were dissolved in normal saline and injected intrathecally followed by a 10- $\mu$ l normal saline flush. Gabapentin or morphine (5  $\mu$ l) was injected immediately followed by normal saline, magnesium chloride, ruthenium red, or spermine (5  $\mu$ l). We did not find gross precipitation when gabapentin was mixed with these three agents in test tubes. The dose of gabapentin was chosen based on our previous study.<sup>2</sup> The doses of magnesium chloride, ruthenium red, and spermine were chosen initially from doses equimolar to 200  $\mu$ g gabapentin and decreased progressively until no motor weakness and antiallodynic effect were noted after given intrathecally with normal saline. The dose of morphine was chosen based on a previous study.<sup>30</sup> Rats showing motor dysfunction, including abnormal ambulation and stepping reflexes, after intrathecal drug injection were excluded from the study.

### *Drugs*

Gabapentin was a gift from Pfizer Inc. (Groton, CT). Hexahydrated magnesium chloride (MgCl<sub>2</sub> · 6H<sub>2</sub>O) was purchased from Wako (Osaka, Japan). Ruthenium red and spermine were from Sigma (Steinheim, Germany). Morphine hydrochloride was purchased from the Narcotics Bureau of the National Health Administration (Taipei, Taiwan).

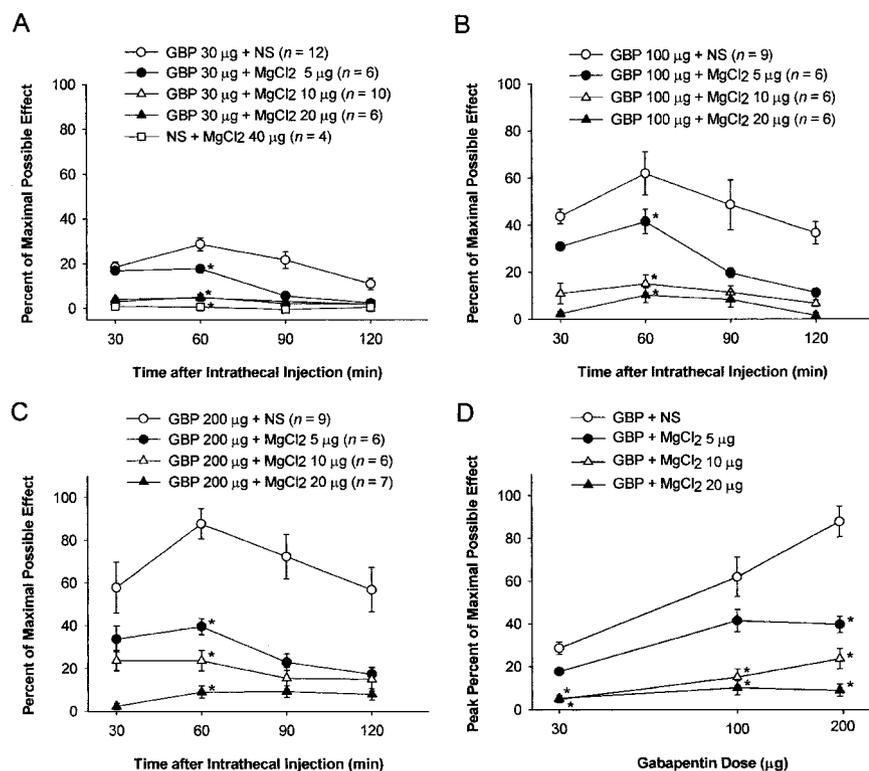
### *Statistical Analysis*

Data are presented as mean  $\pm$  SEM. Two-way analysis of variance with *post hoc* Dunnett test was used to compare the antiallodynic effects between the gabapentin control group or morphine control group and their respective magnesium chloride-, ruthenium red-, and spermine-coadministered groups. A two-tailed Student *t* test was used to compare the peak antiallodynic effect (60 min after intrathecal injection) of each coadministration test group with that of its control group. *P* < 0.05 was considered statistically significant.

## Results

### *Effect of Gabapentin on Incision-induced Allodynia*

As reported previously,<sup>2</sup> intrathecal injection of gabapentin (30, 100, 200  $\mu$ g) reduced incision-induced allodynic response in a dose-dependent manner. The effect



**Fig. 1.** Antiallodynic effects of intrathecal gabapentin (GBP) coadministered with normal saline (NS) or  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ . (A–C) Time courses of antiallodynic effects of 30 (A), 100 (B), or 200  $\mu\text{g}$  gabapentin (C) in the absence (GBP + NS) or presence (GBP +  $\text{MgCl}_2$ ) of 5, 10, or 20  $\mu\text{g}$   $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ . Effect of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  40  $\mu\text{g}$  on the postoperative withdrawal threshold is also shown ( $\square$ , A). \* $P < 0.05$  versus GBP + NS group (two-way analysis of variance with *post hoc* Dunnett test). (D) Dose-response curves of peak (60 min after injection) antiallodynic effects of gabapentin coadministered with normal saline, 5, 10 or 20  $\mu\text{g}$   $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ . \* $P < 0.05$  versus GBP + NS group (two-tailed Student *t* test). Data are expressed as mean  $\pm$  SEM with *n* indicating the number of rats tested in each group.

of gabapentin peaked around 60 min and lasted for at least 2 h after intrathecal injection (fig. 1).

#### Effect of Magnesium Chloride on Gabapentin-induced Antiallodynia

Hind limb paralysis was noted after 400  $\mu\text{g}$  magnesium chloride was given intrathecally. Therefore, we lowered the injected dose of magnesium chloride until no motor weakness was seen (40  $\mu\text{g}$ ). Figure 1 shows the time courses of antiallodynic effects of intrathecal gabapentin (30, 100, and 200  $\mu\text{g}$ ) coadministered with normal saline or magnesium chloride (5, 10, and 20  $\mu\text{g}$ ). Magnesium chloride, at 40  $\mu\text{g}$  or lower, did not affect incision-induced allodynic response *per se* but significantly attenuated the antiallodynic effect of gabapentin (fig. 1). At 5, 10, and 20  $\mu\text{g}$ , magnesium chloride attenuated the effect of 30  $\mu\text{g}$  gabapentin in a dose-dependent manner (fig. 1A). The attenuation by magnesium chloride was still prominent when the dose of gabapentin was increased to 100 or 200  $\mu\text{g}$  (figs. 1B and C). At 20  $\mu\text{g}$ , magnesium chloride almost but not completely abolished the antiallodynic effect of gabapentin (fig. 1). Increasing the dose of magnesium chloride to 40  $\mu\text{g}$  did not produce further attenuation of gabapentin's effect (data not shown). The dose-response curves of peak antiallodynic effects of gabapentin in the presence of various doses of magnesium chloride reveal that the attenuation by magnesium chloride was not completely surmountable by increasing the doses of gabapentin (fig. 1D). It indicates that magnesium chloride inhibited the antiallodynic effect of gabapentin in a noncompetitive manner.

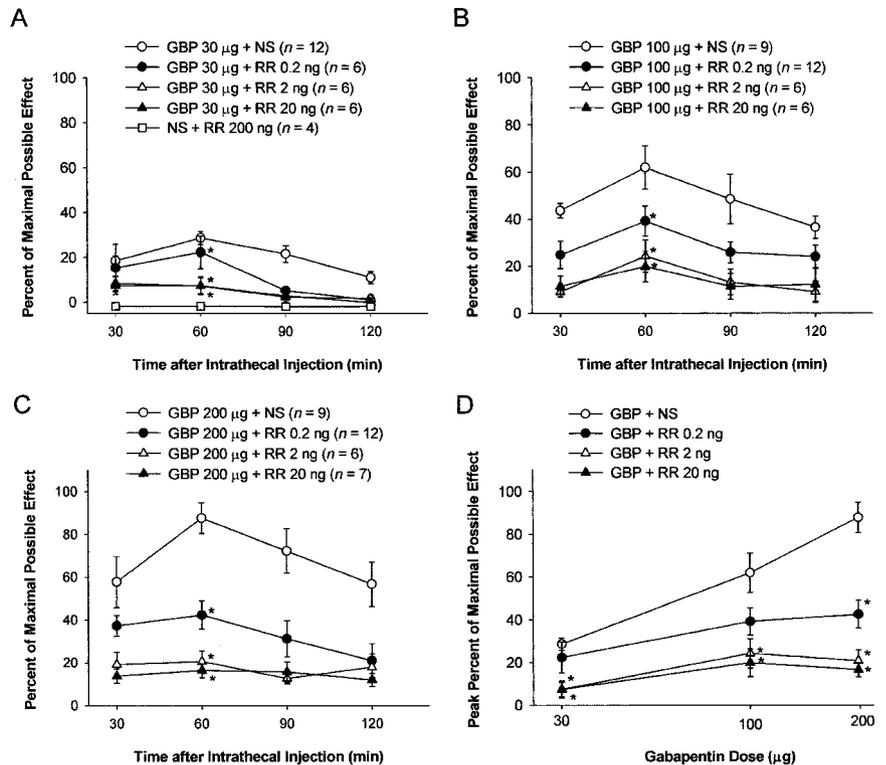
#### Effect of Ruthenium Red on Gabapentin-induced Antiallodynia

After injection of 160  $\mu\text{g}$  ruthenium red, rats showed agitation, tremor, squeaking, paralysis, and eventually death. Similar toxic signs but not death were noted when the dose was reduced to 10  $\mu\text{g}$ , and the toxic signs waned at 200 ng, at which point ruthenium red *per se* had no effect on incision-induced allodynic response (fig. 2A). At 20 ng, ruthenium red effectively attenuated the antiallodynic effect of gabapentin (fig. 2). The magnitude of attenuation by 200 ng ruthenium red is similar to that by 20 ng (data not shown). The time courses of antiallodynic effects of 30, 100, or 200  $\mu\text{g}$  gabapentin coadministered with 0.2, 2, or 20 ng ruthenium red are shown in figures 2A, B, and C, respectively. Ruthenium red at 2 ng seemed to produce saturated attenuation of gabapentin's effect. Increasing the dose of ruthenium red to 20 ng did not produce further attenuation, regardless of the doses of gabapentin (fig. 2). Interestingly, ruthenium red also did not completely abolish the antiallodynic effect of gabapentin at the maximal effective dose. The dose-response curves of peak antiallodynic effects of gabapentin in the absence and presence of ruthenium red also show that ruthenium red inhibited the effect of gabapentin in a noncompetitive manner (fig. 2D).

#### Effect of Spermine on Gabapentin-induced Antiallodynia

Intrathecal spermine, 120  $\mu\text{g}$ , produced motor weakness and a mild antiallodynic effect. Decreasing the dose of spermine to 60  $\mu\text{g}$  still produced a mild antiallodynic

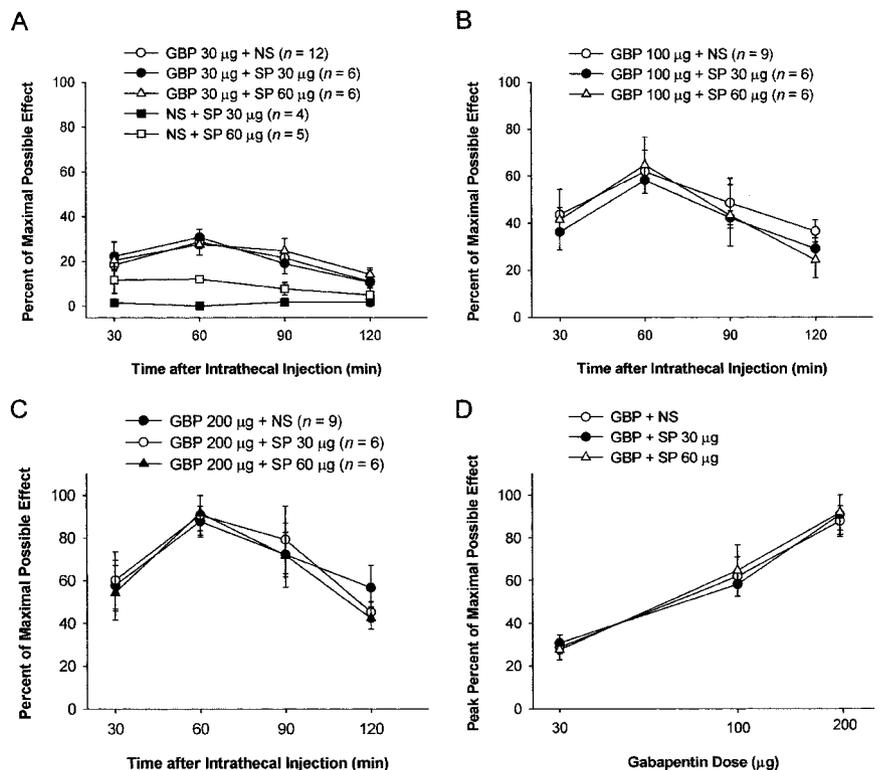
**Fig. 2.** Antialloodynic effects of intrathecal gabapentin (GBP) coadministered with normal saline (NS) or ruthenium red (RR). (A–C) Time courses of antialloodynic effects of 30 (A), 100 (B), or 200  $\mu$ g gabapentin (C) in the absence (GBP + NS) or presence (GBP + RR) of 0.2, 2, or 20 ng ruthenium red. Effect of 200 ng ruthenium red on the postoperative withdrawal threshold is also shown ( $\square$ , A). \* $P < 0.05$  versus GBP + NS group (two-way analysis of variance with *post hoc* Dunnett test). (D) Dose–response curves of peak (60 min after injection) antialloodynic effects of gabapentin coadministered with normal saline, 0.2, 2, or 20 ng ruthenium red. \* $P < 0.05$  versus GBP + NS group (two-tailed Student *t* test). Data are expressed as mean  $\pm$  SEM with *n* indicating the number of rats tested in each group.



effect (fig. 3A) but no motor weakness. At 30  $\mu$ g, spermine alone did not affect incision-induced allodynic response (fig. 3A). In contrast to the results of magnesium chloride and ruthenium red, spermine at 30 or 60  $\mu$ g did not affect the antialloodynic effect of gabapentin (fig. 3).

*Effects of Magnesium Chloride, Ruthenium Red, and Spermine on Morphine-induced Antialloodynia*  
Because the pharmacologic profiles of magnesium chloride, ruthenium red, and spermine are rather nonselective, we also tested whether coadministration of these

**Fig. 3.** Antialloodynic effects of intrathecal gabapentin (GBP) coadministered with normal saline (NS) or spermine (SP). (A–C) Time courses of antialloodynic effects of 30 (A), 100 (B), or 200  $\mu$ g gabapentin (C) in the absence (GBP + NS) or presence (GBP + SP) of 30 or 60  $\mu$ g spermine. Effects of 30 and 60  $\mu$ g spermine on the postoperative withdrawal threshold are also shown ( $\blacksquare$  and  $\square$ , respectively, A). \* $P < 0.05$  versus GBP + NS group (two-way analysis of variance with *post hoc* Dunnett test). (D) Dose–response curves of peak (60 min after injection) antialloodynic effects of gabapentin coadministered with normal saline, 30 or 60  $\mu$ g spermine. \* $P < 0.05$  versus GBP + NS group (two-tailed Student *t* test). Data are expressed as mean  $\pm$  SEM with *n* indicating the number of rats tested in each group.



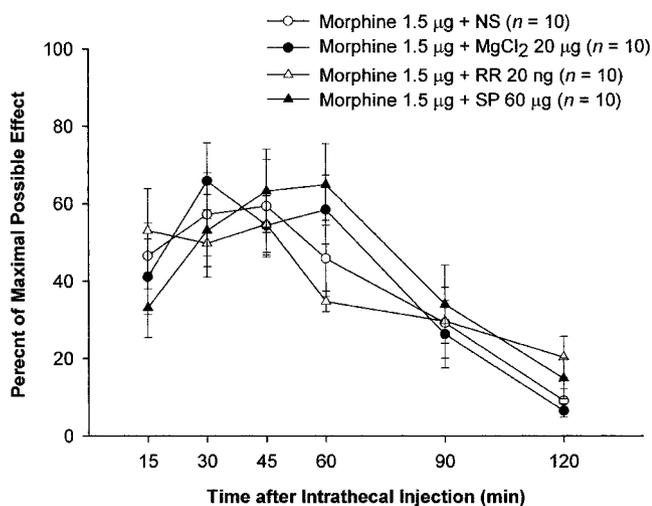


Fig. 4. Time courses of antiallodynic effects of 1.5 µg intrathecal morphine coadministered with normal saline (NS), 20 µg MgCl<sub>2</sub> · 6H<sub>2</sub>O, 20 ng ruthenium red (RR), or 60 µg spermine (SP). The antiallodynic effects are expressed as percent of maximal possible effect. Each point represents mean ± SEM with n indicating the number of rats tested in each group.

three agents could modulate the antiallodynic effect of intrathecal morphine, which is a well-known opioid receptor agonist and structurally unrelated to gabapentin.<sup>31</sup>

Intrathecal injection of 1.5 µg morphine also produced an antiallodynic effect in this postoperative pain model (fig. 4). However, this effect was affected neither by coadministration with 20 µg magnesium chloride or 20 ng ruthenium red, the doses that produced maximal attenuation of gabapentin's effect, nor by coadministration with 60 µg spermine ( $P > 0.05$ , two-way analysis of variance with *post hoc* Dunnett test; fig. 4).

## Discussion

In this study, we found that intrathecal magnesium chloride and ruthenium red but not spermine, all of which have been reported to modulate [<sup>3</sup>H]gabapentin binding to the  $\alpha_2\delta$  subunit of voltage-dependent Ca<sup>2+</sup> channels,<sup>9,16,22</sup> attenuate the antiallodynic effect of intrathecal gabapentin but have no effect on incision-induced allodynia *per se* in the postoperative pain model. On the other hand, all three of these agents did not affect the antiallodynic effect of intrathecal morphine in the same model. These findings suggest that the attenuating effects of magnesium chloride and ruthenium red are specific to the antiallodynic effect of intrathecal gabapentin and that the  $\alpha_2\delta$  subunit of Ca<sup>2+</sup> channels may be involved in the antiallodynic action of intrathecal gabapentin in the postoperative pain model.

### *The Effects of Mg<sup>2+</sup> and Ruthenium Red Are Not Nonspecific*

Mg<sup>2+</sup> and ruthenium red have many effects on the biologic system. Mg<sup>2+</sup> is a blocker of NMDA receptors<sup>32</sup>

and Ca<sup>2+</sup> channels<sup>33</sup> and is also involved in protein phosphorylation.<sup>34</sup> Ruthenium red is a ryanodine<sup>35</sup> and vanilloid<sup>36</sup> receptor antagonist, a mitochondrial Ca<sup>2+</sup> uniporter blocker,<sup>37</sup> and a nonselective Ca<sup>2+</sup> channel blocker.<sup>38</sup> However, it is unlikely that the attenuating effects of magnesium chloride and ruthenium red on gabapentin-induced antiallodynia are nonspecific because these two agents at maximal doses effective in attenuating gabapentin's effect did not reduce the antiallodynic effect of morphine, an analgesic that is structurally and pharmacologically irrelevant to gabapentin. Moreover, we did not observe any antiallodynic effect of magnesium chloride or ruthenium red at doses effective in attenuating gabapentin action, although intrathecal ruthenium red has been shown to inhibit formalin-induced nociceptive response in mice.<sup>39</sup>

In this study, the effective molar ratios of magnesium chloride (20 µg) and ruthenium red (20 ng) to gabapentin (200 µg), respectively, being approximately 0.09 and  $2 \times 10^{-5}$ , are much less than 1. Therefore, it is unlikely that the attenuating effects of magnesium chloride and ruthenium red are attributable to their chemical interactions or chelating with gabapentin. There is also no clue to support such direct chemical interactions in binding studies, because the IC<sub>50</sub> ratios of magnesium chloride and ruthenium red to gabapentin in inhibiting [<sup>3</sup>H]gabapentin binding are approximately 340–1,000 and 320, respectively—much higher than 1.<sup>9,16,22</sup>

### *Roles of N-methyl-D-aspartate Receptors in the Postoperative Pain Model*

Intrathecal NMDA receptor antagonists have been shown to possess little antinociceptive effect in the current postoperative pain model,<sup>40</sup> suggesting that NMDA receptor activation does not play an important role in the maintenance of postoperative pain behaviors. Mg<sup>2+</sup> and spermine are well-known NMDA receptor-channel modulators.<sup>32,41</sup> In this study, we also did not observe any significant antiallodynic effects of magnesium chloride and spermine at the doses tested. In a recent report of Prado *et al.*,<sup>42</sup> they also failed to find magnesium chloride to be effective in the postoperative pain model.

### *Roles of N-methyl-D-aspartate Receptors in Gabapentin Action*

Recently, gabapentin was found to increase the NMDA receptor-mediated current of rat spinal dorsal horn neurons at certain conditions, such as with elevated intracellular protein kinase C concentration,<sup>43</sup> in neurons isolated from inflamed but not normal rats,<sup>43</sup> in GABAergic but not non-GABAergic neurons,<sup>17</sup> or at extrasynaptic but inconsistently at synaptic NMDA receptors.<sup>44</sup> It is not known whether these possible mechanisms could be involved in the antinociceptive action of gabapentin.

The findings of Gu and Huang<sup>17</sup> raise the possibility that gabapentin may exert its antinociceptive action by

increasing the GABAergic neuron activity and subsequently enhancing the release of GABA in the spinal cord. If this is true, then the attenuating effect of magnesium chloride on gabapentin-induced antiallodynia observed in this study may be due to Mg<sup>2+</sup> blockade at NMDA receptors. However, we did not observe any attenuation or enhancement of gabapentin-induced antiallodynia by spermine, which is also a NMDA receptor modulator.<sup>41</sup> In addition, intraperitoneal gabapentin has been shown to prevent, but not to enhance, intrathecal NMDA-induced thermal hyperalgesia.<sup>45</sup> GABA receptor antagonists have been shown to be ineffective in preventing subcutaneous gabapentin-induced antinociception in rat pain models.<sup>46,47</sup> Taken together, all these findings suggest that gabapentin does not act as an NMDA receptor agonist or enhancer to achieve its antinociceptive effect, and the GABA system is not involved in the antinociceptive action of gabapentin. Consequently, it seems unlikely that the attenuation of gabapentin's effect by magnesium chloride observed in this study is due to the blocking effect of Mg<sup>2+</sup> on NMDA receptors.

#### *Roles of the $\alpha_2\delta$ Subunit of Ca<sup>2+</sup> Channels in Gabapentin Antiallodynia*

In the pioneer study revealing the specific binding site of gabapentin in rat brain, which was later identified to be the  $\alpha_2\delta$  subunit of voltage-dependent Ca<sup>2+</sup> channels by Gee *et al.*,<sup>21</sup> Suman-Chauhan *et al.*<sup>9</sup> have shown that magnesium chloride and spermine reduce the maximum binding capacity of [<sup>3</sup>H]gabapentin. Dissanayake *et al.*<sup>16</sup> also demonstrated that magnesium chloride and spermine concentration-dependently inhibit [<sup>3</sup>H]gabapentin binding in detergent-solubilized porcine cerebral cortical membranes but stimulate [<sup>3</sup>H]gabapentin binding to purified  $\alpha_2\delta$  subunit protein. They suggested that there are endogenous heat stable inhibitors of [<sup>3</sup>H]gabapentin binding in the detergent-solubilized membrane fraction.<sup>16</sup> Among various Ca<sup>2+</sup> channel ligands, Taylor *et al.*<sup>22</sup> found that only magnesium chloride, ruthenium red, and spermine significantly modulate [<sup>3</sup>H]gabapentin binding in mouse cerebral cortex, and the interactions are temperature dependent, stimulating [<sup>3</sup>H]gabapentin binding at 30°C but inhibiting it at 4°C in an allosteric manner.

Our finding that magnesium chloride and ruthenium red attenuated the antiallodynic effect of gabapentin agrees with the findings of Suman-Chauhan *et al.*<sup>9</sup> and Dissanayake *et al.*<sup>16</sup> However, it seems go against anticipation from the report by Taylor *et al.*<sup>22</sup> because the normal body temperature of the rat is around 37°C, which is closer to 30°C than 4°C. The reason for this discrepancy is not clear but may be due to different conditions between *in vitro* and *in vivo* studies. Similar change in the direction from inhibition to stimulation of [<sup>3</sup>H]gabapentin binding has been noted when the bind-

ing preparation was changed from solubilized cerebral cortical membranes to purified  $\alpha_2\delta$  subunit protein.<sup>16</sup> The endogenous inhibitors of gabapentin binding<sup>16</sup> may complicate the interaction of gabapentin binding with magnesium chloride and ruthenium red *in vivo*. The noncompetitive inhibitory effects of magnesium chloride and ruthenium red observed in this study agree with their allosteric interactions with [<sup>3</sup>H]gabapentin in the previous binding study.<sup>22</sup> This result also supports the idea that the binding site of Mg<sup>2+</sup> and ruthenium red on the  $\alpha_2\delta$  subunit of Ca<sup>2+</sup> channels is different from that of gabapentin.<sup>22</sup>

In this study, the estimated effective concentrations of gabapentin in the cerebrospinal fluid could be 0.44–2.9 mM, assuming the total cerebrospinal fluid volume of the adult rat is 400  $\mu$ l<sup>48</sup> and the injected drugs were homogeneously distributed in the cerebrospinal fluid. These concentrations are much higher than the K<sub>D</sub> values, 59–153 nM, of gabapentin at the  $\alpha_2\delta$  subunit of Ca<sup>2+</sup> channels.<sup>49</sup> Although we do not know the exact concentration of gabapentin in the spinal cord after intrathecal injection, there remains a possibility that a much higher concentration of gabapentin than its K<sub>D</sub> values at the  $\alpha_2\delta$  subunit of Ca<sup>2+</sup> channels is required *in vivo* to achieve its antiallodynic effect. Penetration barriers or endogenous inhibitors of gabapentin binding,<sup>16</sup> such as L-isoleucine,<sup>50</sup> could partly explain that concentration difference. These barriers or inhibitors may also explain the much lower molar ratios of Mg<sup>2+</sup> and ruthenium red to gabapentin in this study than their respective IC<sub>50</sub> ratios in inhibiting [<sup>3</sup>H]gabapentin binding (approximately 340–1,000 and 320).<sup>9,16,22</sup> This suggests that much less Mg<sup>2+</sup> and ruthenium red are needed *in vivo* than *in vitro* to inhibit gabapentin binding.

The  $\alpha_2\delta$  subunit is an auxiliary subunit of Ca<sup>2+</sup> channels, and its coexpression with the pore-forming  $\alpha_1$  subunit results in a significant increase in whole cell Ca<sup>2+</sup> current.<sup>51</sup> The  $\alpha_2\delta$  subunit of Ca<sup>2+</sup> channels in the spinal cord, and dorsal root ganglia has been shown to be up-regulated in rats with nerve ligation-induced neuropathy.<sup>52</sup> This up-regulation has a time course that is parallel to the duration of allodynia and could be alleviated by intrathecal gabapentin.<sup>52</sup> In the central terminals of primary sensory nerve, Ca<sup>2+</sup> influx through voltage-dependent Ca<sup>2+</sup> channels is generally believed to be responsible for the release of excitatory neurotransmitters, such as glutamate and substance P.<sup>53</sup> Gabapentin has been shown to inhibit glutamatergic synaptic transmission presynaptically in the superficial lamina of spinal cord<sup>18</sup> and whole cell Ca<sup>2+</sup> current in cultured dorsal root ganglion neurons.<sup>50</sup> Through binding to the  $\alpha_2\delta$  subunit of Ca<sup>2+</sup> channels, intrathecal gabapentin may reduce nerve terminal Ca<sup>2+</sup> influx and excitatory neurotransmitter release to achieve its antinociceptive effect.

### The Ineffectiveness of Spermine

Spermine is a naturally occurring polyamine that could interact with NMDA,<sup>41</sup> GABA<sub>A</sub>,<sup>54</sup> A<sub>1</sub> adenosine,<sup>55</sup> and  $\sigma$ -opioid<sup>56</sup> receptors as well as Ca<sup>2+</sup> channels.<sup>57,58</sup> In this study, intrathecal spermine, unlike magnesium chloride and ruthenium red, at the dose without motor blocking effect (60  $\mu$ g), did not modulate the antiallo-dynamic effect of gabapentin. The reason is not clear. The dose of 60  $\mu$ g may be insufficient to modulate gabapentin binding to the  $\alpha_2\delta$  subunit, and the results of using higher doses of spermine were confounded by its motor-blocking effect. Alternatively, spermine may modulate gabapentin binding to the  $\alpha_2\delta$  subunit from the intracellular site, which is exposed and accessible in binding studies but not in this behavioral test. In this regard, spermine has been shown to block neuronal Ca<sup>2+</sup> current from the intracellular site.<sup>59</sup> This may also explain the different results between spermine and ruthenium red, which is a chemical dye and not intracellularly synthesized.

In summary, magnesium chloride and ruthenium red, which *in vitro* modulate [<sup>3</sup>H]gabapentin binding to the  $\alpha_2\delta$  subunit of Ca<sup>2+</sup> channels, attenuate the antiallo-dynamic effect of intrathecal gabapentin in the rat model of postoperative pain. These results provide behavioral evidence to support that binding to the  $\alpha_2\delta$  subunit of Ca<sup>2+</sup> channels is involved in the antiallo-dynamic action of intrathecal gabapentin. Given that complete reversal of gabapentin's effect was not obtained at the maximal effective doses of magnesium chloride and ruthenium red in the current study, which is consistent with their incomplete inhibition of [<sup>3</sup>H]gabapentin binding,<sup>22</sup> it remains to be elucidated whether other mechanisms, such as activation of adenosine 5'-triphosphate-sensitive K<sup>+</sup> channels,<sup>20</sup> also contribute to the antiallo-dynamic action of intrathecal gabapentin.

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