

# Mice with a Melanocortin 1 Receptor Mutation Have a Slightly Greater Minimum Alveolar Concentration than Control Mice

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ANESTHESIA folklore includes a perception that patients with red hair have a greater MAC (the minimum alveolar concentration of anesthetic that prevents movement in response to noxious stimuli in 50% of subjects). In support of this perception, Liem *et al.* found that a greater concentration of the inhaled anesthetic desflurane was required to suppress movement in response to intense electrical stimulation in red haired humans.<sup>1</sup> Such a finding has obvious clinical implications. In addition, a determination of the underlying cause might provide some insight into the mechanisms by which inhaled anesthetics act.

Loss of function mutations in the melanocortin 1 receptor (*MC1R*) gene account for the majority of cases of red hair in humans. Mice with a melanocortin 1 receptor mutation (*MC1R<sup>e-J</sup>*) resulting in a nonfunctional receptor have a yellow coat.<sup>2-9</sup> These observations suggested the hypothesis that *MC1R<sup>e-J</sup>* mice have greater MAC values than control mice. Accordingly, we determined desflurane, isoflurane, halothane, and sevoflurane MAC values for both *MC1R<sup>e-J</sup>* and control mice.

## Materials and Methods

With the approval of the Committee on Animal Research of the University of California, San Francisco, we determined MAC in 22 (14 male, 8 female) 8- to 12-week-old, 20-30 g B6.C-H2<sup>bm12</sup>/KHEg-Mc1r<sup>e-J</sup> congenic mice (obtained from the Jackson Labs, Bar Harbor, Maine, stock no. 003625) harboring a spontaneous mutation in the melanocortin 1 receptor. These mice have deletion of a nucleotide at position 549, which results in a frameshift mutation for 12 amino acids and then termination of the protein. The mice are recessive, with yellow coats and black eyes. The

resulting MAC values were compared with those obtained in 18 (11 male, 7 female) control mice having black coat and eyes, obtained as heterozygotes from the colony, *i.e.*, with *Mc1r<sup>e-J/+</sup>* genotypes because the colony is maintained by breeding homozygotes with heterozygotes. Animals were housed 4 to 5 per cage under 12-h cycles of light and dark for a week before study and had continuous access to standard mouse chow and tap water.

A total of 86 MAC determinations were made. MAC values for halothane (Halocarbon Laboratories, River Edge, NJ), desflurane (Baxter Healthcare Corp, New Providence, NJ), isoflurane (Baxter Healthcare Corp), and sevoflurane (Abbott Laboratories, North Chicago, IL) were determined. Each mouse provided one or more MAC values (some mice died before all MAC values could be obtained), with at least 1 week separating MAC determinations. MAC values were measured as previously described.<sup>10,11</sup> We equilibrated each animal with each halothane concentration for 40 min, with desflurane for 20 min, with isoflurane for 30 min, and with sevoflurane for 30 min.

For study, all animals were kept in individual gas-tight plastic chambers connected to a circle rebreathing system containing a carbon dioxide absorber and fan. Volatile anesthetics were delivered in oxygen using commercial anesthesia vaporizers. Rectal temperature were maintained between 36°C and 38°C. Inhaled anesthetic partial pressures were monitored with an infrared analyzer (Datascop, Helsinki, Finland), but the concentration used in the calculation of MAC was obtained using gas chromatography. After the equilibration period, a tail clamp was applied to the proximal portion of the tail and oscillated 45 degrees at approximately 1 Hz for 1 min or until the animal moved (whichever came first). The anesthetic partial pressure was then increased by 10-20% of the previous step until the anesthetic partial pressures bracketing movement and lack of movement during application of the tail-clamp stimulus were determined.

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## Data Analysis

The null hypothesis of this study was that there was no difference in MAC between mutant and control mice. The data were analyzed using a two-way analysis of variance, with choice of anesthetic (sevoflurane *vs.* desflurane *vs.* isoflurane *vs.* halothane) and genotype (mutant *vs.* control) as the two factors. Differences between in MAC between mutant and control mice were determined using a Student *t* test for individual anesthetics. A value of *P* < 0.05 was taken as the significance threshold.

**Table 1. MAC of Four Inhaled Anesthetics in Melanocortin 1 Receptor Knockout Mice and in Control Mice**

Anesthetic	Number of MC1R* Mutant Mice	MAC†	No. of Control Mice	MAC†	P‡
Isoflurane	12	1.69 ± 0.04	13	1.61 ± 0.04	0.1283
Sevoflurane	9	3.50 ± 0.13	8	3.27 ± 0.14	0.2464
Desflurane	17	8.19 ± 0.19	9	7.61 ± 0.23	0.0659
Halothane	9	1.31 ± 0.02	9	1.27 ± 0.03	0.3108

\* MC1R = melanocortin 1 receptor. † MAC = minimum alveolar concentration. MAC is expressed as mean partial pressure ± standard error in percent atmospheres. ‡ "P" = significance value for a Student T test comparing MAC for MC1R and control mice.

## Results

A two-way analysis of variance showed that MAC values depended on the choice of anesthetic, as expected, with  $P < 0.001$  associated with this factor. There was no significant difference between mutant and control mice for individual anesthetics using a *t* test (table 1). However, the question of a difference in MAC between genotypes was addressed with greater power for the larger sample size comprising all MAC determinations regardless of anesthetic, by examining the significance of the genotype factor in the two-way analysis of variance. Using this analysis, the null hypothesis of no difference in MAC for MC1R mutant mice *versus* control mice was rejected, with  $P = 0.023$  for this factor. That is, there was a significant difference between recessive mice that were homozygous for the MC1R mutation and control mice. This effect was, however, small with only on average a 5.5% increase in MAC in mutant compared to control mice. There was no significant genotype/anesthetic interaction ( $P = 0.200$ ).

## Discussion

We observed a significant ( $P = 0.023$ ) difference in anesthetic requirement between recessive homozygous mice harboring two nonfunctional genes for the melanocortin 1 receptor, and control heterozygous mice with one functional and one nonfunctional gene for the melanocortin 1 receptor. Anesthetic requirement was studied for four clinical anesthetics (isoflurane, desflurane, sevoflurane, and halothane). Taken in aggregate for all MAC determinations and agents, mutant mice had, on average, a 5.5% increase in MAC. This result is consistent with the observation that red-headed people require more desflurane to produce immobility, but is smaller than the difference reported in humans.<sup>1</sup>

The MC1R is one of several melanocortin receptors (MCRs). Five genes that code for melanocortin receptors have been cloned and the properties of the receptors they produce (MC1R, MC2R, MC3R, MC4R, and MC5R) have been characterized.<sup>12-14</sup> All melanocortin receptors are proteins with seven transmembrane domains coupled to G-proteins. MC1Rs are mainly found in the periphery, but they also are expressed in brain glial cells and in neurons of the ventral periaqueductal gray, a region known to modulate nociception.<sup>13-17</sup> MC3R and

MC4R are mainly expressed in the nervous system and may influence nociception, hyperalgesia, and pain. The melanocortins, including adrenocorticotropic hormone (ACTH),  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH),  $\beta$ -MSH, and  $\gamma$ -MSH, are a family of bioactive peptides that share similar structures and bind to the melanocortin receptors to conduct their biologic functions. Whereas all the melanocortins (ACTH,  $\alpha$ -MSH,  $\beta$ -MSH, and  $\gamma$ -MSH) bind to MC1R, MC3R, MC4R, and MC5R to conduct their functions, ACTH binds only to MC2R.<sup>18-20</sup> Among the five melanocortin receptors in humans, MC1R has the highest affinity for  $\alpha$ -MSH.<sup>21</sup> Phenotypic changes (*e.g.*, such as MAC) might result from differences in binding. In the MC1R mutant mice,  $\alpha$ -MSH cannot bind to the MC1R, potentially leaving a higher concentration available to bind to and activate MC3R and MC4R. Unlike the melanocortin 1 receptor, MC3R and MC4R are mainly expressed in the nervous system. Several studies indicate that these two receptors modulate hyperalgesia, pain, behavior, stress, and food intake.

How might MC1R itself mediate the MAC of inhaled anesthetics? MC1R regulates hair and skin pigmentation and immunomodulation and antiinflammatory effects, but it is difficult to see how these might acutely influence anesthetic requirement as defined by MAC. However, as noted, MC1Rs are also expressed in brain glial cells and neurons of the ventral periaqueductal gray and thus may affect nociception,<sup>15-20,22</sup> and through this mechanism might influence MAC. For example, Mogil *et al.* report that the MC1R gene contributes to analgesia in female mice and humans.<sup>23</sup>

A change in central  $\alpha$ -MSH concentrations in MC1R mutant mice may be responsible for the increased MAC. The pituitary gland synthesizes  $\alpha$ -MSH, and synthesis probably is controlled by a negative feedback system.  $\alpha$ -MSH is derived from a precursor protein, proopiomelanocortin (POMC).<sup>19</sup> Injection of  $\alpha$ -MSH into the paraventricular hypothalamic nucleus decreases POMC gene expression in the arcuate nucleus of the hypothalamus (ARC).<sup>24</sup> Thus, the MC1R dysfunction in MC1R mutant mice may increase the concentration of  $\alpha$ -MSH in these mice. Contreras and Takemori reported that  $\alpha$ -MSH antagonized the analgesic effect of morphine.<sup>25</sup> Tail-flick tests showed that  $\alpha$ -MSH could induce hyperalgesia, and  $\gamma$ -MSH has an analgesic effect that may be mediated by

a GABA-ergic mechanism in rats.<sup>26,27</sup> We have shown that the GABA<sub>A</sub> receptor can modulate the MAC of isoflurane.<sup>28-30</sup> In the MC1R mutant mice, MC1R dysfunction may increase  $\alpha$ -MSH and thereby increase MAC.

In summary, MAC in mice with nonfunctional MC1R receptors slightly exceeds that for control mice. This may result from MC1R dysfunction, interactions among MC1R, MC3R, and MC4, or consequent changes in the concentrations of the melanocortins, such as  $\alpha$ -MSH.

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## Delta-opioid Agonist SNC80 Can Attenuate the Development of Dynorphin A-induced Tactile Allodynia in Rats

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DYNORPHIN A is an endogenous opioid peptide with a high degree of selectivity for  $\kappa$ -opioid receptors. It has been reported that high dosages or sustained exposure of dynorphin A may cause neurologic dysfunction including hyper-

algesia, allodynia,<sup>1-3</sup> and paralysis.<sup>4</sup> There is also considerable evidence that levels of dynorphin A increase significantly at the sites of spinal cord injuries<sup>5</sup> and in the spinal cord after nerve injury,<sup>6,7</sup> and the increase of dynorphin levels in the spinal cord was associated with neurologic dysfunction.<sup>8</sup> As neuropathic pain remains a devastating sequela after spinal cord trauma and nerve injury, further understanding regarding pathophysiological mechanisms and treatment of dynorphin-induced behavioral dysfunction may become a key for strategies of pain management in such patients.

Recent evidence suggested the antinociceptive effects of  $\delta$ -opioid agonists in a variety of pain models.<sup>9,10</sup>

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SNC80, (+)-4-[( $\alpha$ R)- $\alpha$ -(2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl-N,N-diethylbenzamide, is a highly selective nonpeptidic delta opioid agonist. Although peptidic opioids may rapidly degrade, nonpeptidic opioids are proteolytically stable and have enhanced bioavailability relative to the peptidic opioids.<sup>11,12</sup> Although several investigators have demonstrated the antinociceptive effects of SNC80 in chronic pain models,<sup>12,13</sup> there has been no data on its antiallodynic effect. We hypothesized that  $\delta$ -opioid agonist SNC80 can attenuate dynorphin A-induced tactile allodynia. First, to determine the dosage of dynorphin and time course of dynorphin-induced allodynia, dose-dependent effects of intrathecally administered dynorphin on the tactile allodynia were evaluated. Second, the effect of *N*-methyl-D-aspartate receptor antagonist MK801 on dynorphin-induced tactile allodynia was assessed. Finally, the effects of SNC80 on dynorphin A-induced tactile allodynia were investigated.

## Materials and Methods

The Animal Experiment Committee of Nara Medical University approved this study. All experimental procedures were performed in accordance with the guidelines established in the Guide for the Care and Use of Laboratory Animals available from the National Academy of Science (Washington, DC).

### Animals

Male Sprague-Dawley rats (300–350 g; Japan SLC, Shizuoka, Japan) were housed in cages with 12–24h light-dark cycle and were allowed free access to food and water.

### Drugs

Dynorphin A(1-13) (Biogenesis Ltd, Poole, England) and MK801 (EMD Biosciences Inc. San Diego, CA) were dissolved in saline, 0.9%. SNC80 (ALEXIS Biochemicals, Lausanne, Switzerland) was prepared in its vehicle (saline, 0.9%, and 100 mM HCl).

### Intrathecal Catheter Implantation

Under isoflurane anesthesia (2% in oxygen-air), the rats were implanted catheters according to the method described by Yaksh and Rudy.<sup>14</sup> A PE-10 polyethylene tube (8.5 cm) was inserted through the atlanto-occipital membrane and to the lumbar enlargement.

### Nociceptive Behavioral Testing

Mechanical allodynia was determined by measuring the paw withdrawal in response to probing with von Frey filaments. A 50% withdrawal threshold was determined by increasing and decreasing stimulus strength eliciting paw withdrawal and estimating the paw withdrawal threshold by a Dixon nonparametric test.<sup>15</sup> Rats with a baseline threshold less than 10 g were excluded from this study.

### Experimental Protocol

Rats were allowed 7 days to recover from the intrathecal implantation, and any rats exhibiting motor deficiency were discarded from testing. We measured the baseline paw withdrawal threshold (control value). In our preliminary study, we demonstrated that even 5 nmol of intrathecal dynorphin A (1-13) produced transient hind limb paralysis in some animals, resulting in a failure of behavioral assessment. Therefore, we used lower doses of dynorphin less than 5 nmol. In the first study, the rats were randomly allocated to one of four groups. Animals received saline (group S;  $n = 8$ ) or dynorphin A (1-13) 0.25 nmol (group D0.25;  $n = 7$ ), 1 nmol (group D1;  $n = 9$ ), or 2 nmol (group D2;  $n = 7$ ). All drugs were injected intrathecally in a volume of 5  $\mu$ l followed by a 9- $\mu$ l flush.<sup>14</sup> In a single blind manner, that is, the observer had no information as to the group designation, we measured paw withdrawal threshold at 10 min, 30 min, 1 h, 2 h, 4 h, 8 h, 1 day, 3 days, and 7 days after intrathecal injection. In the second study, rats were randomly allocated to one of three groups ( $n = 7$  in each group). In the groups M5 and M10, 5 nmol or 10 nmol of MK801, respectively, was intrathecally administered 20 min before intrathecal injection of 2 nmol of dynorphin A (1-13). In the group S, saline was intrathecally administered. In study 3, the rats were randomly allocated to one of three groups ( $n = 9$  in each group). In the groups S25 and S100, 25 nmol or 100 nmol of SNC80, respectively, was intrathecally administered 20 min before intrathecal injection of 2 nmol of dynorphin A (1-13). In the group S, saline with 100 mM HCl was intrathecally administered. In studies 2 and 3, an observer blinded to the drug applications measured paw withdrawal threshold at 10 min, 30 min, 1 h, 2 h, 4 h, and 8 h after intrathecal injection of dynorphin.

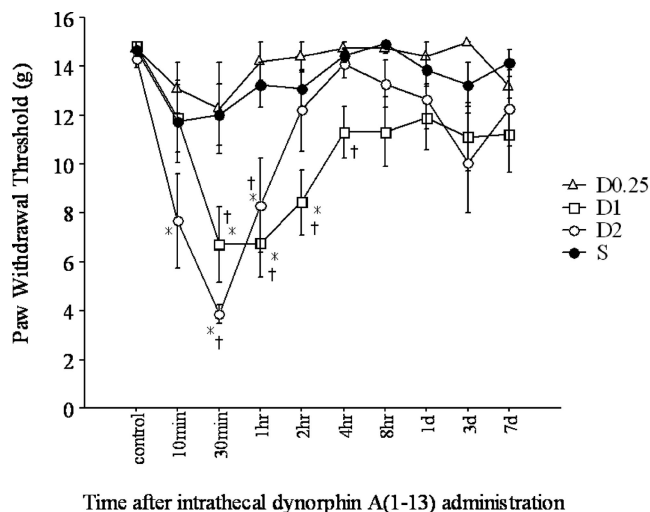
### Statistics

All data are expressed as mean  $\pm$  SEM. Statistical analysis was performed using two-way analysis of variance with repeated measurements followed by Student-Newman-Keuls test for multiple comparisons. A *P* value of  $<0.05$  was considered statistically significant.

## Results

### Dynorphin-Induced Tactile Allodynia

As the withdrawal thresholds of left and right hind paws were similar in each group, mean values of the bilateral thresholds were used for further analysis. As shown in figure 1, the intrathecal administration of 2 nmol of dynorphin A (1-13) produced a significant reduction of the withdrawal threshold at 10 min, 30 min, and 1 h after injection compared with the control values and paw withdrawal threshold at 30 min and 1 h after injection was significantly lower than in the group S. The intrathecal administration of 1 nmol of dynorphin A (1-13) also produced a significant reduction in the withdrawal threshold at 30 min, 1 h, and 2 h after injection



**Fig. 1.** Time-response curves for tactile allodynia induced by intrathecal injections of dynorphin A (1-13). Saline, 0.9%, (group S; closed circle,  $n = 8$ ), 0.25 nmol of dynorphin (group D0.25; triangle,  $n = 7$ ), 1 nmol of dynorphin (group D1; square,  $n = 9$ ), or 2 nmol of dynorphin (group D2; open circle,  $n = 7$ ) was intrathecally administered and paw withdrawal thresholds were assessed by using von Frey filaments. \*  $P < 0.05$  versus control; †  $P < 0.05$  versus group S. Data are expressed as mean  $\pm$  SEM.

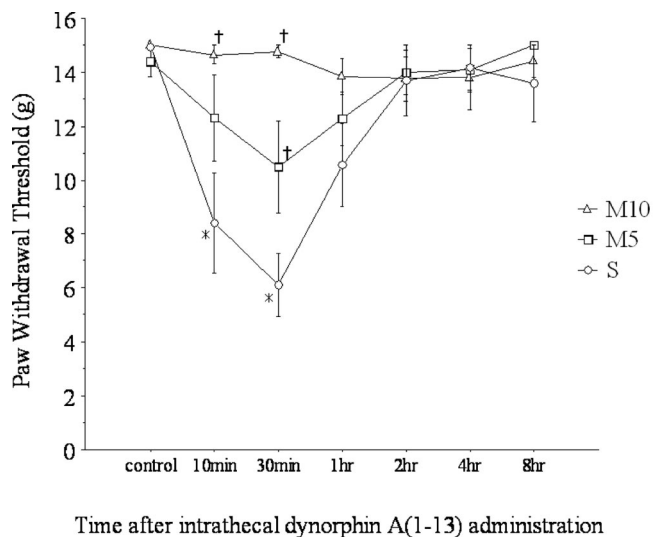
compared with the control values and paw withdrawal threshold at 30 min, 1 h, 2 h, and 4 h after injection was significantly lower than in the group S.

#### Effect of MK801 on Dynorphin-Induced Tactile Allodynia

Changes in withdrawal thresholds after 2 nmol of dynorphin intrathecal injection with or without MK-801 pretreatment are shown in figure 2. Rats pretreated with saline elicited a significant reduction in the withdrawal threshold at 10 min and 30 min after injection. Pretreatment with 5 or 10 nmol of MK801 failed to elicit a significant reduction in the withdrawal threshold compared with the control values. Furthermore, withdrawal thresholds at 10 min and 30 min after injection in the group M10 and at 30 min in the group M5 were significantly higher than in the group S.

#### Effect of SNC80 on Dynorphin-Induced Tactile Allodynia

Changes in withdrawal thresholds after 2 nmol of dynorphin intrathecal injection with or without SNC80 pretreatment are shown in figure 3. Rats pretreated with saline with 100 mM HCl elicited a significant reduction in the withdrawal threshold at 10 min, 30 min, and 1 h after injection. In the group S25, withdrawal thresholds were significantly reduced at 30 min after injection. In the group S100, withdrawal thresholds remained unchanged during the observation period. Withdrawal thresholds at 10 min, 30 min and 1 h after dynorphin injection were significantly higher in the group S100 compared with those in the group S.

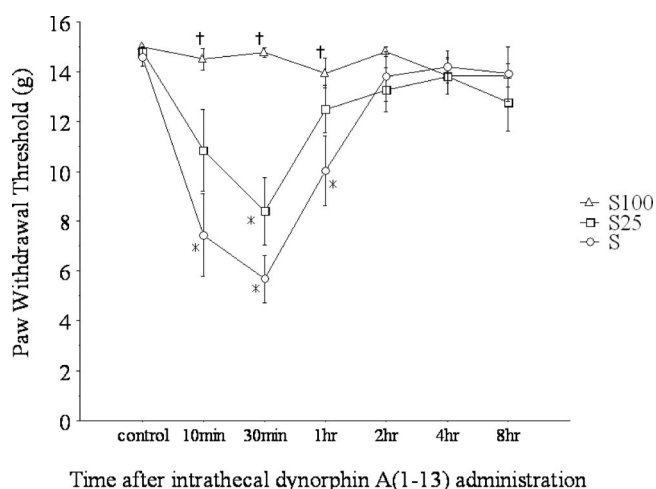


**Fig. 2.** Effect of MK801 on 2 nmol of dynorphin A (1-13)-induced tactile allodynia. Saline, 0.9%, (group S; circle,  $n = 7$ ), 10 nmol of MK801 (group M10; triangle,  $n = 7$ ), or 5 nmol of MK801 (group M5; square,  $n = 7$ ) was intrathecally administered 20 min before intrathecal injection of 2 nmol of dynorphin A (1-13), and paw withdrawal thresholds were assessed by using von Frey filaments. \*  $P < 0.05$  versus control; †  $P < 0.05$  versus group S. Data are expressed as mean  $\pm$  SEM.

## Discussion

This study shows that intrathecally administered dynorphin A (1-13) produced transient tactile allodynia and pretreatment with MK-801 or SNC80 dose-dependently attenuated the development of tactile allodynia induced by dynorphin A.

As reported previously,<sup>1-3</sup> intrathecal administration of dynorphin A produced tactile allodynia. Dynorphin-in-



**Fig. 3.** Effect of SNC80 on 2 nmol of dynorphin A (1-13)-induced tactile allodynia. Saline, 0.9%, with 100 mM HCl (group S; circle,  $n = 9$ ) or 100 nmol of SNC80 (group S100; triangle,  $n = 9$ ), or 25 nmol of SNC80 (group S25; square,  $n = 9$ ) was intrathecally administered 20 min before intrathecal injection of 2 nmol of dynorphin, and paw withdrawal thresholds were assessed by using von Frey filaments. \*  $P < 0.05$  versus control; †  $P < 0.05$  versus group S. Data are expressed as mean  $\pm$  SEM.

duced allodynia peaked at 30 min after injection, but lasted for only a short period. These findings are in contrast to the results in the previous study.<sup>1,2</sup> Vandrah *et al.*<sup>1</sup> reported that intrathecal administration of dynorphin A produced a significant long-lasting tactile allodynia up to 60 days after injection. The reasons of these contradictory results are unknown. However, the dosage of dynorphin might have affected the results.

The mechanisms by which intrathecal administration of dynorphin induces tactile allodynia are unknown. Several possible explanations are as follows. First, dynorphin-induced allodynia may be attributable to ischemic injury after the reduction of spinal cord blood flow.<sup>16</sup> Second, *N*-methyl-D-aspartate receptors may be involved in the development of tactile allodynia after intrathecal administration of dynorphin A.<sup>1-3</sup> In fact, we confirmed that dynorphin-induced allodynia could be reversed by pretreatment with *N*-methyl-D-aspartate antagonist MK-801 in the present study.

Although the classic view has been that neuropathic pain is resistant to opioid therapy, recent evidence suggested an important role of delta opioid receptor agonists in antinociception at the level of the spinal cord.<sup>9,10</sup> These findings were compatible with the results in the present study, in which SNC80 attenuated tactile allodynia. The mechanisms of antiallodynic effect of intrathecally administered SNC80 observed in the present study are unknown. However, glutamate- and glutamate receptor-mediated responses might be at least involved in antiallodynic effect of SNC80. Zhang *et al.*<sup>17</sup> demonstrated that delta opioid receptor agonist, [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]-enkephalin (DADLE), reduced glutamate-induced excitotoxic injury. Wang *et al.*<sup>18</sup> reported that  $\delta$ -opioid agonist, [D-Phe<sup>2</sup>, D-Phe<sup>5</sup>]-enkephalin (DPDPE) attenuated *N*-methyl-D-aspartate-evoked responses of nociceptive neurons. In fact, *N*-methyl-D-aspartate receptor antagonists have been shown to attenuate antinociception induced by  $\delta$ -opioid receptor agonists.<sup>19,20</sup> Considering that dynorphin-induced allodynia was at least in part mediated through the *N*-methyl-D-aspartate receptor activation, SNC80 might exert antiallodynic effects by inhibiting glutamate- and *N*-methyl-D-aspartate-mediated responses in this model. Further investigations would be required to clarify the exact mechanisms of antiallodynic effect of SNC80.

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