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Experimental Research Article

# Involvement of the spinal γ-aminobutyric acid receptor in the analgesic effects of intrathecally injected hypertonic saline in spinal nerve-ligated rats

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ABSTRACT

**Background:** Hypertonic saline is used for treating chronic pain; however, clinical studies that aid in optimizing therapeutic protocols are lacking. We aimed to determine the concentration of intrathecally injected hypertonic saline at which the effect reaches its peak as well as the underlying γ-aminobutyric acid (GABA) receptor-related antinociceptive mechanism.

**Methods:** Spinal nerve ligation (SNL; left L5 and L6) was performed to induce neuropathic pain in rats weighing 250–300 g. Experiment 1: one week after implanting the intrathecal catheter, 60 rats were assigned randomly to intrathecal injection with 0.45%, 0.9%, 2.5%, 5%, 10%, and 20% NaCl, followed by behavioral testing at baseline and after 30 minutes, 2 hours, 1 day, and 1 week to determine the minimal concentration which produced maximal analgesia. Experiment 2: after determining the optimal intrathecal hypertonic saline concentration, 60 rats were randomly divided into four groups: Sham, hypertonic saline without pretreatment, and hypertonic saline after pretreatment with one of two GABA receptor antagonists ($GABA_A$ [bicuculline], or $GABA_B$ [phaclofen]). Behavioral tests were performed at weeks 1 and 3 following each treatment.

**Results:** Hypertonic saline at concentrations greater than 5% alleviated SNL-induced mechanical allodynia and had a significant therapeutic effect, while showing a partial time- and dose-dependent antinociceptive effect on thermal and cold hyperalgesia. However, pretreatment with GABA receptor antagonists inhibited the antinociceptive effect of 5% NaCl.

**Conclusions:** This study indicates that the optimal concentration of hypertonic saline for controlling mechanical allodynia in neuropathic pain is 5%, and that its analgesic effect is related to $GABA_A$ and $GABA_B$ receptors.

**Keywords:** Analgesia; Bicuculline; GABA Antagonists; Hyperalgesia; Hypertonic Saline; Neuralgia; Phaclofen; Receptors, GABA; Injections, Spinal.

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## INTRODUCTION

The first report on using hypertonic saline for pain management was published in 1969 by Hitchcock [1], applied to the treatment of intractable facial cancer pain. Hitchcock and Prandini [2] demonstrated that, although the period of pain relief was short, an intrathecal injection of hypertonic saline is effective, simple, and rarely causes complications. However, further studies have reported fatal adverse effects from intrathecally injected hypertonic saline, such as pulmonary edema, myocardial infarction, paraplegia or quadriplegia, and hearing loss [3,4]. Therefore, the administration of intrathecal hypertonic saline should be avoided in human administration in favor of potential epidural administration. A study of dural permeability in dogs demonstrated that the transdural equilibrium of hypertonic saline occurs very slowly but results in a doubling of the cerebrospinal fluid sodium concentration 20 minutes after the extradural placement of 10% hypertonic saline [5]. Epidural administration of the hypertonic saline is an important component of percutaneous epidural neuroplasty, which was introduced by Racz, and has also been used as an adjunct to epidural blocks [5–13]. Although hypertonic saline is clinically used for the treatment of chronic pain, including neuropathic pain, preclinical studies to help guide physicians with optimal therapeutic protocols are lacking. In addition, little is known about the mechanism underlying the antinociceptive effects of hypertonic saline.

The inhibitory neurotransmitter with the greatest distribution throughout the central nervous system is γ-aminobutyric acid (GABA) [14]. The $GABA_A$ receptor is an ionotropic ligand-gated $Cl^-$ channel, and its activation surges the permeability of $Cl^-$ and hyperpolarizes postsynaptic neurons [15,16]. It has been suggested that one of the mechanisms of action of hypertonic saline is related to its high chloride ion concentration, associated with fast synaptic inhibition mediated by the $GABA_A$ receptor [17].

The present study hypothesized that the hypertonic saline intrathecal injection would alleviate nociceptive behaviors in rat models of spinal nerve ligation (SNL)-induced neuropathic pain. Hypertonic saline is used for treating chronic pain, but clinical studies that aid in optimizing therapeutic protocols are lacking. This study aimed to determine the concentration at which the effect reaches its peak and as well as the GABA receptor-related antinociceptive mechanism of intrathecally injected hypertonic saline. Although hypertonic saline is injected into the epidural space in clinical settings, the site of action is the intrathecal space due to transdural equilibration [5]. Therefore, injecting hypertonic saline intrathecally to investigate its analgesic mechanism, which is the purpose of this study, is logical. These findings will provide basic research evidence for the clinical application of hypertonic saline.

## MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at the Institute for Life Sciences in the Asan Medical Center approved the experiments (No. 2017-13-019), and this committee follows the guidelines of the Institute of Laboratory Animal Resources. The ethical guidelines of the International Association for the Study of Pain and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health were followed during all procedures. Every attempt was made to minimize the number of animals used and their suffering.

### 1. Experimental animals and the SNL model

All trials were conducted using male Sprague-Dawley rats weighing 250–300 g. Conventional experimental preparations followed the protocols of a previous study [18]. The neuropathic model was established as previously described by Kim and Chung [19].

### 2. Implantation of the intrathecal catheter

For the intrathecal injection of drugs, each rat with neuropathy was implanted with a polyethylene-10 catheter seven days after the SNL surgery [20]. A behavioral test was performed one week after the intrathecal catheter implantation surgery to confirm the SNL model status (**Figs. 1, 2**).

### 3. Behavioral tests

Behavioral tests were conducted from 10:00 AM to 4:00 PM each testing day to reduce experimental variation. The experimental animal was placed in a transparent plastic cage with a wire mesh (8 × 8 mm) at the bottom and was kept in the cage for at least 20 minutes to allow it to adjust to the cage. Prior to the drug administration, a baseline behavioral test was conducted to determine whether the neuropathic pain model was successfully established after the SNL surgery. Until the cage investigation and the main grooming activities were terminated,

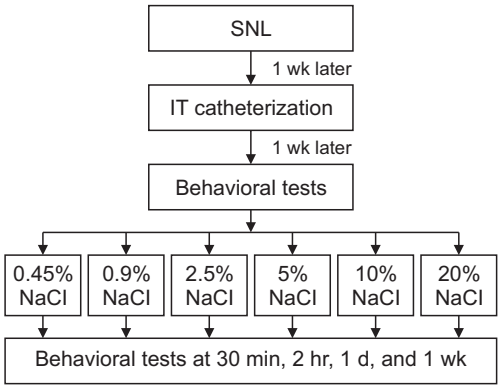


**Fig. 1.** Flowchart of the NaCl concentration experiment. Behavioral tests include the von Frey filaments, hot-plate, and cold-plate tests. NaCl: sodium chloride, SNL: spinal nerve ligation, IT: intrathecal.

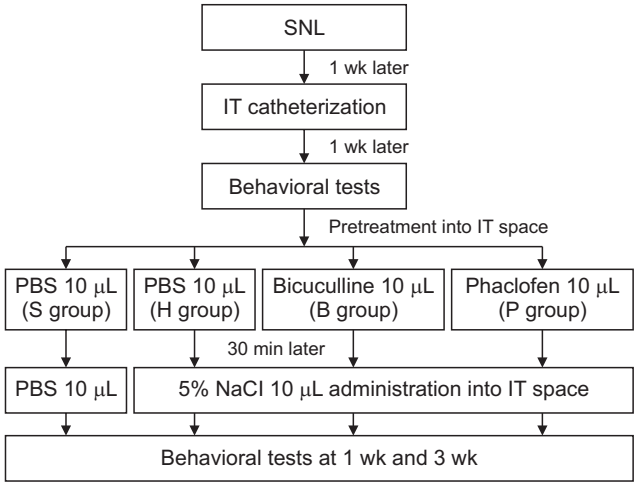


**Fig. 2.** Flowchart of the GABA antagonist experiment. Behavioral tests include the von Frey filaments, hot-plate, and cold-plate tests. GABA: γ-aminobutyric acid, SNL: spinal nerve ligation, IT: intrathecal, PBS: phosphate-buffered saline, S: sham, H: hypertonic, B: bicuculline, P: phaclofen, NaCl: sodium chloride.

behavioral accommodation was permitted for 20 to 30 minutes. The von Frey filament (VFF, Stoelting Co.) test, measuring the anti-allodynic effect, was performed one week after intrathecal catheterization surgery before intrathecal drug administration. The threshold values for mechanical allodynia were determined using eight continuous VFF values. On the plantar surface of the foot, eight calibrated VFF amounts (0.40, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.10 g) were sequentially applied (in either ascending or descending sequence) and lightly pressed against the foot. The stimulation was applied vertically to the center of the left foot for 5–6 seconds to allow the filament to bend slightly. A quick withdrawal or flinching was considered a positive reaction. After a negative reaction, the next heaviest filament was tested. As previously described, the up-down technique was used to determine the 50% withdrawal threshold [18,21,22]. The experiment begins by testing for a response on a filament estimated to be close to the 50% withdrawal threshold. If there is a response, the next filament with a lower force is tested; if there is no response, the next filament with a higher force is tested. At least 6 responses were obtained around the estimated threshold and used a formula to calculate the 50% threshold [23]. A positive response was recorded if the rats exhibited a rapid avoidance response during stimulation, licked their soles, or flinched when the filaments were released. All assessments of the antinociceptive properties of hypertonic saline were performed by blinded observers. When measuring behavioral responses, the contralateral limb was used as the control group for the ipsilateral SNL.

Hot (45°C)- and cold (4°C)-plate tests were performed after each VFF test as described above. For the hot- and cold-plate tests, the rats were separately placed on a hot- or cold-plate apparatus (Ugo-Basile) as previously described [24]. The reaction time was recorded from when the rat was placed on the hot- or cold-plate until it licked its hind paw. To prevent tissue damage without a response, a cutoff period of 30 seconds was set. The average of two attempts separated by a five-minute gap was used to prevent thermal sensitization to calculate the mean withdrawal latencies (sec) for the left hind paw.

## 4. Experiment 1: intrathecal NaCl administration

Based on a computer-generated randomization method, an impartial data manager assigned 10 rats to each of 6 groups (0.45%, 0.9%, 2.5%, 5%, 10%, and 20% NaCl; **Fig. 1**). The person who administered the drug was not blinded, but the evaluator of the behavioral experiment was blinded. One week after implanting the intrathecal catheter, the rats received 10 μL of each concentration of NaCl in their intrathecal space through the intrathecal catheter. Drug administration was immediately followed by a 10 μL phosphate-buffered saline (PBS) infusion to flush the catheter. Behavior tests were performed at baseline (before injecting drugs of different concentrations into the intrathecal space) and 30 minutes, 2 hours, 1 day, and 1 week after the drug administration into the intrathecal space.

### 5. Experiment 2: GABA receptor antagonist administration

One week after the intrathecal catheter implantation surgery in the rats, the previously described behavioral tests (VFF, hot- and cold-plate tests) were performed to prove the SNL model status (**Fig. 2**). Bicuculline ($GABA_A$ receptor antagonist) or phaclofen ($GABA_B$ receptor antagonist) was administered intrathecally 30 minutes before the administration of hypertonic saline [25,26]. The rats were randomly allocated into four groups (15 rats per group): 1) the sham group, which did not receive 5% NaCl (S group); 2) the hypertonic group, which received 5% NaCl (according to the results of Experiment 1) without pretreatment (H group); 3) the bicuculline group, which was pretreated with bicuculline and then received 5% NaCl (B group); and 4) the phaclofen group, which was pretreated with phaclofen and then received 5% NaCl (P group). After the behavioral test, PBS (10 μL) was administered to the sham and hypertonic groups. Bicuculline (2 μg/10 μL) was administered to the B group, and phaclofen (2 μg/10 μL) was administered to the P group. After every drug insertion, PBS (10 μL) was administered to flush the catheter. After 30 minutes, PBS (10 μL) was administered to the S group. To the residual three groups (H, B, and P groups), 5% of NaCl (10 μL) was administered. Behavioral tests were conducted before and 1 and 3 weeks after the drug administration to determine the involvement of spinal GABA receptors in the analgesic effects of the intrathecally administered 5% NaCl.

### 6. Data analysis and statistics

All data manipulations and statistical analyses were conducted using SPSS (version 21; IBM Corp.) and GraphPad Prism version 5.01 (GraphPad Software). Data are presented as the mean ± standard deviation. The data distribution was tested for normality using the Kolmogorov–Smirnov test. Parametric tests were used if the data were normally distributed, whereas nonparametric tests were used if the data were non-normally distributed. To compare the behavioral effects of each hypertonic saline concentration for 1 week, a two-way repeated measure analysis of variance (ANOVA) was used with concentration and time as factors. Bonferroni correction was made for multiple comparisons. One-way ANOVA was used to determine the involvement of spinal GABA receptor antagonists in 5% NaCl administered intrathecally, followed by the post hoc test. Significant *P* values were those less than 0.05.

## RESULTS

All the animals in the current study showed normal weight gain throughout the testing duration. Establishing the SNL model, implantation of the intrathecal catheter, and hypertonic saline treatment did not result in animal deaths.

### 1. Experiment 1: effect of hypertonic saline on SNL-induced mechanical allodynia and thermal and cold hyperalgesia

All the rats showed a baseline withdrawal reaction threshold of 15.10 g (maximum mechanical stimulation) to mechanical stimulation before SNL. After the SNL surgery, the paw withdrawal threshold was significantly reduced in response to mechanical stimulation (**Fig. 3A**, baseline). The rats that received 0.9%, 2.5%, 5%, 10%, and 20% NaCl concentrations showed significant differences in the threshold of mechanical stimulation from 2 hours to the first week, compared to each baseline. The rats that received 0.45% and 2.5% NaCl showed no difference from those that received 0.9% NaCl at each time point.

The withdrawal response to hot and cold stimulation significantly increased in the SNL-induced rats (**Fig. 3B, C**, baseline). SNL produced hot and cold hyperalgesia, which was sustained until one week after the SNL. The rats receiving 2.5%, 5%, and 10% NaCl showed significant differences from 2 hours to the first week, and those receiving 20% NaCl showed significant differences from 30 minutes to the first week, compared to each baseline in thermal hyperalgesia.

The mechanical allodynia and thermal hyperalgesia results in rats receiving 5%, 10%, and 20% NaCl significantly differed from the 2-hour time point to the end of the measurement (first week), compared to those receiving 0.9% NaCl. As shown in **Fig. 3A**, the results related to the effect of NaCl on SNL-induced mechanical allodynia were divided into two groups based on the NaCl concentrations of 5% and above or below this concentration. Therefore, the concentration of NaCl for Experiment 2 was determined to be 5%. The 5%, 10%, and 20% NaCl administration significantly inhibited SNL-induced mechanical allodynia and thermal hyperalgesia from 2 hours to 1 week compared to 0.9% NaCl (**Fig. 3A, B**).

In cold hyperalgesia, the rats receiving NaCl at 10% and 20% concentrations showed significant differences from 2 hours to the first week compared to each baseline. Those with a concentration of 5% showed a significant difference from the first day to the first week compared to

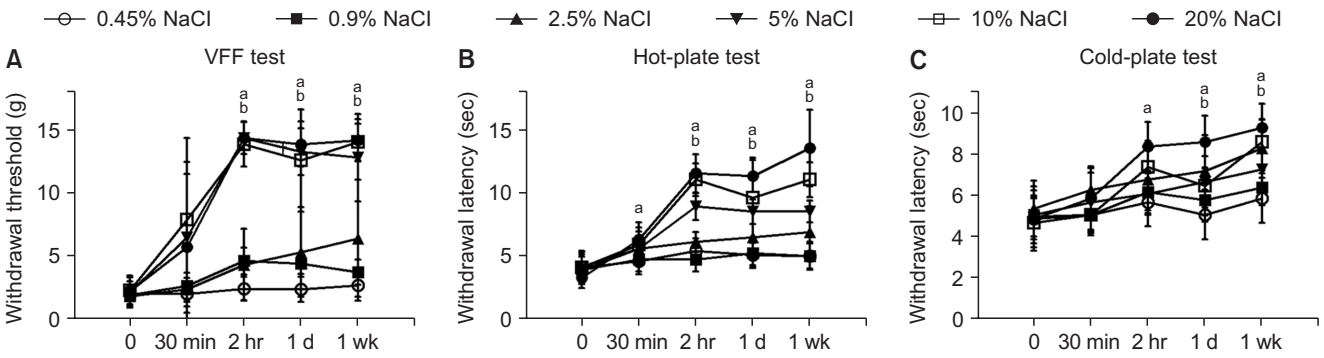


**Fig. 3.** Time course of the anti-allodynic and hyperalgesic effects of NaCl administered intrathecally in the rats with mechanical allodynia and thermal and cold hyperalgesia after L5 and L6 spinal nerve ligation. All the behavioral tests were performed at baseline and after 30 minutes, 2 hours, 1 day, and 1 week of administering 0.45%, 0.9%, 2.5%, 5%, 10%, and 20% NaCl intrathecally. (A) VFF test. The withdrawal threshold (grams) to mechanical stimuli is presented on the y-axis, and time on the x-axis. (B) Hot-plate test. The withdrawal latency (seconds) to hot (45°C) stimuli are presented on the y-axis, and time on the x-axis. (C) Cold-plate test. The withdrawal latency (seconds) to cold (4°C) stimuli is presented on the y-axis and time on the x-axis. Vertical bars indicate the standard deviation of the mean. Ten animals were used in each group. Data represent the mean ± standard deviation. VFF: von Frey filaments. [a]$P < 0.05$ compared to the baseline. [b]$P < 0.05$ compared to the 0.9% NaCl group (0.45% NaCl = open circle; 0.9% NaCl = filled square; 2.5% NaCl = filled up-pointing triangle; 5% NaCl = filled down-pointing triangle; 10% NaCl = open square; 20% NaCl = filled circle).

each baseline. Those with a 2.5% concentration showed a significant difference in the first week compared to the baseline. Rats receiving 0.45% and 2.5% NaCl showed no significant differences from those receiving 0.9% NaCl at any time in hot and cold hyperalgesia. However, those receiving 20% NaCl showed significant differences in cold hyperalgesia from the first day to the first week compared to those receiving 0.9% NaCl. Those with NaCl concentrations of 5% and above showed significant differences in the first week compared to those receiving 0.9% NaCl (**Fig. 3C**).

### 2. Experiment 2: effect of hypertonic saline on SNL-induced mechanical allodynia and thermal and cold hyperalgesia in rats pretreated with $GABA_A$ and $GABA_B$ antagonists

All the rats had a baseline withdrawal reaction threshold of 15.10 g (maximum mechanical stimulation) to mechanical stimulation prior to SNL. The paw withdrawal thresholds to mechanical, thermal, and cold stimuli were significantly reduced after SNL (S group; **Fig. 4**; white box). The mechanical withdrawal threshold and thermal and cold withdrawal latencies were significantly restored (at weeks 1 and 3) to the levels observed prior to the SNL surgery in the 5% NaCl intrathecally injected rat group *versus* the S group (H group; **Fig. 4**; blue box). And these showed more recovery at 3 weeks than at 1 week. The mechanical withdrawal threshold and thermal and cold withdrawal latencies were statistically lower in the B and P groups pretreated with bicuculline or phaclofen before the injection of 5% NaCl than in the H group (B and P groups; **Fig. 4**; purple and green boxes). Pretreatment with GABA antagonist restored mechanical withdrawal threshold and thermal and cold withdrawal latency in the B and P groups at 1 and 3 weeks. The withdrawal latency on thermal hyperalgesia increased at 3 weeks compared to 1 week in group B. The cold withdrawal latencies in the S group increased more at 3 weeks than at 1 week, while they remained similar in the B and P groups.

## DISCUSSION

The present study found that the intrathecal administration of hypertonic saline at a concentration of 5% or more alleviated mechanical allodynia induced by SNL in rats. Additionally, thermal and cold hyperalgesia partially decreased in a time- and dose-dependent manner.

The analgesic effects of intrathecal hypertonic saline were first reported by Hitchcock [27]. However, it is no longer injected intrathecally because of serious complications [3,4]. Although the mechanism of action of hypertonic saline for pain relief is not well understood, it is often administered epidurally to control chronic pain. This is because the transdural equilibrium of hypertonic saline occurs very slowly [5], resulting in analgesia with few serious side effects. However, the site of action is

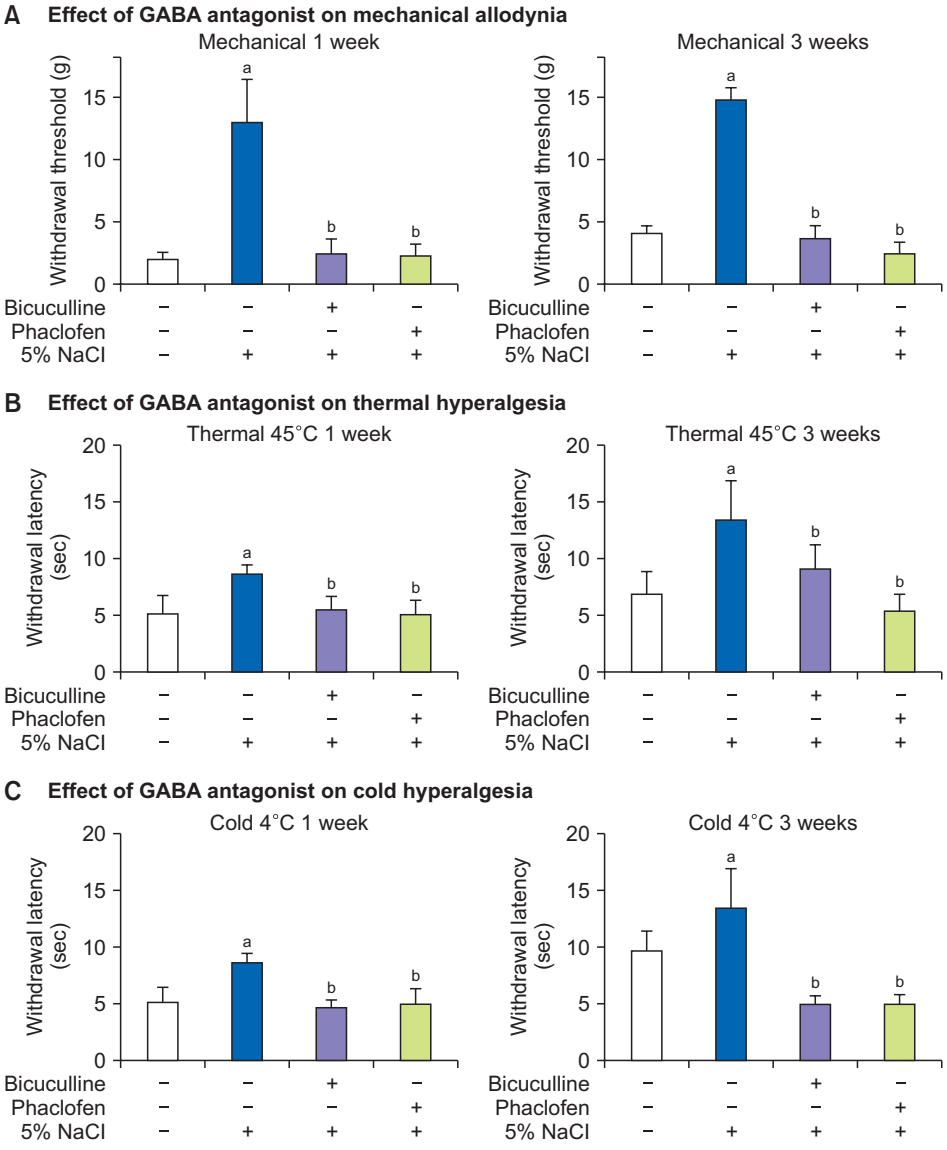


**Fig. 4.** Effect of 5% hypertonic saline on SNL-induced mechanical allodynia and thermal and cold hyperalgesia in rats pretreated with a $GABA_A$ or $GABA_B$ antagonist. (A) Effect of the GABA antagonists on mechanical allodynia. (B) Effect of the GABA antagonists on thermal hyperalgesia. (C) Effect of the GABA antagonists on cold hyperalgesia. The rats in the bicuculline and phaclofen groups were pretreated intrathecally with bicuculline or phaclofen. All the behavioral tests were performed at weeks 1 and 3 following the intrathecal administration of 5% NaCl. Each box represents the following: white (first box) = sham group; blue (second box) = hypertonic group; purple (third box) = bicuculline group; green (fourth box) = phaclofen group. Vertical bars indicate the standard deviation of the mean. Fifteen animals were used in each group. Data represent the mean ± standard deviation. SNL: spinal nerve ligation, GABA: γ-aminobutyric acid receptor. [a]$P < 0.05$ compared to the sham group. [b]$P < 0.05$ compared to the hypertonic group.

intrathecal, so in this experiment, intrathecal administration was used to investigate. Several clinical studies have reported on the analgesic effects of hypertonic saline, and the evidence for its clinical use was thought to be established [6–13]. However, the results of previous clinical studies show that there is a limit to its use in patients with neuropathic pain because the detailed mechanism of action of hypertonic saline is not well known.

Several studies using 7.2%–15% hypertonic saline concentrations have been reported [2,7,10,11,28–30]. Using a neuropathic pain rat model, this study demonstrated that hypertonic saline at concentrations greater than 5% mitigates mechanical allodynia and has a significant therapeutic effect (**Fig. 3A**). In addition, concentrations greater than 5% showed partial time- and dose-dependent effects on thermal and cold hyperalgesia (**Fig. 3B, C**). This suggests that the mechanisms of action of hypertonic saline on mechanical allodynia and thermal and cold hyperalgesia may differ.

The antinociceptive mechanism of hypertonic saline may be related to its chloride ion concentration. In 1972, King et al. [28] demonstrated that a persistent differential C-fiber block is produced by a high chloride ion concentration but not by a high sodium ion concentration. Since then, many studies have been conducted on the effects of chloride ion concentration on the pain pathway. According to a recent study, alterations in the anion equilibrium potential affect the fast synaptic inhibition mediated by $GABA_A$, prototypical ligand-gated anion channels, and glycine receptor activation [17]. Proteins known as cat-

ion-chloride co-transporters have become increasingly important owing to their crucial role in regulating the equilibrium potential of anions. This regulation, in turn, has been linked to the development of allodynia and hyperalgesia following peripheral nerve injury [17]. Another antinociceptive mechanism of hypertonic saline is that it osmotically induces a fluid shift to reduce cell swelling and pressure on nerves, resulting in a local anesthetic-like effect [31].

This study assessed the hypothesis that a loss of spinal GABA tone contributes to the hyperalgesia and allodynia observed after neuropathic pain induced by SNL in rats by examining whether blocking spinal $GABA_A$ and $GABA_B$ receptors is sufficient to lower the tactile and thermal sensory thresholds. GABAergic transmission plays an important role in mediating pain processing in the spinal cord [32,33]. Nerve injury-induced allodynia and hyperalgesia are reduced by $GABA_A$ and $GABA_B$ receptor agonists [25,34]. In the present study, the $GABA_A$ and $GABA_B$ antagonists produced powerful mechanical allodynic and thermal and cold hyperalgesic effects in rats injected intrathecally with 5% hypertonic saline. Because the use of known selective antagonists ($GABA_A$ and $GABA_B$) is effective, the mechanisms underlying the action of hypertonic saline appear to be mediated by $GABA_A$ and $GABA_B$ receptors.

In both control and nerve-injured rodents, the administration of a $GABA_A$ receptor agonist significantly reduced the initial responses to A- and C-fiber stimuli [35]. Mechanical and thermal hypersensitivity, known as allodynia and hyperalgesia, can be induced by intrathecal injection of $GABA_A$ receptor antagonists. These findings suggest that the activation of endogenous $GABA_A$ receptors contributes to inhibitory processes and the modulation of nociceptive thresholds [36–38]. In contrast, the administration of $GABA_A$ receptor agonists via spinal injection resulted in an analgesic effect [37,39,40]. Interestingly, the present study showed that spinal $GABA_A$ and $GABA_B$ receptors control mechanical allodynia in SNL-ligated rats (**Fig. 4**).

This study demonstrated the nociceptive effects of GABA antagonists in rats with SNL-induced neuropathic pain using behavioral tests, even after the administration of 5% NaCl. Mechanical allodynia and thermal hyperalgesia remained significant even after hypertonic saline administration in the pretreatment group at weeks 1 and 3. In thermal hyperalgesia, the effect on $GABA_B$ receptors at week 3 was maintained; however, the effect on $GABA_A$ receptors was reduced (**Fig. 4B**). This indicates that the mechanism by which hypertonic saline acts on thermal hyperalgesia differs from that of mechanical allodynia. This suggests that the GABA receptors acting on thermal hyperalgesia during the three weeks following drug administration are mainly $GABA_B$ receptors rather than $GABA_A$ receptors.

Altogether, it is plausible that GABA receptors play a role in the antinociceptive effects observed during the three weeks following the hypertonic saline administration. This study demonstrates the possible involvement of GABA receptors in the antinociceptive effects of hypertonic saline in rats with SNL-induced neuropathic pain. The importance of GABA receptors in the modulation of neuropathic pain is well established. An important finding of this study was that the minimum concentration of hypertonic saline that had an analgesic effect on mechanical allodynia in SNL-ligated rats was 5%. However, the possibility of the involvement of other inhibitory systems, such as the histamine, serotonin, norepinephrine, adenosine, and opioid systems, cannot be ruled out.

In conclusion, the intrathecal administration of hypertonic saline at concentrations of 5% or higher was found to partially inhibit the mechanical allodynia and thermal and cold hyperalgesia induced by SNL. However, intrathecally injected 5% hypertonic saline did not affect rats pretreated with GABA antagonists such as bicuculline ($GABA_A$) or phaclofen ($GABA_B$). Therefore, the effects of intrathecally injected 5% hypertonic saline in rats with neuropathic pain can be attributed to the involvement of the $GABA_A$ and $GABA_B$ receptors. These results demonstrate that intrathecally injected hypertonic saline is effective at concentrations greater than 5% for treating neuropathic pain, and its effects may be associated with GABA receptors. This study provides a basic background on the analgesic mechanism of hypertonic saline, which can be used in clinical settings.

## DATA AVAILABILITY

The datasets supporting the findings of this study are available from the corresponding author upon reasonable request.

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## CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

## AUTHOR CONTRIBUTIONS

Myong-Hwan Karm: Investigation, Data curation, Analysis and interpretation of data, Writing/manuscript preparation–writing the initial draft; Hyun-Jung Kwon: Analysis and interpretation of data, Writing/manuscript preparation–writing the initial draft; Euiyong Shin: Data curation, Interpretation of data; Honggyoon Bae: Writing–revising a manuscript; Young Ki Kim: Writing–review & editing; Seong-Soo Choi: Study conception, Analysis and interpretation of data, Writing/manuscript preparation–revising it critically for important intellectual content.

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