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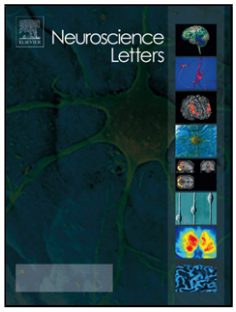

Antinociceptive effects of nefopam modulating serotonergic, adrenergic, and glutamatergic neurotransmission in the spinal cord

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Antinociceptive effects of nefopam modulating serotonergic, adrenergic, and glutamatergic neurotransmission in the spinal cord

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Highlights

- Intrathecal nefopam effectively attenuated formalin-induced pain behavior.
- Intrathecal nefopam inhibited the reuptake of 5-HT and NE and reduced glutamate release after formalin injection.

Abstract

The present study investigated the effects of intrathecal nefopam on the pain behavior and on the extracellular levels of serotonin (5-HT), norepinephrine (NE), and glutamate in the spinal cord, in a rat model of pain induced by formalin. Nefopam was intrathecally administered 10 min prior to the formalin test to assess its antinociceptive effects. In another cohorts of animals, dihydroergocristine, yohimbine, or (RS)-α-Methylserine-O-phosphate (MSOP), a serotonergic, α-2 adrenergic receptor, or group III metabotropic glutamate receptor antagonist, respectively, were administered prior to the application of nefopam in the formalin test. Microdialysis studies were conducted to measure the extracellular levels of 5-HT, NE, and glutamate in the spinal cord following nefopam administration. Intrathecal nefopam reduced formalin-induced behavior in both phases of the test. The blockade of serotonergic or

adrenergic receptors partially reversed the analgesic effects of nefopam in the first phase of the formalin test whereas MSOP reversed these effects in both phases. The microdialysis results revealed that intrathecal nefopam significantly increased 5-HT and NE levels and attenuated the formalin-induced release of glutamate in the spinal cord. Thus, the present data suggest that the increase in the extracellular levels of 5-HT and NE, and reductions in glutamate release in the spinal cord, may have contributed to the analgesic effects of nefopam.

Keywords: Glutamate; microdialysis; nefopam; norepinephrine; serotonin

1. Introduction

Nefopam is widely used for the treatment of moderate to severe pain as an alternative to opioid analgesic pharmacotherapies [1, 2]. This drug is a centrally acting, non-opioid, and non-steroidal analgesic member of the benzoxazocine chemical class that does not bind to opioid receptors or produce anti-inflammatory effects [3]. Compared to other analgesics, nefopam has unique pharmacological properties exhibiting a better tolerability and a favorable side effects profile [3]. Although the detailed mechanisms underlying its analgesic actions remain unclear, previous studies have suggested activities via the serotonergic, noradrenergic, and glutamatergic transmissions [4]. The involvement of serotonergic or noradrenergic pathways in the analgesic effects of nefopam was assessed by antagonism of carrier-dependent depletion of serotonin (5-HT) or norepinephrine (NE) by measuring their total contents in the tissue [5]. A study with an *in vitro* radioligand binding assay reported a potent inhibition of the neuronal uptake of monoamines by nefopam [6]. However, an *in vivo* measurements of the changes in extracellular concentration of these monoamines in response to nefopam administration were not performed. On the other hand, structural similarity of nefopam to orphenadrine which exert unspecific antagonistic activity at the NMDA receptor [7] led a group of researchers to raise the issue regarding the role of glutamatergic neurotransmission in the nefopam-produced antinociception. They reported that nefopam blocks membrane voltage-gated Na^{+} and Ca^{++} channels [8, 9], both of which are necessary for the propagation of action potentials and the synaptic release of glutamate. Consequently, it has been suggested that nefopam could prevent an excessive release of glutamate which plays a significant role in nociceptive processing. However, *in vivo* measurements of

glutamate release from nerve terminals following the administration of nefopam will be necessary to confirm this hypothesis.

Thus, the present study aimed to clarify the roles that spinal serotonergic, adrenergic, and glutamatergic neurotransmission play in the analgesic effects of nefopam. Changes in the extracellular levels of 5-HT, NE, and glutamate in the spinal cord were measured with *in vivo* microdialysis following the intrathecal administration of nefopam. Additionally, the mechanisms underlying the effects of nefopam analgesia were pharmacologically assessed using serotonergic, adrenergic, and group III metabotropic glutamate receptor (mGluR) antagonists, respectively.

2. Materials and Methods

All experiments were reviewed and approved by The Institutional Animal Care and Use Committee of Chonnam National University and the study adhered to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines for reporting animal research [10]. The present study included male Sprague-Dawley rats (225-250 g) that were housed in a room with a constant temperature of 22–23°C, an alternating 12-h light/dark cycle, and ad libitum access to water and food. An intrathecal catheter was placed in each animal as previously described [11] and, following the surgery, the animals were housed in individual cages and allowed to recover for 5 days.

The following drugs were used in this study: nefopam hydrochloride (Acupan®, PharmbioKorea Co. Ltd.; Seoul, Korea), dihydroergocristine (5-HT receptor antagonist, Research Biochemical Internationals; Natick, MA, USA), yohimbine (α-2 adrenergic receptor antagonist, Sigma Aldrich Co.; St. Louis, MO, USA), and (RS)-α-Methylserine-O-phosphate (MSOP, group III mGluR antagonist Tocris Cookson Ltd., Bristol, UK). Nefopam was dissolved in physiological saline while dihydroergocristine and yohimbine were dissolved in 100% dimethyl sulfoxide (DMSO) and MSOP was dissolved in distilled water. All intrathecal drug administration (volume of 10 μl) was performed with a manual gear-operated syringe pump via an intrathecal catheter; the catheter was flushed with 10 μl of saline after each injection considering that the dead capacity of the catheter is 7 to 8 μl.

On the day of the experiments, the rats were acclimatized to the formalin test chamber for at least 30 min and then randomly allocated into one of the experimental groups. Following a 50-μl injection of 5% formalin into the center of the hind paw using a 30-gauge needle, an observer blind to the experimental drug condition counted the number of flinching responses

at 1, 5, and 10 min (Phase 1, 0-10 min) and then at 5-min intervals between 15-60 min (Phase 2, 15-60 min); each duration for counting flinching responses was 1 min.

To assess the analgesic effects of nefopam, this drug was administered intrathecally (3, 10, or 30 μg/10μl) 10 min prior the formalin test. To determine whether the effects of nefopam were mediated via serotonergic, adrenergic, or glutamatergic neurotransmission, either dihydroergocristine (3 μg/10μl), yohimbine (10 μg/10μl), or MSOP (100 μg/10μl), respectively, was administered intrathecally 10 min prior to the delivery of nefopam. The antagonist doses used in the present study were chosen on the basis of a previous report [12] and a pilot study performed by our research group to determine the maximum doses that did not influence the control formalin test.

To measure changes in the extracellular concentrations of 5-HT and NE in the spinal cord, microdialysis studies were performed using previously reported methods [13]. Under anesthesia induced by sevoflurane, the right femoral vein was cannulated for fluid infusion at a rate of 1 ml/h and rectal temperature was maintained at 37-38°C by placing a heating pad under the rat. Following a thoracolumbar laminectomy, the L3-to-L5 segment of the spinal cord was exposed and a microdialysis probe (AI-8-01; Eicom Co.; Kyoto, Japan) was inserted at an angle of 30° to a depth of 1 mm using a micromanipulator (model MM-3, Narishige; Tokyo, Japan). Ultimately, the probe was presumably located in the dorsal horn of the spinal cord and was perfused with Ringer's solution (147.0 mmol/L NaCl, 4.0 mmol/L KCl, and 2.3 mmol/L $CaCl_2$) at a flow rate of 1 μl/min. After 120 min of constant perfusion, two consecutive samples were collected for baseline measurements of 5-HT and NE concentrations in the dialysate [13]. To investigate changes in 5-HT and NE levels after the intrathecal administration of nefopam, nefopam (0.1 mM) in Ringer's Solution was perfused

into the spinal cord and perfusate fractions (15 μl) were collected over a 15-min period. These fractions were automatically injected by an autoinjector (EAS-20, Eicom Co.) into the HTEC-500 system (Eicom Co.) to analyze 5-HT and NE concentrations via a high-performance liquid chromatography (HPLC) with electrochemical detection under the following conditions: the mobile phase was comprised of 0.1 mol/l ammonium acetate buffer (pH 6.0), methanol (7:3 vol/vol) containing 0.05 mol/l sodium sulfonate, and 50 mg/l EDTA-2Na; the column was an EICOMPAC CAX (Eicom Co.); the working electrode was glassy carbon (WE-3G, Eicom Co.).

To measure changes in the extracellular levels of glutamate in the spinal cord, an intrathecal microdialysis probe was constructed using a technique modified and adapted from previous reports [14]. The probe was constructed with two 6-cm polyurethane tubes (OD: 356 μm; ID: 178 μm) and a 4.2 cm cellulose hollow fiber (18 kDa molecular weight cut-off; Spectrum Laboratories, Inc.; Rancho Dominguez, CA, USA). A Nichrome-Formvar wire (diameter: 66.04 μm, A-M Systems, Inc.; Sequim, WA, USA) was passed through the hollow fiber (active dialysis region) and bent to form a U-shaped loop. Both ends of the dialysis loop were connected to polyurethane tubes using cyclohexanone and the other ends of the two polyurethane tubes were attached to 4-cm PE-10 catheters. To collect cerebrospinal fluid (CSF) samples after intrathecal injections of the drug, both the constructed microdialysis probe and the intrathecal catheter were placed intrathecally. The intrathecal microdialysis studies were performed as previously reported [15]. On the day of microdialysis, one of the externalized PE-10 tubes (inflow) was connected to a microsyringe pump (ESP 64, Eicom Co.) and perfused with Ringer's solution (147.0 mmol/l NaCl, 4.0 mmol/l KCl, and 2.3 mmol/l $CaCl_2$) at a constant flow rate of 5 μl/min while the other was connected to a tube that

served as an outflow. Baseline samples were collected after a 30-min washout period and then nefopam was administered intrathecally (30 μg/10μl) 10 min prior to the formalin injection. Next, two samples were collected at 5-min intervals and then five samples were collected at 10-min intervals, for up to 60 min (n = 5 in each group). Each sample was collected in a polyethylene tube on ice and then frozen at -80°C until assayed. Glutamate concentrations were measured via HPLC with electrochemical detection (HTEC-500, Eicom Co.) under the following conditions: the mobile phase was comprised of 60 mM NH_4Cl-NH_4OH containing HDTA with 1.0 mg/L EDTA-2Na; the columns were GU-GEL and E-ENZYMPAK (Eicom Co.); the precolumn was an EICOMPAK CH-GEL in PC-04 (Eicom Co.); the working electrode was WE-PT Gasket GS-25P (Eicom Co.).

All data are expressed as mean (95% confidence interval). The time-response data of the formalin-evoked behaviors are presented as the number of flinches whereas the dose-response data are presented as a percentage of the control for each phase (% of control = total flinching number with drug in phase 1[2]/total flinching number of control in phase 1[2] × 100). The dose-response data were analyzed with one-way analysis of variance (ANOVA) tests and a T3 Dunnett adjustment for the post hoc analyses, the antagonistic effects of nefopam were compared with unpaired *t*-tests. For the microdialysis data, a two-way mixed ANOVA was performed. If a significant interaction effect between the independent variables has been detected, a one-way repeated measure ANOVA was conducted to analyze simple effects. All statistical analyses were performed using PASW Statistics software (version 18.0; SPSS Inc., Chicago, IL) and P values < 0.05 were considered to indicate statistical significance.

3. Results

In the saline group, the subcutaneous injection of formalin into the hind paw evoked a biphasic pattern of flinching. Figure 1A illustrates the time courses of the effects of nefopam following its administration at 10 min prior to the formalin injection. One-way ANOVA to explore the effect of nefopam on pain behavior showed a statistically significant difference for the four groups; F (3, 16) = 7.60, P = 0.002 and F (3, 16) = 8.121, P = 0.002, for phase I and II, respectively (Fig. 1B and 1C). Post-hoc comparisons using Dunnett T3 test indicated that nefopam 10 μg reduced the number of flinching responses to 46 (29 - 63) % in phase I and to 53 (31 - 75) % in Phase II of the formalin test (P = 0.006 and 0.016, respectively), compared to the saline group. In the 30-μg nefopam-administered group behavioral response was decreased to 38 (22 - 54) % of that in the saline group during Phase I (P = 0.001) and to 52 (34 - 69) % of that in the saline group in Phase II (P = 0.002).

Dihydroergocristine administered 10 min prior to the delivery of nefopam increased flinching count to 89 (62 - 116) % of the saline group in Phase I (P = 0.005, compared to nefopam 30 μg) but did not in Phase II. Similarly, pretreated yohimbine increased flinching count to 76 (56 - 96) % of the control group only in Phase I of the test (P = 0.011). Whereas, pretreatment with MSOP attenuated the analgesic effects of nefopam 30 μg in both phases of the formalin test (P = 0.045 and P < 0.001, respectively, Figs. 1B and 1C).

The baseline concentrations of 5-HT in the saline- and nefopam-treated groups were 16.49 (5.89 - 27.09) fg/μl and 10.97 (7.58 - 14.36) fg/μl, respectively; however, the differences between these groups were not significant. Two-way mixed ANOVA assessing the effect of two groups (Saline, Nefopam) on 5-HT concentration across time periods showed a significant interaction between Group and Time, F (8, 64) = 18.31, P < 0.001, partial eta

squared = 0.70. The main effect comparing the two groups suggested a significant difference between saline and nefopam administration, F (df 1, 8) = 26.63, *P* = 0.001, partial eta squared = 0.77. The analysis of simple effects by one-way repeated measure ANOVA revealed a significant effect for Time in nefopam group, F (8, 32) = 19.10, *P* < 0.001, partial eta squared = 0.83, but non-significant effect in saline group, F (8, 32) = 1.92, *P* = 0.09, partial eta squared = 0.32. Pairwise comparisons indicated significant differences at 30, 45, 60, 75, and 90 min of nefopam administration, compared to baseline (*P* = 0.007, 0.002, 0.013, 0.004, and 0.009, respectively, Fig. 2A). The baseline concentrations of NE in the saline- and nefopam-treated groups were also statistically similar; 30.31 (16.34 - 44.28) fg/µl and 27.58 (9.57 - 45.59) fg/µl, respectively. Two-way mixed ANOVA revealed a significant interaction between Group and Time, F (8, 64) = 3.96, *P* = 0.001, partial eta squared = 0.33, and a significant main effect of Group, F (df 1, 8) = 5.77, *P* = 0.043, partial eta squared = 0.42. The analysis of simple effects revealed a significant effect for Time in nefopam group, F (8, 32) = 4.05, *P* = 0.002, partial eta squared = 0.50, but non-significant effect in saline group, F (8, 32) = 1.11, *P* = 0.39, partial eta squared = 0.22. Pairwise comparisons indicated significant differences at 15, 30, 45, and 60 min of nefopam administration, compared to baseline (*P* = 0.013, 0.001, 0.006, and 0.014, respectively, Fig. 2B). The baseline glutamate concentrations of the groups did not significantly differ; 137.80 (126.32 - 149.28) fmol/µl and 133.47 (96.49 - 170.44) fmol/µl, for the saline- and nefopam-treated group, respectively. Two-way mixed ANOVA showed a significant main effect of Group*Time interaction; F (8, 64) = 12.63, P < 0.001, partial eta squared = 0.61, but not significant main effect of Group; F (df 1, 8) = 0.52, *P* = 0.49, partial eta squared = 0.06. Pairwise comparisons exhibited a significant difference at 5 min of formalin injection in saline (*P* = 0.035) but did not in nefopam group (*P* = 1.00, Fig.

3C).

4. Discussion

The present study demonstrated that nefopam attenuated flinching responses induced by an injection of formalin into the hind paw of rats. Microdialysis analyses revealed that nefopam increased extracellular levels of 5-HT and NE in the spinal cord, and its antinociceptive effects were attenuated by the blockades of serotonergic and adrenergic receptors. In addition, nefopam inhibited glutamate release during the formalin test, and the inhibition of group III mGluRs, which act as presynaptic autoreceptors that mediate negative feedback control during the release of glutamate [16], reversed nefopam-induced antinociception. Taken together, these observations suggest that the increase in extracelluar concentration of 5-HT and NE and the attenuated release of glutamate in the spinal cord may contribute to the analgesic effects of nefopam.

Monoamines in the spinal cord, including 5-HT and NE, mediate the descending modulation of pain signals [17, 18]. Although several studies have demonstrated the involvement of serotonergic and/or noradrenergic descending pathways in the analgesic action of nefopam [19-21], there are conflicting results regarding the inhibition of the uptakes of 5-HT or NE by nefopam. For example, Hunskarr et al. [19] found that the intraperitoneal administration of nefopam (15 mg/kg) does not alter 5-HT concentrations in the mouse frontal cortex or spinal cord, whereas Fuller and Snoddy [5] reported that intraperitoneal injections of nefopam effectively inhibit 5-HT and NE uptake after a 32-mg/kg dose but not a 3- or 10-mg/kg dose. However, these studies were based on monoamine measurements obtained using the total contents of the brain or spinal cord tissues rather than the extracellular levels of neurotransmitters released from neurons. In the present study, microdialysis analyses clearly indicated that nefopam increased the extracellular levels of 5-

HT and NE in the spinal cord. Nevertheless, the mechanism of the increase in 5-HT and NE level by nefopam could not be determined by the current study. Although an *in vitro* radioligand binding assay showed that nefopam was a potent inhibitor of monoamine uptake [6], we could not exclude the involvement of an inhibition of monoamine degradation in the effects of nefopam.

The present study also found that nefopam-induced antinociception was partially reversed in Phase I but not in Phase II of the formalin test by serotonergic and adrenergic antagonists. Additionally, the present findings indicated that the inhibition of glutamate release may have accounted for, at least in part, the mechanisms underlying nefopam analgesia. Previous studies have shown that formalin injections into the hind paw induce acute increases in extracellular glutamate levels during the first phase of the test [15, 22], which is consistent with the present findings. This formalin-evoked glutamate release may be responsible for the first-phase behavioral effects and may also mediate increases in the extracellular levels of pronociceptive mediators, such as prostaglandin E_2, that are likely to facilitate nociceptive processing during the second phase of the formalin test [23]. The present study showed that the formalin-induced release of glutamate in Phase I of the test was blocked by nefopam and that pretreatment with group III mGluR antagonist reversed nefopam-induced antinociception in both phases of the test. Group III mGluRs are localized on presynaptic terminals in the dorsal horns of the spinal cord, and when activated, inhibit the evoked release of glutamate primarily through the modulation of calcium (Ca^{++}) channel activity [16, 24].

These findings are consistent with those of Fernandez-Sanchez et al. [8] showing that nefopam effectively prevented the signs of veratridine-induced neurotoxicity. The capability of depolarizing stimuli by veratridine to release glutamate from cerebellar neurons has been

demonstrated and the amount of glutamate released is sufficient to produce excitotoxicity [25]. Because nefopam failed to display an affinity for any of the subtypes of ionotropic glutamate receptors [8, 26], the authors suggested that nefopam effects were related to a reduction of the release of glutamate from neurons. The current study confirmed this hypothesis by *in vivo* microdialysis analyses of glutamate release levels in the extracellular space of the spinal cord following nefopam administration.

However, the mechanisms that underlie the effects of nefopam on glutamate release could not be elucidated in the present study. Nefopam blocks voltage-gated Na^{+} and/or Ca^{++} channels on the presynaptic membrane, which are necessary for the synaptic release of glutamate [8, 9]. These ion channels represent major components of the cycle linking NMDA receptor-mediated glutamate release [9, 27]. The blockades of these channels are the recognized mechanisms of action underlying the effects of commonly used anticonvulsants for managing neuropathic pain such as carbamazepine or lamotrigine [28, 29]. Similarly, nefopam may also block Na^{+} and/or Ca^{++} channels to inhibit formalin-induced glutamate release, which should be verified in future research.

The present study has some limitations that should be considered. The amount of nefopam delivered to the spinal cord during the microdialysis study depends on the recovery rate of the probe. However, the recovery rate of the probe used in the current study for nefopam is unknown and we determined the concentration of nefopam for perfusion arbitrarily. Consequently, the actual amount of nefopam delivered into the spinal cord are unknown, which might have affected the magnitude of change in extracellular monoamine level. Additionally, we could not exclude the possibility of the involvement of supraspinal action by an intrathecal administration of the experimental drugs. However, it is noteworthy that

intrathecal injections in a volume of 10 μl were reported to diffuse up to 25mm from the tip [30], thus the drugs might be confined to the lumbar spinal cord rather than spread more proximally than the basal cistern. Finally, we need to consider that the current study used a small sample size (n=5) which: gives a relatively low power to the study; inflates the probability of false positives by sampling effect; tends to artificially give larger effect sizes.

In conclusion, the present findings suggest that nefopam may be useful for the management of inflammatory pain. Additionally, the analgesic effects of nefopam may be associated with the increase in extracellular level of 5-HT and NE, as well as the modulation of glutamate release.

Declarations of interest: none

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CRediT authorship contribution statement

Joo Wung Chae : Investigation, Writing-original investigation

Dong Ho Kang : Conceptualization, Formal anaylsis, Investigation

Yaqun Li : Investigation

Seung Hoon Kim : Visualization

Hyung Gon Lee: Methodology

Jeong Il Choi : Supervision

Myung Ha Yoon : Resources

Woong Mo Kim : Conceptualization, Methodology, Supervision, Funding aquisition

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Figure legends

Figure 1. Time-response (A) and dose-response (B, C) data showing the effects of nefopam on flinching during the formalin test. Data are presented as the number of flinches or the percentage of control. Each line or bar represents the mean (95% CI) of five rats. * $P < 0.05$ compared to the saline group. § $P < 0.05$ compared to the nefopam (30 μg)-only group.

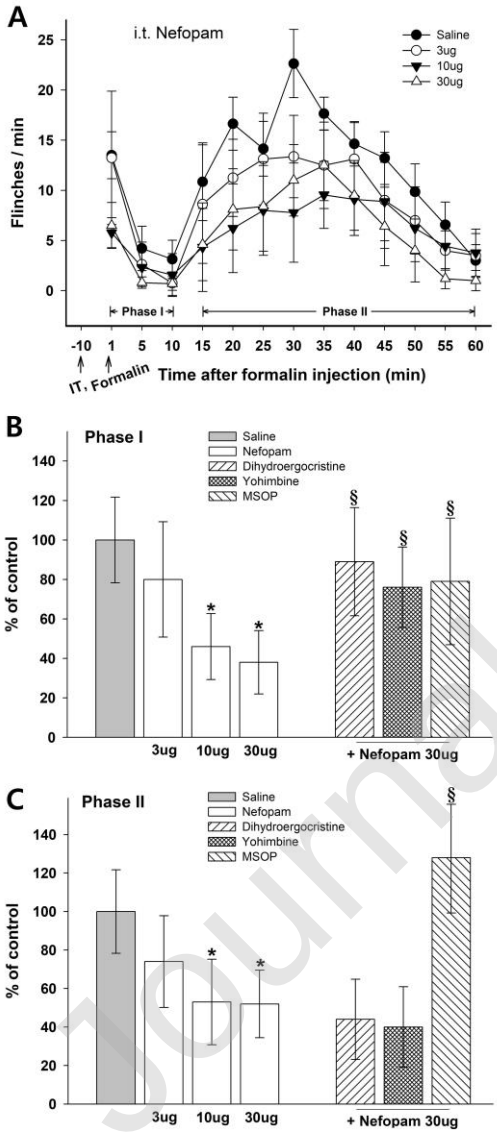

Figure 2. Microdialysis measurements of spinal 5-HT (A), NE (B), and glutamate (C) levels after nefopam administration (30 μg). Data are presented over time as a mean (95% CI) percentage of the baseline (n = 5 in each group). * $P < 0.05$ compared to baseline value.