Title	Neurotropin inhibits neuronal activity through potentiation of sustained Kv currents in primary cultured DRG neurons
Author(s)	Kawai, Hiroyuki; Asaoka, Nozomi; Miyake, Takahito; Nagayasu, Kazuki; Nakagawa, Takayuki; Shirakawa, Hisashi; Kaneko, Shuji
Citation	Journal of pharmacological sciences (2018), 137(3): 313-316
Issue Date	2018-07
URL	http://hdl.handle.net/2433/241622
Right	© 2018 The Authors. Production and hosting by Elsevier B.V. on behalf of Japanese Pharmacological Society. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Type	Journal Article
Textversion	publisher

Journal of Pharmacological Sciences 137 (2018) 313–316

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Short Communication

Neurotropin inhibits neuronal activity through potentiation of sustained K_v currents in primary cultured DRG neurons

Hiroyuki Kawai [a], Nozomi Asaoka [a], Takahito Miyake [a], Kazuki Nagayasu [a], Takayuki Nakagawa [b], Hisashi Shirakawa [a,*], Shuji Kaneko [a]

[a] Department of Molecular Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto, 606-8501, Japan
[b] Department of Clinical Pharmacology and Therapeutics, Kyoto University Hospital, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto, 606-8507, Japan

ARTICLE INFO

Article history:
Received 18 January 2018
Received in revised form
28 April 2018
Accepted 22 May 2018
Available online 31 May 2018

Keywords:
Neurotropin
DRG neuron
Voltage-dependent K^+ channel

ABSTRACT

Neurotropin (NTP) is a Japanese analgesic agent for treating neuropathic pain; however, its method of action remains unclear. This study examined the effects of NTP on the activity of small dorsal root ganglion (DRG) neurons using whole-cell patch clamp recordings. After 3 days of treatment, NTP decreased current injection-induced firing activity of cultured DRG neurons by raising the current threshold for action potential generation. Additionally, NTP increased the sustained component of voltage-gated potassium (K_v) channel currents without affecting other K^+ currents. These results suggest that NTP inhibits the firing activity of DRG neurons through augmentation of sustained K_v current.

Neurotropin (NTP), a non-protein extract from inflamed rabbit skin inoculated with vaccinia virus, is widely used in Japan as an analgesic drug for neuropathic pain.[1] One of the main consequences of NTP treatment is activation of the descending pain inhibitory system.[1] In addition, NTP exerts an anti-nociceptive effect in a central neuropathic pain model induced by noradrenergic lesions,[2] suggesting that neuronal pathways other than the descending pain inhibitory systems are involved in NTP-induced anti-nociception. The effects of NTP on the peripheral nervous system have not been fully elucidated.

Most analgesic agents currently used to treat neuropathic pain (*e.g.* opioids) act on the central nervous system. It is widely recognized that hyperactivity of dorsal root ganglion (DRG) neurons causes neuropathic pain.[3] Nerve injury and inflammatory stimuli cause dysfunction of K^+ channels in DRG neurons, resulting in their hyperactivity.[3] Abnormal modulation of K^+ channels is one of the pathophysiological mechanisms underlying neuropathic pain, highlighting the therapeutic potential of K^+ channel activation.

To test that NTP affects the activity of DRG neurons, we examined its effects on cultured DRG neurons using electrophysiological recordings.

All experimental procedures were performed in accordance with the ethical guidelines of the Kyoto University Animal Research Committee. Male Wistar/ST rats (6–9 weeks; Nihon SLC, Shizuoka) were housed under constant ambient temperature (22 ± 2 °C) and a light–dark cycle. NTP was obtained from Nippon Zoki Pharmaceutical (Osaka).

Primary cultures of DRG neurons were prepared as previously described with several modifications.[4] Briefly, rats were euthanized with CO_2 and the thoracic and lumbar DRGs were dissected out. Dissociated DRG neurons were cultured on Poly-L-lysine (PLL)/laminin-coated coverslips in Neuro Medium (Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with 2% NeuroBrew-21 (Miltenyi Biotec), 0.5% GlutaMAX supplement (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin (Nacalai Tesque, Kyoto), 100 g/mL streptomycin (Nacalai Tesque), and 0.05 g/mL 2.5S nerve growth factor (NGF; Sigma–Aldrich, Saint Louis, MO, USA). On the next day, the culture medium was replaced by NTP-containing medium. After 3 days of treatment with NTP, whole-cell recordings were performed of small DRG neurons (membrane capacitance < 35 pF)[5] using an EPC-10 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) and Patchmaster software (HEKA). Data were filtered at 2.9 kHz and access resistances were compensated by 70%.

* Corresponding author. 46-29 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto, 606-8501, Japan. Fax: +81 75 753 4548.
E-mail address: shirakaw@pharm.kyoto-u.ac.jp (H. Shirakawa).
Peer review under responsibility of Japanese Pharmacological Society.

https://doi.org/10.1016/j.jphs.2018.05.005

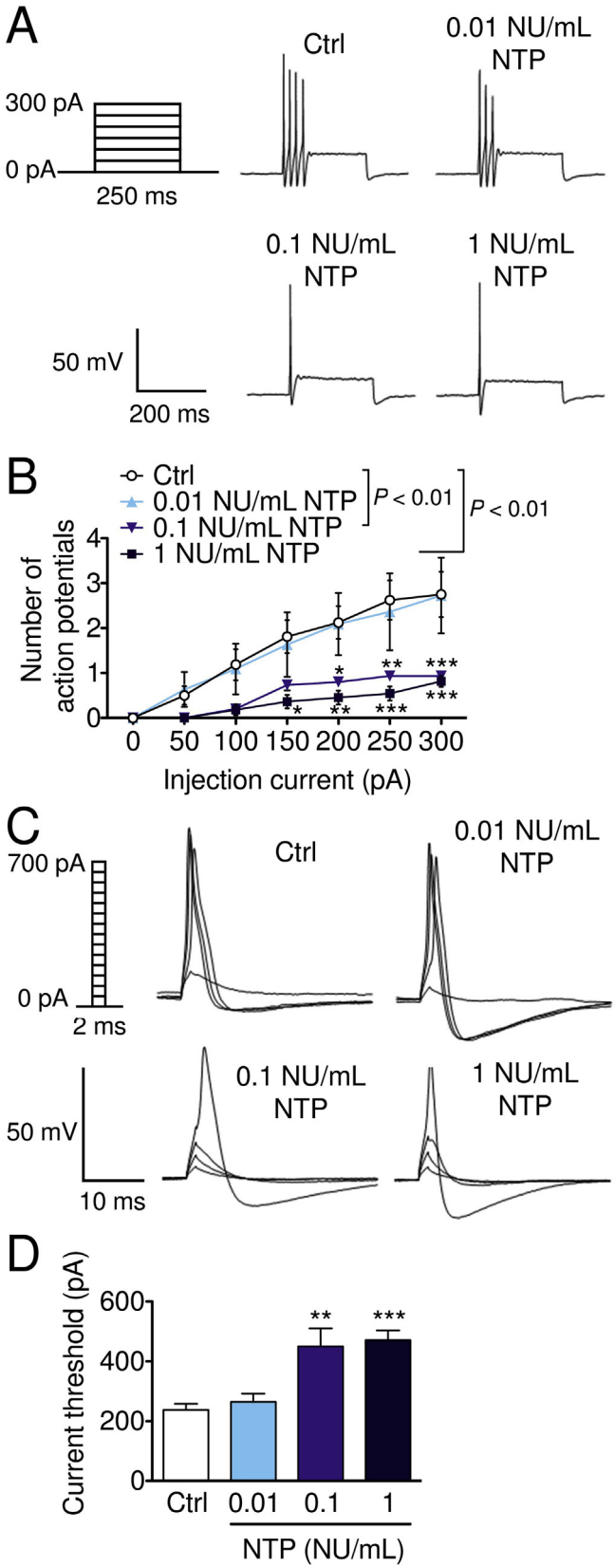

Fig. 1. Effects of NTP on the firing activity of DRG neurons. (A) Protocol for current injection (upper left; 0–300 pA in 50 pA increments, 250 ms duration) and representative traces of action potentials evoked by 300 pA injection (right). Between each pulse, the holding current was set at 0 pA. (B) Number of evoked action potentials recorded from control (Ctrl) and NTP-treated DRG neurons. n = 11–16. Ctrl vs. 0.01 NU/mL; $F_{1,\ 25} = 0.01$, $P = 0.9141$, Ctrl vs. 0.1 NU/mL; $F_{1,\ 29} = 11.20$, $P = 0.0023$, Ctrl vs. 1 NU/mL; $F_{1,\ 25} = 10.96$, $P = 0.0028$; two-way ANOVA. (C) Protocol for determining current threshold (upper left; 0–700 pA in 50 pA increments, 2 ms duration) and representative responses evoked by 100, 200, 300, and 400 pA injection (right). (D) Current threshold for action potential generation recorded from control (Ctrl) and NTP-treated DRG neurons. n = 6–11. $F_{3,\ 28} = 12.10$, $P < 0.001$; one-way ANOVA. Data are expressed as means ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. Ctrl.

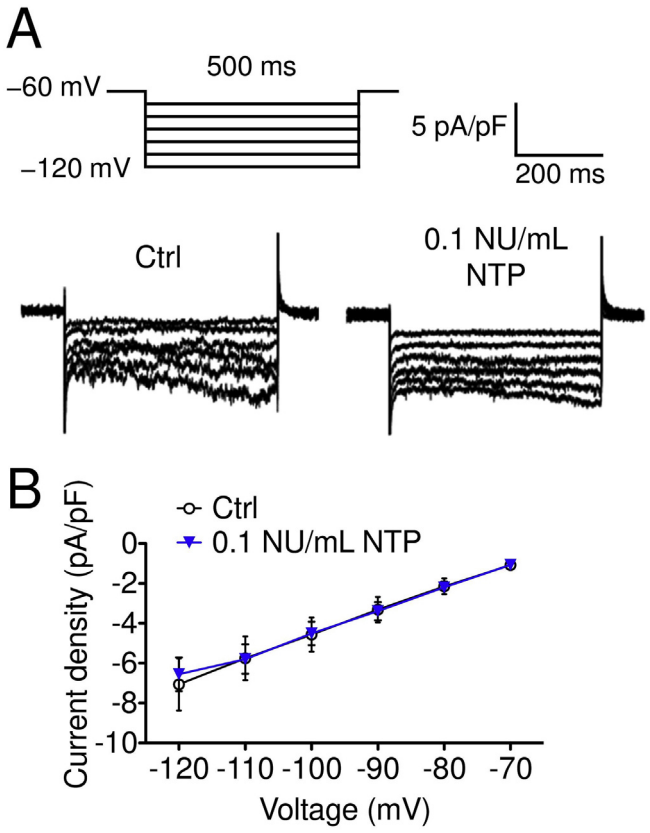

Fig. 2. Effect of NTP on voltage-independent K^+ channels in DRG neurons. (A) Representative traces of inward current elicited by hyperpolarization pulses (−120 to −70 mV in 10 mV increments; 500 ms duration). Between each pulse, the membrane potential was held at −60 mV. (B) Current–voltage relationships for K^+ currents recorded from control (Ctrl) and 0.1 NU/mL NTP-treated DRG neurons. n = 6–8. Ctrl vs. 0.1 NU/mL; $F_{1,\ 12} = 0.00$, $P = 0.9457$; two-way ANOVA. Data are expressed as means ± S.E.M.

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Differences between groups were analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA followed by the Bonferroni *post hoc* test. Differences of $P < 0.05$ were considered statistically significant.

To examine the effects of NTP treatment on the firing activity of DRG neurons, current injection-evoked action potentials were recorded. Recording conditions were as follows: bath solution (composition in mM; 124 NaCl, 3 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 10 HEPES, 26 $NaHCO_3$, 1 NaH_2PO_4, and 10 D-glucose, pH 7.3 adjusted with NaOH) and pipette solution (composition in mM; 140 K-gluconate, 5 KCl, 2 $MgCl_2$, 10 HEPES, 0.2 EGTA, and 2 ATP-Na_2, pH 7.2 adjusted with KOH). Under control conditions (Ctrl), DRG neurons showed multiple spikes, while neurons treated with NTP (0.1 and 1 neurotropin unit (NU)/mL) exhibited a significant decrease in the number of action potentials (Fig. 1A and B). The current threshold for action potential generation was significantly higher in the 0.1 and 1 NU/mL NTP-treated groups than in the control group (Fig. 1C and D), suggesting that NTP dose-dependently inhibits DRG neuron action potential generation.

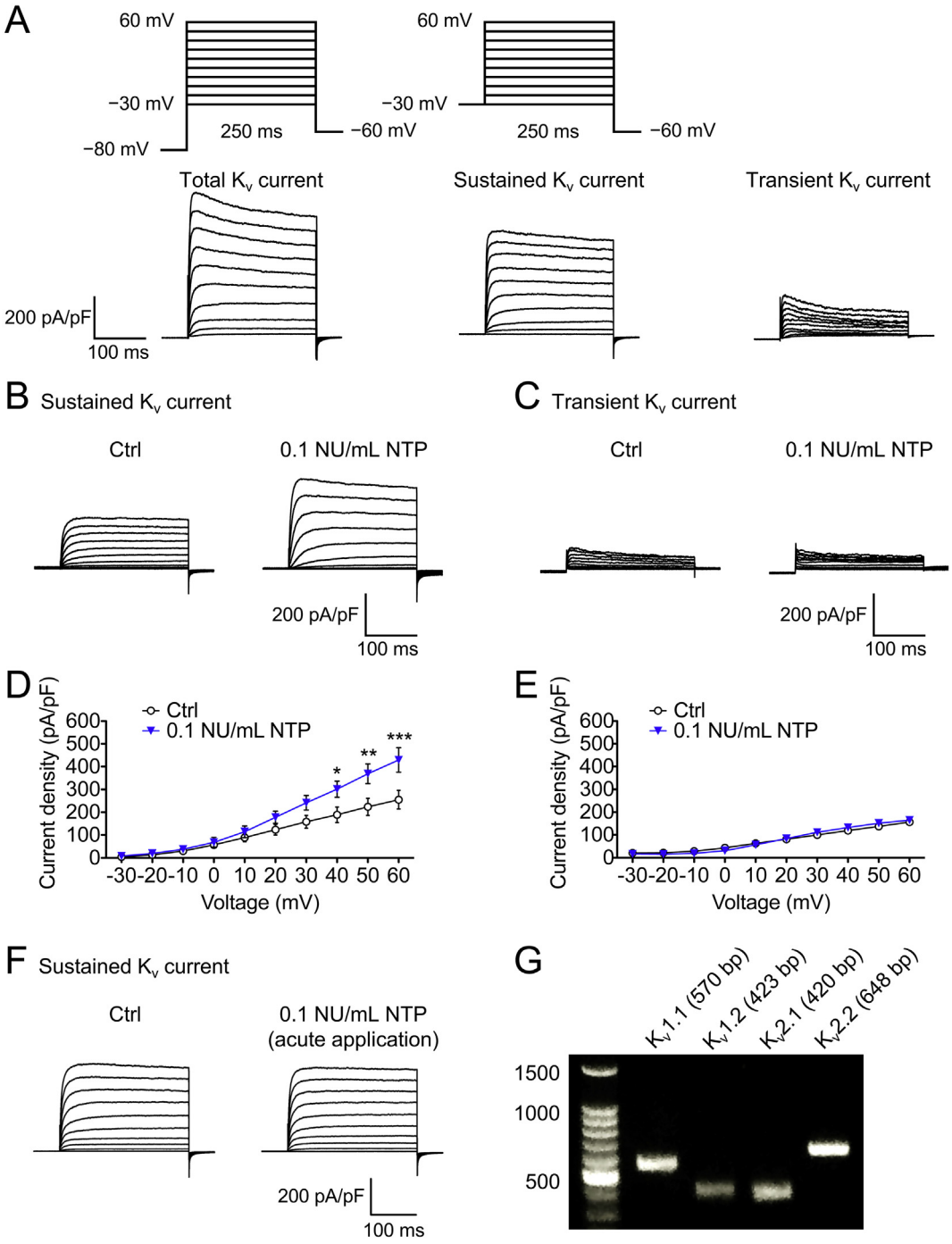

Fig. 3. Effect of NTP on K_v channels in DRG neurons. (A) Protocols for recording total K_v current (left) and sustained K_v current (middle). Transient K_v currents were obtained by subtracting sustained K_v currents from total K_v currents (right). Representative traces of sustained K_v currents (B) and current–voltage relationships for sustained K_v currents (D) recorded from control (Ctrl) or 0.1 NU/mL NTP (3-day)-treated DRG neurons. $n = 6–7$. $F_{1,\ 11} = 4.27$, $P = 0.0632$; two-way ANOVA. Representative traces of transient K_v currents (C) and current–voltage relationships for transient K_v currents (E) recorded from control (Ctrl) or 0.1 NU/mL NTP (3-day)-treated DRG neurons. $n = 6–7$. $F_{1,\ 11} = 0.02$, $P = 0.8843$; two-way ANOVA. Data are expressed as means ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. Ctrl. (F) Representative traces of sustained K_v currents recorded from DRG neurons with control (Ctrl) or 0.1 NU/mL NTP acutely-treated DRG neurons. (G) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of K_v α subunit mRNAs expression in DRG neuron of normal male Wistar/ST rats. Total RNA was isolated from DRG neurons, and first strand cDNA was synthesized by reverse transcription (RT) with random primers. The RT products were amplified with each of the primer pairs for K_v genes during 30 cycles. The products were electrophoresed and stained with ethidium bromide in 2% agarose gel. Molecular weight markers were indicated on the left-hand side of the figure. The oligonucleotide sequences used for the primers were as follows: 5′-TTTACGAGTTGGGCGAGGAG-3′ and 5′-AGTGGCGGGAGAGTTTGAAG-3′ for K_v1.1 (570 bp); 5′-GTCTATCACCCAGGAACATGGAG-3′ and 5′-GCTCCTGTAGGAAAGACCCAG -3′ for K_v1.2 (423 bp); 5′-ACGAGTACTTCTTCGACCGC-3′ and 5′-CGAACTCGTCTAGGCTCTGC-3′ for K_v2.1 (420 bp); 5′-GAACCGGAGTGAGGGATGTA-3′ and 5′-CATGTGGAGCTTTCCCGTCC-3′ for K_v2.2 (648 bp).

It is widely accepted that K^+ channels in DRG neurons play an important role in abnormal pain processing and its relief.[3] To determine the mechanisms regulating the inhibitory effect of NTP on DRG neuronal activity, we focused on K^+ currents. The K^+ current was isolated using a modified bath solution (composition in mM; 150 choline-Cl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 1 $CoCl_2$, 10 HEPES, and 10 D-glucose, pH 7.3 adjusted with KOH), which blocks Na^+ and Ca^{2+} currents. The voltage-independent K^+ current did not significantly differ between the control and NTP-treated groups (Fig. 2). We next examined whether NTP treatment affects voltage-dependent K^+ (K_v) channels. K_v currents are divided into sustained and transient K_v currents by the kinetics.[6] To evaluate these components separately, transient K_v currents were obtained by subtracting the sustained K_v currents from the total K_v currents (Fig. 3A). The total and sustained K_v currents were elicited by the voltage steps from −80 and −30 mV, respectively. Sustained K_v currents were significantly larger in NTP-treated DRG neurons than in control neurons (Fig. 3B, D), whereas NTP did not affect transient K_v currents (Fig. 3C, E). By contrast, acute application of NTP did not affect sustained K_v currents (Fig. 3F). The mRNA levels of K_v channel subunits were not changed in NTP (3-day)-treated DRG neurons (data not shown), while sustained K_v channel subunits were expressed in non-treated cultured DRG neurons (Fig. 3G). These results indicate that 3-day treatment with NTP selectively potentiates a sustained K_v current in DRG neurons.

In the present study, we showed that NTP decreased the firing activity of DRG neurons by raising the current threshold for firing. Additionally, NTP selectively increased sustained K_v currents. Due to their slow deactivation, sustained K_v currents are involved in relatively long-term inhibition following action potentials (*e.g.* generation of large after-hyperpolarization), resulting in a prolonged refractory period.[7] Considering the inhibitory mechanisms of sustained K_v channels, NTP might inhibit strong stimulation-induced repetitive firing in DRG neurons. In support of this hypothesis, a previous study reported that NTP decreases capsaicin-induced neuropeptide release without affecting basal release.[8] While the effects of NTP on the descending pain inhibitory system are well-accepted,[1] our results indicate that NTP also affects the peripheral nervous system.

In this study, we showed that 3-day, but not acute, treatment with NTP increased sustained K_v current without affecting mRNA expression levels. These observations suggest that NTP potentiates K_v channels by post-translational modification. For example, NGF treatment increases membrane expression of $K_v2.1$ without changing its mRNA expression.[9] Phosphorylation is one of the main post-translational modification of K_v channels,[10] and phosphorylation of serine residues in delayed rectifier K^+ channels, such as $K_v2.1$, positively regulates their surface expression.[11] Recent research demonstrated that NTP promotes NGF signaling and the downstream activation of several protein kinases,[12] suggesting that enhanced NGF signaling is involved in the NTP-induced increase in sustained K_v current. Considering that NTP directly elicits inward current in noradrenergic neurons in the central nervous system,[13] it might modulate the descending pain inhibitory system and peripheral sensory system via distinct mechanisms. Further studies are needed to clarify the precise cellular mechanisms underlying these dual effects of NTP.

In conclusion, the present study demonstrated that NTP inhibits the activity of cultured DRG neurons through potentiation of sustained K_v currents. These findings support the hypothesis that NTP acts on both the central and peripheral nervous systems, highlighting the unique anti-nociceptive mechanisms of NTP.

Conflict of interest

This study was funded by Nippon Zoki Pharmaceutical.

Acknowledgements

This work was supported by MEXT/JSPS KAKENHI Grant Numbers 17K19486 (to H.S.), 17KT0052 (to S.K.), and also supported in part by grants from Nippon Zoki Pharmaceutical.

References

1. Miura T, Okazaki R, Yoshida H, Namba H, Okai H, Kawamura M. Mechanisms of analgesic action of neurotropin on chronic pain in adjuvant-induced arthritic rat: roles of descending noradrenergic and serotonergic systems. *J Pharmacol Sci*. 2005;97:429–436.
2. Kudo T, Kushikata T, Kudo M, Kudo T, Hirota K. Antinociceptive effects of neurotropin in a rat model of central neuropathic pain: DSP-4 induced noradrenergic lesion. *Neurosci Lett*. 2011;503:20–22.
3. Tsantoulas C, McMahon SB. Opening paths to novel analgesics: the role of potassium channels in chronic pain. *Trends Neurosci*. 2014;37:146–158.
4. Imai S, Koyanagi M, Azimi Z, et al. Taxanes and platinum derivatives impair Schwann cells via distinct mechanisms. *Sci Rep*. 2017;7:5947.
5. Hong S, Wiley JW. Early painful diabetic neuropathy is associated with differential changes in the expression and function of vanilloid receptor 1. *J Biol Chem*. 2005;280:618–627.
6. Johnston J, Forsythe ID, Kopp-Scheinpflug C. Going native: voltage-gated potassium channels controlling neuronal excitability. *J Physiol*. 2010;588:3187–3200.
7. Tsantoulas C, Zhu L, Yip P, Grist J, Michael GJ, McMahon SB. K_v2 dysfunction after peripheral axotomy enhances sensory neuron responsiveness to sustained input. *Exp Neurol*. 2014;251:115–126.
8. Taneda K, Tominaga M, Tengara S, Ogawa H, Takamori K. Neurotropin inhibits both capsaicin-induced substance P release and nerve growth factor-induced neurite outgrowth in cultured rat dorsal root ganglion neurones. *Clin Exp Dermatol*. 2010;35:73–77.
9. Sharma N, D'Arcangelo G, Kleinlaus A, Halegoua S, Trimmer JS. Nerve growth factor regulates the abundance and distribution of K^+ channels in PC12 cells. *J Cell Biol*. 1993;123:1835–1843.
10. Cerda O, Trimmer JS. Analysis and functional implications of phosphorylation of neuronal voltage-gated potassium channels. *Neurosci Lett*. 2010;486:60–67.
11. Redman PT, He K, Hartnett KA, et al. Apoptotic surge of potassium currents is mediated by p38 phosphorylation of $K_v2.1$. *Proc Natl Acad Sci U S A*. 2007;104:3568–3573.
12. Fukuda Y, Fukui T, Hikichi C, et al. Neurotropin promotes NGF signaling through interaction of GM1 ganglioside with Trk neurotrophin receptor in PC12 cells. *Brain Res*. 2015;1596:13–21.
13. Okai H, Okazaki R, Kawamura M, Yoshimura M. Excitatory effect of Neurotropin® on noradrenergic neurons in rat locus coeruleus. *Life Sci*. 2015;136:79–86.