Journal of Anesthesia (2019) 33:495–500
https://doi.org/10.1007/s00540-019-02655-z

ORIGINAL ARTICLE

Neuroprotective effects of neurotropin in a mouse model of hypoxic–ischemic brain injury

Sohei Hishiyama[1] · Masakazu Kotoda[1] · Tadahiko Ishiyama[2] · Kazuha Mitsui[2] · Takashi Matsukawa[1]

Received: 24 January 2019 / Accepted: 14 May 2019 / Published online: 21 May 2019

Abstract

Purpose Ischemic–hypoxic insult leads to detrimental effects on multiple organs. The brain is especially vulnerable, and it is hard to regenerate once damaged. Currently, therapeutic options are very limited. Previous studies have reported neuroprotective effects of neurotropin, a non-protein extract derived from the inflamed skin of rabbits inoculated with vaccinia virus, using a murine model of peripheral nerve injury and cultured cell lines. However, whether neurotropin might have protective effects against brain injuries remains unclear. We, therefore, investigated the neuroprotective effect of neurotropin and possible underlying mechanisms, using a mouse model of hypoxic–ischemic brain injury.

Methods Hypoxic–ischemic brain injury was induced via a combination of the left common carotid artery occlusion and exposure to hypoxic environment (8% oxygen) in adult male C57BL/6 mice. Immediately following induction of hypoxia–ischemia, mice received either saline or 2.4 units of neurotropin. The survival rate, neurological function, infarct volume, and expression of inflammatory cytokines were evaluated.

Results Compared to the control group, the neurotropin group exhibited a significantly higher survival rate (100% vs. 62.5%, $p < 0.05$) and lower neurological deficit scores (1; 0–2 vs. 3; 0–5, median; range, $p < 0.05$) after the hypoxic–ischemic insult. The administration of neurotropin also reduced infarct volume ($18.3 \pm 5.1\%$ vs. $38.3 \pm 7.2\%$, $p < 0.05$) and mRNA expression of pro-inflammatory cytokines.

Conclusions The post-treatment with neurotropin improved survival and neurological outcomes after hypoxic–ischemic insult. Our results indicate that neurotropin has neuroprotective effects against hypoxic–ischemic brain injury by suppressing pro-inflammatory cytokines.

Keywords Neurotropin · Hypoxic–ischemic brain injury · Inflammatory cytokine

Introduction

Hypoxic–ischemic insult leads to detrimental effects in multiple organs. Among various organs, the brain is especially vulnerable, and it is hard to regenerate once damaged. Stroke is currently the second leading cause of death worldwide [1]. Despite accumulated efforts on establishing novel therapeutic methods for the treatment of hypoxic–ischemic brain injury, the therapeutic options are still very limited [2]. New drugs that can improve neurological outcomes after hypoxic–ischemic brain injury are, therefore, desperately needed.

Neurotropin, a non-protein extract derived from the inflamed skin of rabbits inoculated with vaccinia virus, has been used to treat chronic pain and peripheral inflammation. Neurotropin consists of multiple physiologically active substances and has wide-ranging pharmacological effects. It has been reported that neurotropin exerts its analgesic and anti-inflammatory effects via the promotion of the descending pain inhibitory pathway [3] and the inhibition of leukocyte infiltration into inflammation sites [4]. In addition to these analgesic and anti-inflammatory activities, neuroprotective effects of neurotropin on the peripheral nerves have been reported in the literature. Nishimoto et al. demonstrated that neurotropin prevents demyelination in a rat model of sciatic nerve injury by suppressing pro-inflammatory cytokines [5].

✉ Masakazu Kotoda
mkotoda@yamanashi.ac.jp

[1] Department of Anesthesiology, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 4093898, Japan

[2] Surgical Center, University of Yamanashi Hospital, University of Yamanashi, Chuo, Yamanashi, Japan

However, the neuroprotective effects of neurotropin on the central nervous system remains unclear, and the evidence for its neuroprotective effect against hypoxic–ischemic brain injury is very limited [2]. We, therefore, investigated the effect of neurotropin on hypoxic–ischemic brain injury and possible underlying mechanisms using a mouse model of hypoxic–ischemic brain injury. Our hypothesis was that neurotropin attenuates brain injury and improves neurological outcomes after hypoxic–ischemic insult by suppressing pro-inflammatory cytokines. As the main outcomes, we assessed survival rates and cerebral infarct volumes. The secondary outcomes included neurological function and pro-inflammatory cytokine expression.

Methods

All experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. The experimental protocol was reviewed and approved by the University of Yamanashi Animal Care Committee.

Animals

Male C57BL/6 mice (8–10 weeks old, weight: 20–25 g) were purchased from Japan SLC (Tokyo, Japan). The mice were housed at 23 ± 2 °C under a 12-h light–dark cycle with free access to standard food and water. All experiments were performed between 09:00 and 17:00 under normal room light and temperature (23 ± 2 °C) conditions.

Hypoxic–ischemic brain injury

Hypoxic–ischemic brain injury was induced via a combination of permanent left common carotid artery (CCA) occlusion and exposure to a low-oxygen environment, as previously described by others [6, 7]. Briefly, mice were placed in a dorsal position, and a middle neck incision was made under isoflurane anesthesia. The left CCA was isolated from the valgus nerve, and then ligated and cut. One hour after the CCA occlusion, mice were exposed to a low-oxygen environment (8% O_2 balanced with nitrogen) for 15 min. During the procedure, the rectal temperature was monitored and maintained at 37 ± 0.5 °C using a heating pad.

To verify that the left CCA occlusion had reduced the cerebral blood flow (CBF) in the experimental model, another set of three mice were subjected to CBF measurement. Under isoflurane anesthesia, a 1-cm midline incision was made over the scull, and a laser Doppler flowmeter (FLO-C1; Omegaflo, Tokyo, Japan) was attached perpendicularly to the left parietal bone surface, 1-mm posterior and 5-mm lateral from the bregma, to monitor CBF in the left middle cerebral artery region [8, 9]. After the Doppler flowmeter was installed, the skin incision was closed and the mice were subjected to left CCA occlusion. The CBF decreased by 50.6 ± 18.6%, confirming that the left CCA occlusion alone induces brain ischemia in the left middle cerebral region.

Treatment

Immediately after the hypoxic–ischemic insult, a total of 40 mice that survived the exposure were randomly assigned to two groups that received either saline (control group) or 2.4 units of neurotropin (neurotropin group, Nippon Zoki, Osaka, Japan), by intraperitoneal injection. The neurotropin dose was chosen based on previously published data [10, 11].

Survival rates and neurological function

Survival rates over 7 days after the hypoxic–ischemic injury were assessed in 20 mice (10 mice per each group). Global neurological function was evaluated at the end of the 7 days using the neurological deficit scores (0: no deficit; 1: flexion of the torso; 2: spontaneous circling; 3: longitudinal circling or leaning; 4: no spontaneous movement; 5: death) [12].

Measurement of infarct volume

Another group of 20 mice with the same hypoxic–ischemic brain injury were euthanized 24 h after the procedure for the evaluation of infarct volumes and inflammatory cytokine expression levels. Twenty-four hours after injury induction, the mice were deeply anesthetized with 5% isoflurane and euthanized by cervical dislocation. The brains were removed and coronal slices with a thickness of 1 mm were prepared. Brain slices were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Aldrich, St. Louis, MO) solution, and incubated at 37 °C for 15 min. The area of infarction was traced and measured using image analysis software (ImageJ; National Institutes of Health, Bethesda, MD). The infarct area was calculated as follows to correct for edema: [1—(total ipsilateral hemisphere—infarct region)/total contralateral hemisphere] × 100% [13]. Total infarct volume was calculated as the sum of all infarct areas multiplied by section thickness.

Real-time polymerase chain reaction (PCR)

Real-time PCR was used to measure the mRNA expression levels of interleukin-6 (IL-6), tumor necrosis factor α (TNF-α), and interleukin-1β (IL-1β). Total mRNA was extracted from the brain slices using a RNeasy Mini Kit (Qiagen, Hilden, Germany). 1 μg of mRNA was reverse transcribed using a QuantiTect Reverse Transcription Kit

(Qiagen). PCR was performed on a StepOneTM Real-time PCR system (Life Technologies, Carlsbad, CA) using the PowerSYBR® Green PCR Master Mix and corresponding primers to quantify target genes. The relative changes were normalized to the GAPDH mRNA levels of the same sample. The expression levels of the inflammatory cytokines were reported as % expression by defining the level of the control group as 100%.

Hemodynamic measurements

To test the effects of neurotropin on hemodynamic condition, heart rate and non-invasive blood pressure were measured using an electronic sphygmomanometer (Softron, Tokyo, Japan) in another set of mice ($n=8$). The heart rate and blood pressure were measured and recorded 10 min before the hypoxic–ischemic insult (baseline), during the insult (20 min after the onset of exposure to the low-oxygen environment, immediately after the insult, 1 h and 24 h after the insult.

Statistical analysis

Statistical analysis was performed using Prism 6 software (GraphPad Software, San Diego, CA). A log-rank test was used to analyze the survival rate; two-tailed *t* test was used to analyze infarct volumes, cytokine expression levels; Mann–Whitney test was used to analyze neurological deficit scores; and two-way analysis of variance for repeated measures were used to analyze heart rate and blood pressure. Values are presented as mean ± standard error of the mean for infarct volumes and cytokine expression levels; as median and range for the neurological deficit scores. The sample size of 10 mice per group was sufficient to detect a mean difference of 10% in infarct volume with a power of 80% and an α level of 0.05. A p value of less than 0.05 was considered statistically significant.

Results

As shown in Fig. 1, the neurotropin group mice exhibited a higher survival rate over 7 days than the control group treated with saline (100% vs. 60%, $n=10$ each, $p<0.01$). The neurological deficit scores 7 days after insult were significantly lower in the neurotropin group mice (1; 0–2 vs. 3; 0–5, median; range, $p<0.05$).

To further investigate the mechanisms underlying the improved outcomes in neurotropin-treated mice, the infarct volume analysis and inflammatory cytokine mRNA expression analysis were conducted in the additional set of mice with brain injuries 24 h after the insult.

As shown in Fig. 2, the neurotropin group mice exhibited smaller infarct volumes 24 h after hypoxic–ischemic insult. (18.3 ± 5.1 vs. 38.3 ± 7.2 mm^3, $n=10$ each, $p<0.05$) The neurotropin group mice also showed significantly lower IL-6 mRNA expression levels 24 h after insult (33.8 ± 7.4 vs. 100 ± 26.7%, $p<0.05$). Although the differences in TNF-α and IL-1β mRNA expression levels between the control and neurotropin groups did not reach the statistical significance, there was a trend toward lower mRNA expression levels of both cytokines in the neurotropin-treated mice (TNF-α 23.7 ± 6.2 vs. 100 ± 43.2%, $p=0.1202$, IL-1β 50.3 ± 8.0 vs. 100 ± 22.9%, $p=0.067$) (Fig. 3).

Heart rate and blood pressure were similar during and after hypoxic–ischemic insult. In addition, no changes in heart rate or blood pressure were observed between the groups (Fig. 4).

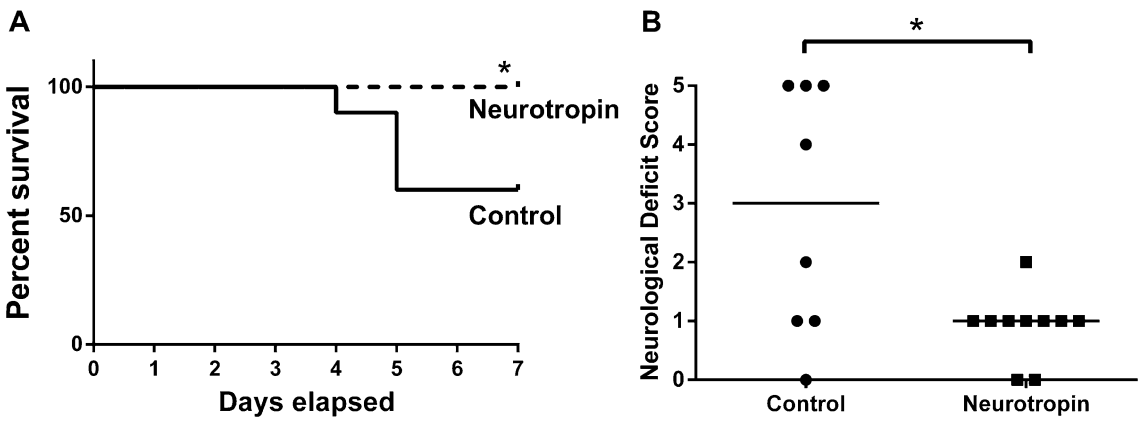

Fig. 1 **a** Survival rate over 7 days after hypoxic–ischemic injury. **b** Neurological deficit score 7 days after hypoxic–ischemic injury. The neurotropin group mice exhibited a higher survival rate over 7 days and lower neurological deficit scores. *$p<0.05$

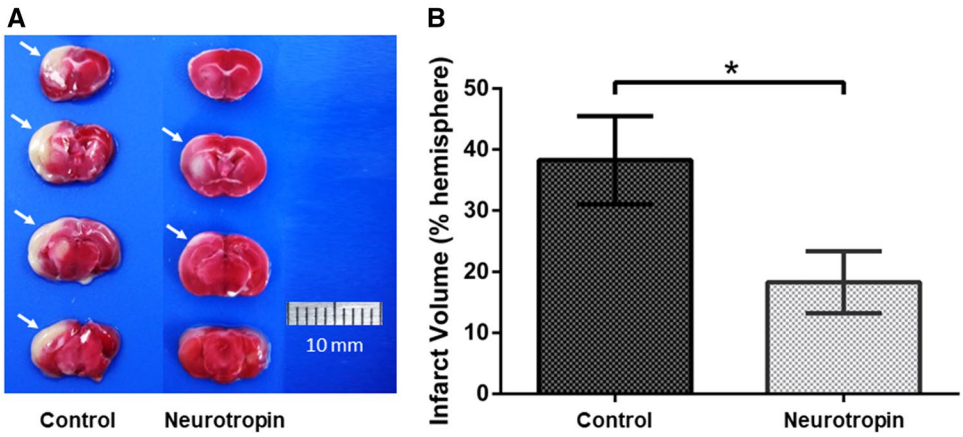

Fig. 2 **a** Representative 2,3,5-triphenyltetrazolium chloride (TTC)-stained corresponding coronal brain sections. Arrows indicate the infarct area (white). **b** Infarct volume 24 h after hypoxic–ischemic injury. The neurotropin group mice exhibited smaller infarct volumes. *$p < 0.05$

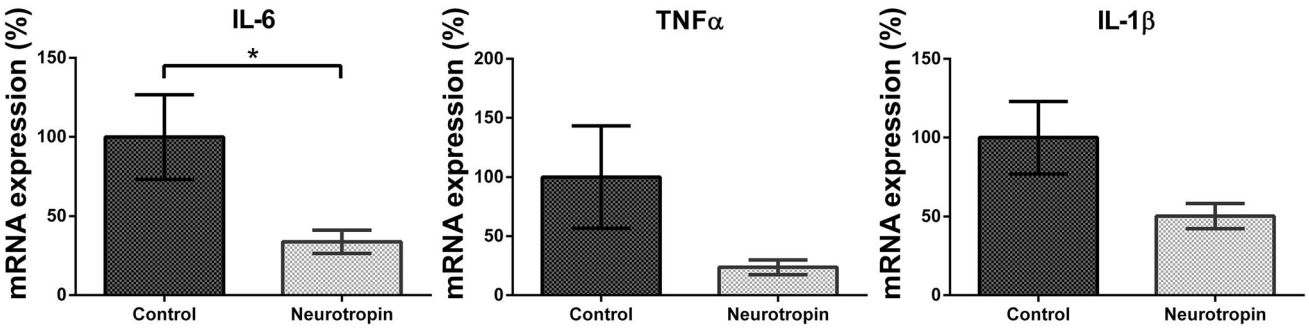

Fig. 3 Inflammatory cytokine expression levels 24 h after hypoxic–ischemic injury. The expression levels are reported as % expression relative to the control group (expression level of 100%, by definition). Neurotropin group mice exhibited lower levels of inflammatory cytokine expression. *$p < 0.05$

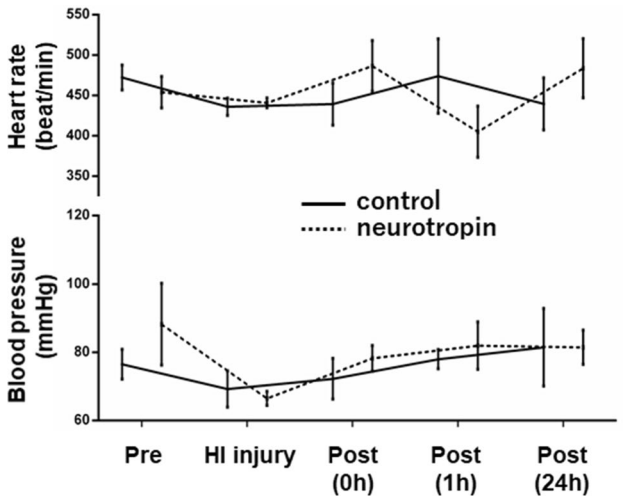

Fig. 4 Heart rate and blood pressure before, during, and after hypoxic–ischemic brain injury. Heart rate and blood pressure did not change significantly during and after the procedure. In addition, no changes in heart rate or blood pressure were observed between the groups

Discussion

In the present study, exposure to hypoxic–ischemic insult caused cerebral infarction and led to a 40% mortality rate in untreated mice. The administration of neurotropin after hypoxic–ischemic insult successfully attenuated the brain injury and improved survival rate and neurological function, demonstrating the neuroprotective effect of post-treatment with neurotropin.

Administration of neurotropin systemically did not affect the heart rate or blood pressure, suggesting cerebral blood flow was not affected by neurotropin [14]. Thus, the neuroprotective effects of neurotropin observed in this study were unlikely to be a result of changes in hemodynamics.

Although evidence for the neuroprotective effect of neurotropin on the central nervous system is so far lacking, Nakajo et al. have reported that oral administration of neurotropin starting 3 weeks prior to ischemic insult promotes the production of brain-derived neurotrophic factor and improves neurological outcomes by suppressing pro-inflammatory

cytokine production in a mouse model of ischemic stroke [2]. The results of this earlier as well as present study suggest that both pre- and post-treatment with neurotropin can induce neuroprotective effects against hypoxic–ischemic brain injuries by suppressing brain inflammation.

Inflammation plays pivotal roles in the pathogenesis of hypoxic–ischemic brain injury. Following direct neuronal damage due to discontinuation of oxygen and glucose supply, activation or infiltration of inflammatory cells enhances local inflammation, resulting in further tissue damage [15]. Among various cytokines and chemokines, IL-1β, IL-6, and TNFα are considered to play crucial roles in cytotoxicity and local inflammation after hypoxic–ischemic brain injury [16–18]. Although there is conflicting evidence for both neuroprotective and detrimental effects of IL-6 in experimental models of hypoxic–ischemic brain injuries, the expression level of IL-6 mRNA has been shown to correlate with infarct volume [19]. Furthermore, Wei et al. recently demonstrated that the blockade of IL-6 receptors reduces infarct volume and improves cognitive function in mice with stroke [20]. These studies support the present findings of the significantly lower IL-6 expression levels in neurotropin-treated mice associated with smaller infarct volumes and improved neurological outcomes. In addition, IL-6 has also been shown to be involved in the mechanism underlying the expansion of ischemic stroke [21]. In the present study, the neurological function of neurotropin-treated mice at 7 days after hypoxic–ischemic insult was found to be almost fully recovered, possibly indicating that neurotropin attenuates hypoxic–ischemic brain injury by suppressing not only the initial local inflammation but also the expansion of the inflammation.

Mechanisms underlying the anti-inflammatory profile and neuroprotective effects of neurotropin have been reported and discussed in the literature [2, 5, 22, 23]. A previous study using a murine model of sciatic nerve injury showed that neurotropin prevents demyelination and promotes differentiation of the Schwann cells by suppressing pro-inflammatory cytokine production [5]. Fukuda et al. reported that neurotropin promotes nerve growth factor signaling and neurite outgrowth of rat pheochromocytoma PC12 cells [22]. It has also been reported that neurotropin inhibits the nuclear factor kappa B-mitogen-activated protein kinases cascade and suppresses the production of pro-inflammatory cytokines [23].

In experimental models of hypoxic–ischemic brain injury, infarct volume increases after the insult and reaches a peak at 24 h, and no further expansion of cerebral infarction occurs afterwards [24]. Overall neurological function shows dramatic recovery over the course of 7 days, indicating that the animals are already in the recovery state [24]. The mRNA expression of pro-inflammatory cytokines also increases in response to hypoxic–ischemic insult and reaches a peak between 3 and 24 h, while the local inflammation diminishes over the course of 5–7 days [25–28]. In this study, we, therefore, harvested the brains 24 h after hypoxic–ischemic insult, during the period when the peak values of infarct volume and cytokine expression levels can be correctly evaluated, and assessed the survival and neurological function at day 7, when the animals are already in the recovery state.

The present study has several limitations. First, because neurotropin contains multiple physiologically active substances and because its pharmacological profile is considered to be based on the combination of the actions of those substances [29], we could not determine which substance is mainly involved in the anti-inflammatory effects observed in this study. Second, we used relatively young male mice, which showed no degenerative vascular changes. Future studies should use aged mice and female mice at different menopausal states. Third, as mentioned above, because neurotropin contains multiple substances and has a wide-ranging pharmacological profile, we cannot exclude the possible involvement of effects other than the anti-inflammatory action in the neuroprotective effect observed in this study. Finally, although this study demonstrated decreased mRNA expression levels of pro-inflammatory cytokines in neurotropin-treated mice, future expansive studies using protein assay would help unveil the mechanisms underlying the neuroprotective effects of neurotropin.

In conclusion, we found that post-treatment with neurotropin-attenuated brain injury and improved neurological outcomes in a mouse model of hypoxic–ischemic brain injury. Inhibition of pro-inflammatory cytokine production is a likely mechanism underlying the neuroprotective effect of neurotropin.

Funding This work was supported by the Japan Society for Promotion of Science (JSPS KAKENHI Grant number 15K10507 and 17K11044).

Compliance with ethical standards

Conflict of interest The author declares that they have no conflict interests.

References

1. Hankey GJ. The global and regional burden of stroke. Lancet Glob Health. 2013;1:e239–40.
2. Nakajo Y, Yang D, Takahashi JC, Zhao Q, Kataoka H, Yanamoto H. ERV enhances spatial learning and prevents the development of infarcts, accompanied by upregulated BDNF in the cortex. Brain Res. 2015;1610:110–23.
3. Ishikawa T, Yasuda S, Minoda S, Ibuki T, Fukuhara K, Iwanaga Y, Ariyoshi T, Sasaki H. Neurotropin((R)) ameliorates chronic pain via induction of brain-derived neurotrophic factor. Cell Mol Neurobiol. 2015;35:231–41.

4. Yoshii H, Fukata Y, Yamamoto K, Naiki M, Suehiro S, Yanagihara Y, Okudaira H. Neurotropin inhibits accumulation of eosinophils induced by allergen through the suppression of sensitized T-cells. Int J Immunopharmacol. 1995;17:879–86.
5. Nishimoto S, Okada K, Tanaka H, Okamoto M, Fujisawa H, Okada T, Naiki M, Murase T, Yoshikawa H. Neurotropin attenuates local inflammatory response and inhibits demyelination induced by chronic constriction injury of the mouse sciatic nerve. Biologicals. 2016;44:206–11.
6. Rice JE 3rd, Vannucci RC, Brierley JB. The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann Neurol. 1981;9:131–41.
7. Cowper-Smith CD, Anger GJ, Magal E, Norman MH, Robertson GS. Delayed administration of a potent cyclin dependent kinase and glycogen synthase kinase 3 beta inhibitor produces long-term neuroprotection in a hypoxia-ischemia model of brain injury. Neuroscience. 2008;155:864–75.
8. Hedna VS, Ansari S, Shahjouei S, Cai PY, Ahmad AS, Mocco J, Qureshi AI. Validity of laser doppler flowmetry in predicting outcome in murine intraluminal middle cerebral artery occlusion stroke. J Vasc Interv Neurol. 2015;8:74–82.
9. Kotoda M, Ishiyama T, Mitsui K, Hishiyama S, Matsukawa T. Nicorandil increased the cerebral blood flow via nitric oxide pathway and ATP-sensitive potassium channel opening in mice. J Anesth. 2018;32:244–9.
10. Toda K, Muneshige H, Ikuta Y. Antinociceptive effects of neurotropin in a rat model of painful peripheral mononeuropathy. Life Sci. 1998;62:913–21.
11. Suzuki T, Li YH, Mashimo T. The antiallodynic and antihyperalgesic effects of neurotropin in mice with spinal nerve ligation. Anesth Analg. 2005;101:793–9.
12. Wang P, Xu TY, Wei K, Guan YF, Wang X, Xu H, Su DF, Pei G, Miao CY. ARRB1/beta-arrestin-1 mediates neuroprotection through coordination of BECN1-dependent autophagy in cerebral ischemia. Autophagy. 2014;10:1535–48.
13. Llovera G, Roth S, Plesnila N, Veltkamp R, Liesz A. Modeling stroke in mice: permanent coagulation of the distal middle cerebral artery. J Vis Exp. 2014;89:e51729.
14. Rosner MJ, Rosner SD, Johnson AH. Cerebral perfusion pressure: management protocol and clinical results. J Neurosurg. 1995;83:949–62.
15. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci. 1999;22:391–7.
16. Sobowale OA, Parry-Jones AR, Smith CJ, Tyrrell PJ, Rothwell NJ, Allan SM. Interleukin-1 in Stroke: from bench to bedside. Stroke. 2016;47:2160–7.
17. Cojocaru IM, Cojocaru M, Tanasescu R, Iliescu I, Dumitrescu L, Silosi I. Expression of IL-6 activity in patients with acute ischemic stroke. Rom J Intern Med. 2009;47:393–6.
18. Doll DN, Rellick SL, Barr TL, Ren X, Simpkins JW. Rapid mitochondrial dysfunction mediates TNF-alpha-induced neurotoxicity. J Neurochem. 2015;132:443–51.
19. Gredal H, Thomsen BB, Boza-Serrano A, Garosi L, Rusbridge C, Anthony D, Moller A, Finsen B, Deierborg T, Lambertsen KL, Berendt M. Interleukin-6 is increased in plasma and cerebrospinal fluid of community-dwelling domestic dogs with acute ischaemic stroke. Neuroreport. 2017;28:134–40.
20. Wei J, Sun C, Liu C, Zhang Q. Effects of rat anti-mouse interleukin-6 receptor antibody on the recovery of cognitive function in stroke mice. Cell Mol Neurobiol. 2018;38:507–15.
21. Armstead WM, Hekierski H, Pastor P, Yarovoi S, Higazi AA, Cines DB. Release of IL-6 after stroke contributes to impaired cerebral autoregulation and hippocampal neuronal necrosis through NMDA receptor activation and upregulation of ET-1 and JNK. Transl Stroke Res. 2019;10:104–11.
22. Fukuda Y, Fukui T, Hikichi C, Ishikawa T, Murate K, Adachi T, Imai H, Fukuhara K, Ueda A, Kaplan AP, Mutoh T. Neurotropin promotes NGF signaling through interaction of GM1 ganglioside with Trk neurotrophin receptor in PC12 cells. Brain Res. 2015;1596:13–21.
23. Zheng Y, Fang W, Fan S, Liao W, Xiong Y, Liao S, Li Y, Xiao S, Liu J. Neurotropin inhibits neuroinflammation via suppressing NF-kappaB and MAPKs signaling pathways in lipopolysaccharide-stimulated BV2 cells. J Pharmacol Sci. 2018;136:242–8.
24. Liu F, Schafer DP, McCullough LD. TTC, fluoro-Jade B and NeuN staining confirm evolving phases of infarction induced by middle cerebral artery occlusion. J Neurosci Methods. 2009;179:1–8.
25. Ohtaki H, Yin L, Nakamachi T, Dohi K, Kudo Y, Makino R, Shioda S. Expression of tumor necrosis factor alpha in nerve fibers and oligodendrocytes after transient focal ischemia in mice. Neurosci Lett. 2004;368:162–6.
26. Clausen BH, Lambertsen KL, Meldgaard M, Finsen B. A quantitative in situ hybridization and polymerase chain reaction study of microglial-macrophage expression of interleukin-1beta mRNA following permanent middle cerebral artery occlusion in mice. Neuroscience. 2005;132:879–92.
27. Suzuki S, Tanaka K, Nogawa S, Nagata E, Ito D, Dembo T, Fukuuchi Y. Temporal profile and cellular localization of interleukin-6 protein after focal cerebral ischemia in rats. J Cereb Blood Flow Metab. 1999;19:1256–62.
28. Gelderblom M, Leypoldt F, Steinbach K, Behrens D, Choe CU, Siler DA, Arumugam TV, Orthey E, Gerloff C, Tolosa E, Magnus T. Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. Stroke. 2009;40:1849–57.
29. Itoh ET. Hata, Analgesic mechanism of neurotropin: relation to the serotonergic system and influence of spinal cord transection. Jpn J Pharmacol. 1989;51:267–72.