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Neuronostatin induces hyperalgesia in formalin test in mice

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ABSTRACT

Neuronostatin, a newly identified peptide encoded by the somatostatin (SST) gene, was proved to produce significant antinociceptive effect in mouse tail immersion test. However, the effect of neuronostatin on tonic pain was still not clear. The aim of this study was to investigate the effect of neuronostatin in the formalin test and its possible mechanism. We found that intracerebroventricular (i.c.v.) administration of neuronostatin (1, 3, 6, 12 nmol/mouse) increased licking in a dose-related manner during the late phase, but did not affect the early phase of formalin test in mice. In addition, the hyperalgesic effect during the late phase was completely reversed by melanocortin 3/4 receptor antagonist SHU9119 (50 pmol/mouse) or opioid receptor antagonist naloxone (5 nmol/mouse), but not GABAA receptor antagonist bicuculline (1086 pmol/mouse). These data suggested that the hyperalgesic response induced by neuronostatin was dependent upon the central melanocortin system and endogenous opioid system. In conclusion, these results indicated that neuronostatin may be a new neuropeptide with important role in the modulation of acute and tonic pain.

1. Introduction

Neuronostatin, a 13-amino acid peptide with C-terminal amidation, is recently discovered from pro-somatostatin protein [22]. Unlike somatostatin, neuronostatin fails to activate any of the five endogenous somatostatin receptors, and it neither stimulates Gi signaling mediated by the somatostatin receptors nor modulates growth hormone release from pituitary cells [8,22]. In addition, somatostatin is a cyclic polypeptide while neuronostatin is amidated [22].

Neuronostatin possesses important physiological functions in neuronal, metabolic, and other tissues [22]. Intracerebroventricular (i.c.v.) administration of neuronostatin induces an inhibition of both food and water intake, and the actions are dependent upon the central melanocortin system [31]. Neuronostatin leads to a biphasic, dose-related increase in mean arterial pressure, the hypertensive effect seems due to increase of sympathetic nervous system activity and vasopressin secretion, which acts also through the central melanocortin system [30]. It also depresses the contractile function of both whole hearts and cardiomyocytes [12]. In addition, previous work from our laboratory has demonstrated that i.c.v. administration of neuronostatin produces a dose- and time-related anti-nociceptive effect in the tail immersion test in mice [31].

The tail immersion test is based on a phasic stimulus of high intensity [3,9]. This nociceptive stimulus could only measure the responses that are not contaminated by simultaneous perturbations related to other functions [5]. In contrast, the responses to noxious stimuli by tonic pain tests are short-lasting and of moderate intensity. Because of their longer duration and association with tissue injury, tonic pain test is believed to provide a more valid model for clinical pain than the tests with phasic stimuli [28,3]. The formalin test is a classic model of the tonic pain test and the response shows a biphasic behavioral reaction. This behavior consists of an early phase, occurring about 3 min after injected formalin, and then after a quiescent period, a second phase between the 10th and 30th minutes. The early phase seems to be caused predominantly by C-fibre activation due to the direct stimulation of peripheral nociceptors, while the late phase appears to be dependent on the combination of an inflammatory reaction in the peripheral tissue and function changes in the dorsal horn of the spinal cord [28,1,3,23]. However, to our knowledge, the nociceptive effect of neuronostatin in the tonic pain test has not been reported. Therefore, it seems necessary to investigate the effect of neuronostatin on tonic pain. In the present work, we intend to determine whether neuronostatin was involved in nociception effect in inflammatory model.

2. Materials and methods

2.1. Animals

Male Kunming mice, weighing 18–22 g, were obtained from the Experimental Animal Center of Lanzhou University (Lanzhou,

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China). The animals were maintained (5–6/cage) at room temperature of 22–24 °C and 50–60% relative humidity with free access to water and food, under a 12 h light–dark cycle (light on 7:30 a.m.–7:30 p.m.). The animals were allowed to adapt to this environment for a period of 3–5 days before the experiments. All testing procedures were approved of by the guidelines of the Ethics Committee of Animal Experiments of Lanzhou University.

2.2. Drugs

Neuronostatin (Leu-Arg-Gln-Phe-Leu-Gln-Lys-Ser-Leu-Ala-Ala-Ala-Ala-NH_2) was synthesized by manual solid-phase synthesis using standard Fmoc-chemistry as described in our previous report [31]. Naloxone hydrochloride dehydrates and bicuculline methiodide were purchased from Sigma–Aldrich Chemical Company (USA). SHU9119 was bought from Genscript Corporation (USA). All reagents were dissolved in normal saline (NS).

2.3. Intracerebroventricular injection

Intracerebroventricular (i.c.v.) administration was performed following the method described by Haley and McCormick [11]. The injection site was 1.5 mm from the middle, 1 mm from the bregma and 3 mm from the surface of the skull. Drugs were administered in a volume of 5 μl at a constant rate of 10 μl/min using a 25 μl Hamilton microsyringe. The proper injection site was verified in pilot experiments by administration and localization of methylene blue dye.

2.4. Formalin test

The method was performed as previously described [28,19]. Briefly, mice were placed in a Plexiglas box (15 cm in diameter and 20 cm in height) with a mirror placed under the floor at a 45° angle to allow observation of the paws of the mice. After each mouse was habituated to the chamber about 5 min, drug or saline was given intracerebroventricularly, after another 5 min the formalin (1% formaldehyde solution, 20 μl) was injected subcutaneously (s.c.) into the ventral surface of the right hind paw, then mice were placed back and observed for 30 min and the time(s) spent licking the injected hind paw was recorded in 5 min intervals. The licking time was collected manually by a trained observer, only scoring the time spent licking and biting the injected paw. The time spent licking the formalin-injected paw was an indicator of the nociceptive response. The response is biphasic: the first 10 min after formalin injection are referred as the acute phase and the period between 10 min and 30 min as the second phase. After experiment, mice were immediately sacrificed by cervical dislocation.

2.5. Experimental design

The experimental scheme was divided into two sections: (1) neuronostatin (1, 3, 6, 12 nmol/mouse) or saline were given i.c.v. in mice, in experimental and vehicle groups, respectively; (2) in order to determine the mechanisms of the effect elicited by neuronostatin, melanocortin 3/4 (MC3/4) receptor antagonist SHU9119 (50 pmol/mouse) [31], γ-aminobutyric acid (GABAA) receptor antagonist bicucullin (1086 pmol/mouse) [17] or opioid receptor antagonist naloxone (5 nmol/mouse) [31] was i.c.v. co-administered with neuronostatin.

2.6. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Differences between treatment groups were analyzed by one-way analysis of variance (ANOVA). Post hoc analyses were performed using Dunnett's test. In all statistical comparisons, differences with $P < 0.05$ were considered significance.

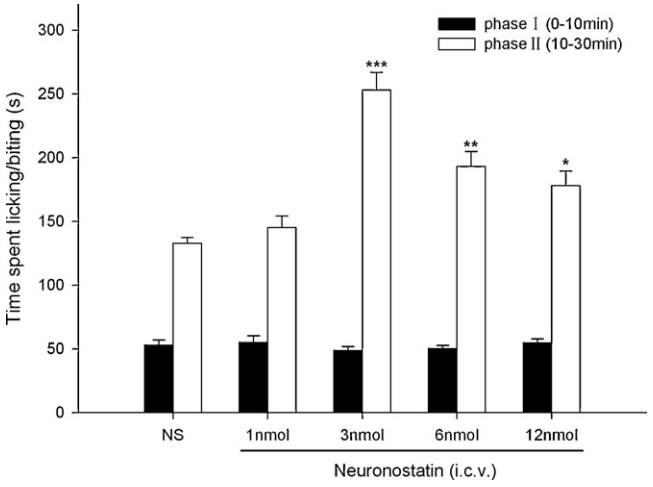

Fig. 1. The hyperalgesic effect of neuronostain in the formalin test in mice. The first phase represents the cumulative NR in the first 10 min after formalin injection whereas the second phase represents the cumulative NR 10–30 min after formalin injection. Neuronostain were injected i.c.v. 5 min before the s.c. administration of 1% formaldehyde solution on the right ventral paw as described in material and methods. All data are presented mean ± SEM for $n = 8$–12 per group. The statistical significance of differences between the groups was assessed with a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. $^{**}P < 0.01$ and $^{***}P < 0.001$, statistically significant differences between neuronostain vs. NS.

3. Results

3.1. Hyperalgesia induced by neuronostatin in formalin test

The dose-related hyperalgesic effect of i.c.v. injection of neuronostatin was illustrated in Fig. 1. Compared to the saline, i.c.v. administration of neuronostatin (1, 3, 6 or 12 nmol/mouse) produced a dose-related increase in the paw-licking time of the second phase, and no significant difference was detected in the first phase (vs. NS group, each $P > 0.855$). The paw-licking time after i.c.v. administration of neuronostatin (1, 3, 6 or 12 nmol/mouse) in the second phase was 145.06 ± 9.24 s, 252.92 ± 13.84 s, 192.94 ± 11.88 s, 178.04 ± 11.58 s (vs. NS group, respectively, $P = 0.841$, $P < 0.001$, $P = 0.002$, $P = 0.017$). 1 nmol neuronostatin failed to produce significant effect in comparison with the saline group, the most effective hyperalgsia was evoked by 3 nmol/mouse neuronostatin.

3.2. Effect of SHU9119 on the hyperalgesia induced by neuronostatin

As showed in Fig. 2A, i.c.v. co-injection of the selective MC3/4 receptor antagonist SHU9119 (50 pmol/mouse) and neuronostatin (3 nmol/mouse) completely blocked the hyperalgesia induced by neuronostatin, the licking time was 137.62 ± 9.69 s (vs. neuronostatin group, $P < 0.001$). However, 50 pmol SHU9119 alone failed to alter the licking time compared with vehicle treatment (vs. NS group, $P = 0.760$).

3.3. Effect of bicuculline on the hyperalgesia induced by neuronostatin

Bicuculline, γ-aminobutyric acid (GABAA) receptor antagonist, was chosen in our experiment to test whether GABAA receptor participates in the hyperalgesic effect of neuronostatin. As