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Research paper

Intranasal administration of neuromedin U derivatives containing cell-penetrating peptides and a penetration-accelerating sequence induced memory improvements in mice

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ABSTRACT

Neuromedin U (NMU) is a neuropeptide that is expressed and secreted in the brain and gut. We previously demonstrated that the intracerebroventricular (i.c.v.) administration of NMU inhibited inflammation-mediated memory impairment in mice. In order to utilize NMU as a clinical treatment tool for inflammation-mediated amnesia, we herein focused on non-invasive intranasal delivery because the i.c.v. administration route is invasive and impractical. In the present study, we prepared two NMU derivatives containing cell-penetrating peptides (CPPs), octaarginine (R8), and each penetration-accelerating sequence, namely FFLIPKG (PASR8-NMU) and FFFFG (F4R8-NMU), for intranasal (i.n.) administration. In the Y-maze test, the i.c.v. administration of lipopolysaccharide (LPS) (10 μg/mouse) significantly decreased spontaneous alternation behavior, and this was prevented by the prior administration of PASR8-NMU or F4R8-NMU (5.6 μg/mouse, i.n.). Moreover, the administration of PASR8-NMU or F4R8-NMU (5.6 μg/mouse, i.n.) just before the Y-maze test also improved LPS-induced memory impairment. Indocyanine green (ICG)-labeled PASR8-NMU (i.n.) was significantly observed in the hippocampus and paraventricular hypothalamic nucleus 30 min after its i.n. administration. PASR8-NMU, but not F4R8-NMU guaranteed the stability of the administration liquid for 24 h. These results suggest that PASR8-NMU is effective for i.n. delivery to the brain, and may be useful in the clinical treatment of inflammation-mediated amnesia.

1. Introduction

Neuromedin U (NMU) is a neuropeptide that was originally isolated from the porcine spinal cord and named after its ability to cause constriction of rat uterine smooth muscle [1]. NMU mRNA expression has been detected in several organs with the highest levels being observed in the brain and gastrointestinal tract in rodents [2,3] and in human antigen-presenting cells, particularly monocytes and dendritic cells [4]. Two G-protein-coupled receptors for NMU have been identified and designated as NMU-receptor 1 (NMUR1) and NMU-receptor 2 (NMUR2) [5–9]. NMUR1 is mainly expressed in the peripheral tissues with the strongest expression being detected in the gastrointestinal tract [8,9] and in T-cells and natural killer cells [4]. In contrast, NMUR2 is predominantly expressed in the central nervous system (CNS), in which its expression has been found in the hippocampus and paraventricular hypothalamic nucleus (PVH) [5]. NMU was previously reported to induce an increase in blood pressure, smooth muscle contraction, pronociception, stress responses, a decrease in food intake and body weight, and immune regulation [5,10–13].

Neuroinflammatory responses are induced in a number of neurodegenerative disorders including Alzheimer's disease [14,15], in the early stage of which the production of several cytokines in glia play an

Abbreviations: BLA, basolateral amygdala; CNS, central nervous system; CPPs, cell penetrating peptides; DMH, dorsomedial hypothalamic nucleus; F4, FFFFG (N terminus as a simplified version of the PAS segment); FITC, fluorescein isothiocyanate; FOS12, n-dodecylphosphocholine; Hip, hippocampus; ICG, indocyanine green; IL, infralimbic cortex; i.n., intranasal; i.c.v., intracerebroventricular; LPS, lipopolysaccharide; NMU, neuromedin U; NMUR1, NMU receptor 1; NMUR2, NMU receptor 2; OB, olfactory bulb; PAS, penetration-accelerating sequence; PBN, parabrachial nucleus; PBS, phosphate-buffered saline; PVH, paraventricular hypothalamic nucleus; R8, octaarginine

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important role [16]. Lipopolysaccharide (LPS), an endotoxin found in the outer leaflet of the outer membrane of Gram-negative bacteria, has been used experimentally to induce inflammatory responses [17]. We previously reported that the central administration of NMU inhibited LPS-induced memory impairment and neuronal cell-death [18]. However, central administration is invasive, costly, and impractical for the delivery of drugs into human brains, and thus we need to develop a non-invasive and effective route by which to deliver NMU into the brain.

In the present study, we prepared two NMU derivatives containing cell-penetrating peptides (CPPs), octaarginine (R8), and a penetration-accelerating sequence (PAS) for intranasal (i.n.) administration. CPPs including arginine-rich peptides have been shown to deliver various bioactive molecules with low membrane permeability into cells, and led to the regulation of cell functions [19–21]. Macropinocytosis is transient, actin-driven fluid-phase endocytosis that involves membrane ruffling and the formation of large vacuoles called macropinosomes [22,23], which plays an important role in the cellular uptake of arginine-rich CPPs, resulting in highly efficient intracellular delivery [24,25]. The addition of a PAS to CPPs has been reported to enhance the efficiency of the intracellular delivery of bioactive peptides by promoting endosomal escape [26,27], and we recently demonstrated that a glucagon-like peptide-2 derivative containing a PAS and R8 was effectively delivered into the brain by the i.n. administration [28]. We herein examined i.n. delivery to the brain, behavioral effects, and the stability of NMU derivatives, and also assessed how a difference in PAS may affect delivery.

2. Materials and methods

2.1. Preparation of NMU derivatives

PASR8-NMU (FFLIPKG-RRRRRRRR-GG-YKVNEYQGPVAPSGGFFL FRPRN), F4R8-NMU (FFFFG-RRRRRRRR-GG-YKVNEYQGPVAPSGGFF LFRPRN), PASR8 and F4R8 (Patent pending: WO 2016/035820 A1; USA: 15/507, 403; EU: 15837573.3), which were labeled with fluorescein isothiocyanate (FITC) or indocyanine green (ICG), were synthesized by SCRUM Inc (Tokyo, Japan) with a peptide synthesizer (433A: Applied Biosystems) following the standard 9-fluorenylmethoxycarbonyl (Fmoc) method.

2.2. Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee at Tokyo University of Science, and were conducted according to the guidelines of the National Institute of Health and Japan Neuroscience Society. We used 5- to 7-week-old male ddY mice (Japan SLC, Shizuoka, Japan), and attempted to minimize the number of animals used and their suffering. A total of 240 ddY mice were used in experiments. All animals were kept in a controlled environment, with a 12:12-h light schedule, temperature (23 °C), and relative humidity (55 ± 5%) for at least 5 days before experiments, and were provided *ad libitum* access to food and water.

2.3. The administration of NMU derivatives or native NMU

NMU derivatives and native NMU (Peptide Inc., Osaka, Japan) were dissolved in 0.5% *n*-dodecylphosphocholine (FOS12) (GE Healthcare Japan Inc., Tokyo, Japan) at 4 °C. Mice were anesthetized using isoflurane, and i.n. administered a NMU derivative (a total amount of 5.6 μg/mouse), native NMU (5.6 μg/mouse), PASR8, F4R8, or vehicle (0.5% FOS12 in phosphate-buffered saline) (a total volume of 4 μl/mouse) via a micropipette in each nostril 15 min before the intracerebroventricular (i.c.v.) administration of lipopolysaccharide (LPS) or 30 min before the behavioral test. Nose drops administered to animals lying on their backs resulted in consistent deposition in the olfactory or respiratory epithelium [28]. Although our previous studies showed that the native NMU (1.3 μg equivalent to 0.5 nmol/mouse, i.c.v.) significantly prevented the impairment of spontaneous alternation performance induced by LPS in the Y-maze test, the NMU derivative (0.5 nmol/mouse, i.n.) did not affect them [$F(3,20) = 0.3569$, $P = 0.7847$, one-way ANOVA; $P > 0.05$ for LPS-vehicle v.s. LPS-F4R8-NMU, Bonferroni's post hoc test]. We thus employed the two-fold dose for the i.c.v. administration of native NMU accordingly (5.6 μg equivalent to 0.97 nmol of PASR8-NMU and 1.0 nmol of F4R8-NMU).

2.4. Y-maze test

The experimental schedule is shown in Figs. 1 A and 2 A. The Y-maze test was performed under bright (fluorescent room light) conditions and as described previously with some modifications [18,29]. Each mouse, new to the maze, was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The series of arm entries were recorded visually. Alternation was defined as successive entries into the three arms on overlapping triplet sets. The effect was calculated as the percent alternation according to the following formula: Percent alternation = {(number of alternations)/(total number of arm entries − 2)} × 100 (%). The arms were wiped down with paper between sessions.

2.5. Distribution of i.n. administrated NMU derivatives in the mouse brain

Mice were transcardially perfused with 0.1 M phosphate buffer (PB) (pH 7.4), followed by 50–100 ml of 4% (w/v) paraformaldehyde 30 min after the i.n. administration of NMU derivatives or vehicle (0.5% FOS12 in phosphate-buffered saline, PBS). Brains were removed and postfixed at 4 °C overnight in the same fixative. After cryoprotection with 30% (w/v) sucrose in PBS, brains were sectioned using a cryostat (CM1560S; Leica Microsystems, Wetzlar, Germany), at a thickness of 30 μm into five series. Tissue sections were washed twice in PB, and fluorescence patterns were analyzed microscopically (BZ-9000, Keyence, Osaka, Japan). ICG-positive dots per each tissue section were counted using software Dynamic cell count BZ-HIC (Keyence). In order to ascertain brain regions, alternate sections were stained with 0.2% cresyl violet for the Nissl substance in the following areas according to Paxinos & Franklin [30]: the olfactory bulb (OB; bregma 3.92 mm), infralimbic cortex (IL; bregma 1.54 mm), paraventricular hypothalamic nucleus (PVH; bregma −0.82 mm), basolateral amygdala (BLA), hippocampus (Hip) (bregma −1.34 mm), dorsomedial hypothalamic nucleus (DMH; bregma −1.46 mm), and parabrachial nucleus (PBN; bregma −5.33 mm).

2.6. In vitro stability test of NMU derivatives

NMU derivatives were dissolved in 0.5% FOS12 at 4 °C. Stability was assessed using high-performance liquid chromatography (HPLC) (LC-20A series; Shimadzu, Kyoto, Japan). The amount of NMU derivatives present at 0 h was defined as 100%. HPLC conditions were as follows: separation was achieved using a reversed-phase column (C18, 100 × 4.6 mm; Sigma-Aldrich, St. Louis, Mo, USA). Phase A was 0.1% trifluoroacetic acid (TFA) in water and phase B was 0.085% TFA solution in acetonitrile. Samples were eluted with a linear gradient of acetonitrile (25%–90%) delivered at 1.0 ml/min at 40 °C, and UV absorbance was detected at 214 nm.

2.7. Data and statistical analyses

All values are given as means ± SEM. The significance of differences was evaluated using the one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. In all cases, significance was set at $P < 0.05$. Statistical analyses were performed using Graphpad Prism (Graphpad Software, USA).