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Neuromedin U inhibits food intake partly by inhibiting gastric emptying

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ABSTRACT

Neuromedin U (NMU) is a gut-brain peptide, implicated in energy and glucose homeostasis via the peripherally expressed NMU receptor 1 (NMUR1) and the central NMUR2. We investigated the effects of a lipidated NMU analog on gastric emptying (GE), glucose homeostasis and food intake to evaluate the use of a NMU analog as drug candidate for treatment of obesity and diabetes. Finally mRNA expression of NMU and NMUR1 in the gut and NMUR2 in the hypothalamus was investigated using a novel chromogen-based in situ hybridization (ISH) assay. Effects on food intake (6 and 18 h post dosing) were addressed in both mice and rats. The effects on GE and glycaemic control were assessed in mice, immediately after the first dose and after seven days of bidaily (BID) dosing. The lipidated NMU analog exerted robust reductions in GE and food intake in mice and improved glycaemic control when measured immediately after the first dose. No effects were observed after seven days BID. In rats, the analog induced only a minor effect on food intake. NMU mRNA was detected in the enteric nervous system throughout the gut, whereas NMUR1 was confined to the lamina propria. NMUR2 was detected in the paraventricular (PVN) and arcuate nuclei (ARC) in mice, with a reduced expression in ARC in rats. In summary, the anorectic effect of the lipidated NMU is partly mediated by a decrease in gastric emptying which is subject to tachyphylaxis after continuous dosing. Susceptibility to NMU appears to be species specific.

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Introduction

Neuromedin U (NMU) is a neuropeptide, which is expressed and secreted in both the brain and gut [1–3]. Two G-protein-coupled receptor subtypes for NMU have been identified: the neuromedin U receptor 1 (NMUR1) and neuromedin U receptor 2 (NMUR2) [2,4–7]. NMUR1 is mainly expressed peripherally with highest expression in the gastrointestinal tract [2,4,5,8]. In contrast, NMUR2 is predominantly expressed in the central nervous system where expression is mainly found in the hippocampus, spinal cord and hypothalamus [2,4–7].

The amino acid sequence of NMU is highly conserved across species indicating physiological importance of this peptide [6]. Despite this, the exact role of NMU and the NMU receptors still needs to be clarified. Studies have indicated that NMU could play a role in regulation of blood pressure, smooth muscle contraction, nociception, stress and inflammation [6]. Furthermore, both genetic and pharmacological data indicate that NMU could be involved in energy and appetite homeostasis. Mice lacking the gene encoding NMU become obese, and in humans, a genetic variation in the NMU gene has been associated with overweight and obesity [9,10]. Central administration of NMU has been shown to decrease food intake and body weight in rodents by increasing energy expenditure, locomotor activity, core body temperature, and oxygen consumption [3,11–14]. In addition, it has been shown that subcutaneous (s.c.) administration of NMU to diet induced obese (DIO) mice decreases food intake, body weight, and increases energy expenditure. These data support a role for peripherally administered NMU in regulation of appetite, body weight and glucose homeostasis [15]. Even though several studies involving NMU have been conducted, the mechanism of action of NMU on food intake and appetite modulation is still poorly defined and only a few studies focus specifically on unraveling the mechanisms behind the anorectic effect of peripheral administered NMU. The distribution of NMUR2 in key hypothalamic areas may indicate a direct central

Abbreviations: ARC, arcuate nucleus; AUC, area under the curve; DIO, diet induced obesity; FFPE, formalin fixated paraffin embedded; GE, gastric emptying; GE-OGTT, gastric emptying and oral glucose tolerance test; GLP-1, gastrointestinal (GI) tract, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; NMU, neuromedin U; NMUR1, neuromedin U receptor 1; NMUR2, neuromedin U receptor 2; PVN, paraventricular nucleus; s.c., subcutaneous.

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effect. However, many gut brain peptides have also been shown to reduce food intake, at least partly, due to an inhibitory effect on gastric emptying [16,17].

The present study was initiated to get a more in-depth knowledge about the pharmacological and physiological effects of NMU and its involvement in food intake. We used a long-acting lipidated NMU peptide analog (GUB07-007) to investigate effects on food intake, glucose homeostasis and gastric emptying immediately after the first dose and after seven days of bidaily (BID) dosing. The effects were compared to the long acting Glucagon-like peptide-1 (GLP-1) analog liraglutide to evaluate the use of a lipidated NMU analog as a potential drug candidate for treatment of obesity and diabetes. Finally, we investigated the mRNA expression of NMU and NMUR1 in the gastrointestinal tract, as well as the expression of NMUR2 in the hypothalamus, by means of a novel chromogen-based in situ hybridization technique.

Materials and methods

Peptide synthesis

The lipidated NMU analog (GUB07-007) (H-FRVDEEFQK(Pam-γE-OH)PFASQSRGYFLFRPRN-NH_2) was prepared by automated solid-phase peptide synthesis using the Fmoc/*t*Bu strategy on Rink amide TentaGel resin (Rapp polymere GmbH, Tuebingen, Germany). The couplings were performed using Fmoc-N^{α}-protected amino acids, *N,N′*-diisopropylcarbodiimide and ethyl cyanoglyoxylate-2-oxime (oxyma) in *N,N*-dimethylformamide (Iris Biotech GmbH, Marktredwitz, Germany) for 2×2 h. The N^{α}-deprotections were performed using 40% piperidine in *N*-methyl-2-pyrrolidione (Iris Biotech GmbH, Marktredwitz, Germany) for 3 min followed by 20% piperidine in *N*-methyl-2-pyrrolidione for 22 min. The lipidation was performed selectively on-resin on the N^{ε} amine of a Lys-9 using the alloc protecting group. Following linear assembly of the peptide backbone, the alloc protecting group was removed using tetrakis(triphenylphosphine)palladium(0) [$Pd(PPh_3)_4$] (Sigma–Aldrich, Brøndby, Denmark) and borane dimethylamine complex (Sigma–Aldrich, Brøndby, Denmark) as scavenger in dichloromethane (Sigma-Aldrich, Brøndby, Denmark). The mixture reacted for 2.5 h at room temperature. Fmoc-Glu-O*tBu* (4 eq.) (Iris Biotech GmbH, Marktredwitz, Germany) was coupled to the free amine using *N*-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) (Iris Biotech GmbH, Marktredwitz, Germany) as coupling reagent and DIEA (Iris Biotech GmbH, Marktredwitz, Germany) as base for 2 h at room temperature. Palmitic acid (Sigma–Aldrich, Brøndby, Denmark) was incorporated using HATU and DIEA for 2 h at room temperature. Finally, the peptide was simultaneously side-chain deprotected and released from the solid support by a TFA cocktail containing trifluoro acetic acid (TFA) (Iris Biotech GmbH, Marktredwitz, Germany), triethylsilane (Sigma–Aldrich, Brøndby, Denmark) and H_2O (95/2.5/2.5) as scavengers for 2 h. The peptide was precipitated by the addition of diethylether (Sigma–Aldrich, Brøndby, Denmark). The peptide was purified by RP-HPLC and identified by LC-MS. The final products were obtained with >95% purity. For a detailed description see [18].

Animal studies

All animal experiments were conducted in accordance with internationally accepted principles for the care and use of laboratory animals and were covered by a personal license issued for Jacob Jelsing (approved by the Danish Committee for Animal Research, permit number: 2013-15-2934-00784).

In a recent paper, we have described the development and specific effects on food intake of a library of long-acting lipidated NMU analogs [18]. Based on these data, GUB07-007 (0.3 μmol/kg) was selected as one of the most efficacious for the present study.

Effect of a single dose of GUB07-007 on cumulative food intake in male NMRI mice and SPD rats

The effect on cumulative food intake was measured following a single dose of GUB07-007 using a fully automated food intake monitoring system (HM-2; MBRose ApS, Faaborg, Denmark), allowing for advanced synchronous real-time monitoring of food intake behavior of individual animals as previously described [18]. A total of 24 male NMRI mice, 6–7 weeks old (approx. 25–30 g body weight) at the time of arrival were obtained from Taconic (Denmark). Upon arrival, the animals were uniquely identified with s.c. implantable microchips (Pet ID Microchip, E-vet, Haderslev, Denmark), transferred to the HM-2 system and acclimatized to their new environment. The animals were housed in groups of 4 in a light-, temperature-, and humidity-controlled room (a 12/12 LD cycle, lights on at 02:00 AM; 22 ± 2 °C; 50% relative humidity). The mice had ad libitum access to regular chow diet (Altromin 1324, Brogaarden A/S, Lynge, Denmark) and domestic quality tap water. Mice arrived at day-7, and a minimum of 5 days of habituation to the system was allowed prior to beginning of the study. During these days the animals were handled daily to accustom them to the experimental paradigm. On the day of dosing, the animals were randomized into three groups according to body weight ($n = 8$), which received vehicle (0.9% NaCl (Fresenius Kabi, Uppsala, Sweden) + 0.1% BSA (Roche Diagnostics, Mannheim, Germany) + 5% DMSO (Sigma-Aldrich, St. Louis, USA)), the positive control liraglutide (0.05 μmol/kg, (Liraglutide, Lot. AP52177, Maaloev, Denmark)) or the lipidated NMU analog GUB07-007 (0.3 μmol/kg), respectively. Animals were dosed s.c. in the lower back (dose volume 10 ml/kg) in the afternoon just prior to lights out, and food intake data were collected for 18 h post dosing using the HM-2 system with automatic food recordings every 5 min.

The efficacy of GUB07-007 on food intake was tested in male Sprague Dawley rats (8 weeks of age, Taconic, Denmark) using a similar setup, except the rats were housed only 2 per cage, and the dose volume was 5 ml/kg.

Effect of seven days BID of GUB07-007 in male NMRI mice

The effect of seven days BID of GUB07-007, on food intake, body weight, oral glucose tolerance and gastric emptying was investigated in a total of 24 male NMRI mice. The mice were obtained from Taconic (Denmark) and were 8 weeks old (approx. 35–40 g body weight) at the time of arrival. They were acclimatized to their new environment for at least one week. Animals were single housed in a light-, temperature-, and humidity-controlled room (a 12/12 LD cycle, lights on at 06:00 AM; 22 ± 2 °C; 50% relative humidity). All animals had free access to standard chow (Altromin 1324, Brogaarden A/S, Lynge, Denmark) and domestic quality tap water. Body weight, food and water intake were measured daily from day-5 to day-7 in the morning between 8 and 9 AM. One day before the experiment the mice were randomized according to body weight into three experimental groups ($n = 8$), which received vehicle s.c. bi-daily (BID) (0.9% NaCl (Fresenius Kabi, Uppsala, Sweden) + 0.1% BSA (Roche Diagnostics, Mannheim, Germany) + 5% DMSO (Sigma–Aldrich, St. Louis, USA)), the positive control liraglutide 0.05 μmol/kg s.c. BID or the lipidated NMU analog GUB07-007 0.3 μmol/kg s.c. BID, respectively.

The test period was initiated with a gastric emptying and oral glucose tolerance test (GE-OGTT) performed in the morning on experimental day 0. Clean cages were provided for the mice on the day prior to the test, and the animals were semi-fasted (50% of their average 24-h intake was given in the afternoon the day before

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