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The neuromedin U system: Pharmacological implications for the treatment of obesity and binge eating behavior

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ABSTRACT

Neuromedin U (NMU) is a bioactive peptide produced in the gut and in the brain, with a role in multiple physiological processes. NMU acts by binding and activating two G protein coupled receptors (GPCR), the NMU receptor 1 (NMU-R1), which is predominantly expressed in the periphery, and the NMU receptor 2 (NMU-R2), mainly expressed in the central nervous system (CNS). In the brain, NMU and NMU-R2 are consistently present in the hypothalamus, commonly recognized as the main "feeding center". Considering its distribution pattern, NMU revealed to be an important neuropeptide involved in the regulation of food intake, with a powerful anorexigenic ability. This has been observed through direct administration of NMU and by studies using genetically modified animals, which revealed an obesity phenotype when the NMU gene is deleted. Thus, the development of NMU analogs or NMU-R2 agonists might represent a promising pharmacological strategy to treat obese individuals. Furthermore, NMU has been demonstrated to influence the non-homeostatic aspect of food intake, playing a potential role in binge eating behavior. This review aims to discuss and summarize the current literature linking the NMU system with obesity and binge eating behavior, focusing on the influence of NMU on food intake and the neuronal mechanisms underlying its anti-obesity properties. Pharmacological strategies to improve the pharmacokinetic profile of NMU will also be reported.

Abbreviations: ACTH, adrenocorticotropic hormone; AgRP, agouti-related peptide; ARC, arcuate nucleus of the hypothalamus; BAT, brown adipose tissue; BED, Binge Eating Disorder; BMI, body mass index; CART, cocaine and amphetamine regulated transcript; CCK, cholecystokinin; CNS, central nervous system; CRF, corticotropin-releasing factor; CRF-R, CRF receptor; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats; DMH, dorsomedial hypothalamus; DRN, dorsal raphe nucleus; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide 1 receptor; GPCR, G protein coupled receptor; HFD, high fat diet; HPA, hypothalamic pituitary adrenal axis; HPF, highly palatable food; ICV, intracerebroventricular; KO, knock-out; LEP-Rs, leptin receptors; LHA, lateral hypothalamic area; MC4R, melanocortin 4 receptor; MCH, melanin-concentrating hormone; NAc, nucleus accumbens; NEAT, non-exercise activity thermogenesis; NMS, Neuromedin S; NMU, Neuromedin U; NMU-R1, NMU receptor 1; NMU-R2, NMU receptor 2; $NMU^{-/-}$, mutant mice lacking the NMU peptide; $NMU\text{-}R2^{-/-}$, NMU-R2 deficient mice; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; SCN, suprachiasmatic nucleus; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus of the hypothalamus; SNP, single nucleotide polymorphism; TH, tyrosine hydroxylase; VMH, ventromedial hypothalamus; VTA, ventral tegmental area; WAT, white adipose tissue; WHO, World Health Organization; WT, wild-type.

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1. Introduction

The World Health Organization (WHO) defines overweight and obesity as conditions characterized by abnormal or excessive fat accumulation that may impair health [1]. According to the WHO, overweight is a body mass index (BMI) greater than or equal to 25 kg/m^2; while obesity is a BMI greater than or equal to 30 kg/m^2 [1]. Obesity results as a consequence of a sustained positive energy balance, in which the excessive calories consumed exceed the energy expenditure [2]. Obesity is associated to an increased incidence of pathological conditions, including type 2 diabetes, cardiovascular diseases, and cancer, and significantly decreases life-expectancy [3–6]. A significant association of infection and mortality rate due to COVID-19 with concomitant obesity has also been highlighted [7]. With an increased understanding of the neurobiological mechanisms that drive appetite, the search for innovative anti-obesity medications is strongly required, in association with lifestyle and behavioral interventions [8–10]. Failures in the development of anti-obesity drugs have been reported, mostly because of the side effects observed with long-term administration regimens, including cardiovascular problems (e.g. sibutramine) [11] or increased suicidality rates (e.g. rimonabant) [12]. Recently, the US Food and Drug Administration (FDA) approved semaglutide, a glucagon-like peptide 1 receptor (GLP-1R) agonist, for chronic weight management in adults with obesity or overweight with at least one weight-related condition, in addition to a reduced calorie diet and increased physical activity [13]. The use of semaglutide has been additionally extended in adolescents ($\geq$ 12 years old) with an initial BMI at or above the 95th percentile for age and sex along with a reduced calorie meal plan and increased physical activity to lose weight and keep it off [14]. However, the development of innovative, effective, and safe pharmacological approaches with weight-loss efficacy combined with the ability to decrease cardiovascular problems is strongly necessary [8].

In this context, preclinical and clinical data suggest that the bioactive peptide Neuromedin U (NMU) might represent a promising candidate for the management of obesity, considering its powerful anorexigenic effect. The central NMU system proved to be implicated even in the non-homeostatic aspect of food intake and recent findings linked a dysregulation of the NMU system to binge eating behavior. This also makes NMU an appealing candidate for the treatment of Binge Eating Disorder (BED), the most common eating disorder, characterized by recurrent episodes of binge eating, during which individuals consume in a discrete period of time amounts of food larger than most people would eat in a similar period of time under similar circumstances. These binge eating episodes are commonly accompanied by a sense of lack of control with inability to refrain from eating or to stop once started [15].

Currently, only one drug (lisdexamfetamine dimesylate) has been approved by FDA, in 2015 for the treatment of moderate to severe BED in adults [16]. However, its s use is limited by multiple side effects, including high risk of abuse and dependence, and an increased incidence of adverse cardiovascular events [17]. Therefore, the research for treatment approaches which combine clinical efficacy in BED with a good safety profile are strongly required, and the NMU system recently emerged as a promising target.

The aim of this review is to summarize the current literature linking the NMU system to obesity and binge eating behavior. Specifically, after an introduction about the biology of the NMU system, the neuronal mechanisms, the endocrine signals, and the pharmacological implications evidencing the appetite-suppressant potential of NMU will be discussed. The strategies used to improve the pharmacokinetic profile of NMU in the context of the obesity management will also be mentioned. These data should encourage the research to further investigate the role of NMU in the control of feeding behavior, and to test the efficacy of compounds that act on this system in obese and/or BED humans.

1.1. Biology of NMU

In 1985, Minamino et al. reported the isolation of the bioactive peptide NMU from the porcine spinal cord, evidencing its potent stimulatory activity on smooth muscle contraction [18]. The name "NMU" was designated referring to its uterus stimulating effect, but it was additionally characterized by a hypertensive ability in anesthetized rats [18].

NMU belongs to the neuromedin superfamily, divided into four subgroups: the bombesin-like (NMB and NMC), kassinin-like (NMK and NML), neurotensin-like (NMN) peptides, and the NMU group (NMU and NMS) [19,20].

NMU is an amidated peptide, produced by the enzymatic cleavage occurring at the C-terminal region of a 174 amino acids precursor [21]. The sequence of this precursor is characterized by 74 % homology between human beings and rats, indicating evolutionary conservation between these two species [22]. The sequence of NMU is strongly conserved across many species, from mammals to amphibians, with an almost complete conservation of the amidated C-terminal pentapeptide, suggesting a strong evolutionary pressure to retain this peptide, and its physiological relevance [19,20,23,24].

The porcine NMU was initially isolated in two forms: NMU-8 (8 amino acids long) and NMU-25 (25 amino-acids long). A biosynthetic relationship for these peptides has been proposed, since NMU-8 represents the C-terminal region of NMU-25, and is located after two Arg residues, a typical processing signal [18].

After the identification from the porcine spinal cord, different forms of NMU have been isolated from several species, including fishes [25–27], amphibians [28–30], avian species [31,32], guinea-pigs [33], rabbits [34], rats [35], dogs [36] and humans [22]. Interestingly, the amidated C-terminal pentapeptide (-Phe-Arg-Pro-Arg-Asn-NH_2) is fully conserved in most vertebrates, except carp and goldfish [24]. Most of the NMU peptides isolated are icosapentapeptides, except for the rat NMU (NMU-23), or nonapeptides (NMU-9). Also, as with porcine NMU-8, this analog was found in the dog [19,24]. In humans, the peptide NMU is an icosapentapeptide (NMU-25) [22]. The high degree of conservation of NMU among many species suggests a tight relationship between the structure and function of this peptide [19].

Initially, the sequence of the NMU-8 was demonstrated essential for the biological activity, while the N-terminal part proved to be important for the reinforcement and prolongation of the effects, given that NMU-25 is three-times more potent than NMU-8 in stimulating the rat uterus contraction [18]. Probably, alterations in the N-terminal region modify the three-dimensional architecture of the peptide, favoring a conformation with an increased activity [20]. Thus, interspecies differences in terms of peptide/receptor interaction are mostly due to variations in the N-terminal regions of the various NMU-analogs [24]. Further analyses highlighted that the C-terminal amidation is essential for exerting the biological activities [18,37]. Indeed, a synthetic form of NMU-8, which lacks the C-terminal asparagine-amide structure, was not able to influence both the uterus activity and blood pressure in rats [18].

Subsequent structure-activity relationship investigations supported the previous findings, evidencing the vital importance of the amino acids 16–23 (representing the C-terminal octapeptide) of the rat NMU, in chicken crop smooth-muscle and rat uterus contraction assays [37]. A critical role for the amidation of the core peptide was further supported by in vitro studies, in which the non-amidated form of NMU-8 did not activate and did not produce the intra-cellular Ca^{2+} signaling by both the recombinant human NMU receptor GPR66/FM-3 (NMU receptor 1, NMU-R1) [38], and by the recombinant mouse homologues of the NMU-R1 and NMU receptor 2 (NMU-R2) [39]. It was also shown that the Ala-7 of NMU-8 is essential for the functional activity, since Ala-substituted peptides in this position completely failed to induce Ca^{2+} mobilization and receptor activation [39].

Regarding the distribution of NMU in the body, several techniques (including chromatographic and immunological techniques, in

particular radioimmunoassay and immunocytochemistry using antibodies, and mRNA detection) have demonstrated that NMU is widely expressed throughout the body, with the highest levels found in the brain and in the gastrointestinal tract [40,41]. Specifically, in the gastrointestinal tract, high levels of NMU were detected in the duodenum, jejunum, ileum, caecum, colon, and rectum, localized within Auerbach's and Meissner's plexi [42]. NMU-positive cells are exclusively confined to nerve fibers [43], with an important regulatory activity on gastrointestinal motility, mediated via the NMU-R1 [42,44].

In the brain, the highest levels of NMU expression are observed in the pituitary gland [45]. However, moderate levels were detected in the striatum, hypothalamus, medulla oblongata, and spinal cord [45]. In the mouse hypothalamus, NMU expression has been observed in the arcuate nucleus (ARC), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH) and suprachiasmatic nucleus (SCN). The most abundant levels were in the DMH and SCN [46].

Conversely, in the rat hypothalamus, NMU is weakly expressed in the ARC and DMH, and absent in the VMH, while abundant expression is reported in the pars tuberalis, suggesting the presence of species differences [46]. NMU has been additionally detected in the nucleus accumbens (NAc), septum, amygdala, medulla oblongata and globus pallidus [41]. In the spinal cord, the levels of NMU are higher in the dorsal horn rather than in the ventral horn. Considering the high concentrations in the dorsal root ganglia, this distribution pattern is consistent with a sensory role for NMU [41]. Circulating NMU-like immunoreactivity has not been detected and its absence was also reported in endocrine cells, supporting that NMU acts predominantly as a neuropeptide or a neuromodulator rather than a circulating hormone [41,47].

1.2. Neuromedin S (NMS)

In 2005, Mori et al. reported the identification of a novel neuropeptide structurally related to NMU, the Neuromedin S (NMS) purified from rat brain extracts and identified as the endogenous ligand for the same receptors of NMU, using a reverse pharmacological approach [48].

The term "NMS" was chosen because of the specific expression of this neuropeptide in the SCN [48,49].

NMS is a 36 amino acid residues neuropeptide with a nucleotide sequence homology with NMU of about 53 %. NMS also shares a C-terminal core structure with NMU [48,49]. Indeed, the seven-residue C-terminal amidated sequence of NMS is the same of NMU; this structure was demonstrated essential for NMU-Rs binding [18,48,49].

NMS and NMU display a quite similar efficacy and potency for NMU-R1 and NMU-R2 [48]. Using quantitative RT-PCR, the expression of NMS mRNA was predominantly observed in the central nervous system (CNS), spleen and testis. Specifically, in the brain, NMS expression was found restricted in the SCN, with very low levels in other brain regions [48].

Through a highly sensitive radioimmunoassay for rat NMS, it has been demonstrated the presence of a high content of NMS in the hypothalamus, midbrain, pons and medulla oblongata, suggesting that nerve fibers originating from the hypothalamic NMS-producing neurons might be directed to the brainstem, where NMU-R2 expression has been reported [50].

Functionally, NMS exerts physiological activities similar to NMU influencing the regulation of circadian rhythm [48], feeding behavior [51,52], oxytocin release [53], gonadotropin axis activity [54] and urinary output [55].

1.3. The NMU-R1 and NMU-R2

NMU exerts its functions through the interaction with two structurally related receptors, the NMU-R1 (previously known as GPR66 or FM-3) and the NMU-R2 (previously known as TGR-1 or FM-4), encoded by genes located in the human chromosomes 2 and 5, respectively. They are typical G-protein-coupled receptors (GPCR), with the characteristic seven-transmembrane domains [19,56].

The NMU-R1 was initially isolated in 1998 from human and murine cDNA libraries, in light of its moderate sequence homology with the Growth Hormone Secretagogue receptor and with the Neurotensin receptor [57].

Following the isolation of this GPCR, a "reverse pharmacological" approach was used to identify putative ligands, leading to the discovery of NMU as the specific ligand for this previously orphan GPCR [38,45,56,58,59]. However, the presence of a second receptor specific for NMU has been hypothesized, and the isolation of human and rat cDNA encoding this novel subtype has been subsequently realized, with the identification of the NMU-R2, showing considerable homology with the NMU-R1 subtype (approximately 50 %) [56,58,60,61].

Upon stimulation by NMU, either NMU-R1 and NMU-R2 promote intracellular Ca^{2+} signaling with a potency in the nanomolar range [38,45]. This effect is mediated by the activation of a $G_{q/11}$, with some evidence of G_i coupling [19,45]. In particular, the NMU-R1 and NMU-R2 show a certain degree of specificity, since the NMU-R1 signals mainly via $G_{q/11}$ and NMU-R2 signals via G_i [62]. Additionally, the activation of either NMU-R1 or NMU-R2 stimulates the release of arachidonic acid, through a Ca^{2+} dependent activation of phospholipase A2 [45,60].

Generally, the expression of NMU-R1 has predominantly been found in peripheral tissues and organs (especially the gastrointestinal tract), while the highest level of expression of the NMU-R2 was observed in the brain [19,63].

Consistent expression of NMU-R1 has been detected in the stomach and small intestine, but relatively high levels were observed even in the pancreas, testis, adrenal cortex, liver, spleen, peripheral blood leukocytes, adipose tissue, placenta, lung, heart and mammary gland [38,56,58,59]. The presence of a considerable amount of NMU-R1 mRNA in the gastrointestinal system is consistent with the high levels of NMU detected, supporting the role of NMU and NMU-R1 as potent smooth muscle constrictors [56]. The expression of the NMU-R1 in the spleen, lung and lymphocytes, coupled with the presence of NMU in dendritic cells, monocytes and B cells, [38,56] indicates its involvement in immune responses, as recently reviewed [64]. In particular, the NMU-R1 is detected in natural killer cells, T cells and mast cells [38,65], and its stimulation by NMU leads to pro-inflammatory activities [64,66–68].

The expression of NMU-R2 appears restricted to the CNS, in specific brain regions, such as the hypothalamus, hippocampus, thalamus, substantia nigra, cerebral cortex, medulla oblongata, pontine reticular formation and spinal cord [56,58,61]. Analyzing the distribution of the NMU-R2 in the mouse hypothalamus, this receptor was observed in the ARC, DMH, around the VMH, ependymal layer of the third ventricle and in the paraventricular nucleus of the hypothalamus (PVN). By contrast, in the rat hypothalamus, NMU-R2 expression is confined to the ependymal layer of the third ventricle and PVN [46]. Considerable differences were detected in the expression of both NMU and NMU-R2 in rats and mice, suggesting possible distinct functions between the two species [46]. In the human CNS, only limited information is available regarding the NMU-R2 distribution, whose expression was observed in the medulla oblongata, thalamus, pontine reticular formation, spinal cord, hippocampus, hypothalamus, cerebral cortex, amygdala and cerebellum [56]. Despite being predominantly found in the CNS, NMU-R2 has also been detected in some peripheral organs, including kidney, lung, trachea, testis, and uterus [56,60,61].

2. The role of the NMU system in feeding behavior and energy homeostasis

2.1. Effect of NMU on food intake and energy homeostasis

In the brain, the hypothalamus is recognized as the major center in regulating food intake, body weight and energy homeostasis. Specifically, a large number of evidence identified the VMH as the "satiety

center", while the lateral hypothalamic area (LHA) as the "hunger center" [69]. In the hypothalamus, specific subpopulations of neurons are implicated in energy homeostasis, including the orexigenic neuropeptide Y (NPY)/Agouti-related peptide (AgRP)-producing neurons and the anorexigenic pro-opiomelanocortin (POMC)/cocaine and amphetamine regulated transcript (CART)-producing neurons, located in the ARC [70].

These first-order neurons send projections to second-order neurons located in the PVN, LHA and perifornical area, influencing the activity of different neuropeptides implicated in the control of food intake, such as the corticotropin-releasing factor (CRF) and oxytocin (which decrease food intake), and melanin-concentrating hormone (MCH) and orexins (stimulators of food intake) [69,71–75]. The activity of these populations of hypothalamic neurons is also responsive to hormonal signals derived from the peripheral system, including the appetite stimulator ghrelin or the satiety stimulators glucagon-like peptide-1 (GLP-1), peptide tyrosine, cholecystokinin (CCK), leptin and insulin [69,76–81].

In light of the anatomical distribution of NMU and NMU-R2 in the hypothalamus, numerous studies investigated the role of this neuropeptide in the regulation of energy homeostasis and feeding behavior. Initial evidence reported that intracerebroventricular (ICV) administration of NMU in rats and mice led to a suppression of food intake, accompanied by a decrease in body weight and an increase in body temperature and locomotor activity [58,82–87]. On the other hand, central injection of anti-NMU IgG resulted in an increased food intake in free-feeding rats [84,85].

To investigate the specific site of action of NMU in suppressing feeding behavior, Wren et al. directly injected NMU in different hypothalamic nuclei that receive innervation by NMU-immunoreactive fibers [88]. The authors observed that food intake was significantly decreased in fasted rats in the first hour after injection of NMU in the ARC and PVN [88]. The anorectic effect of NMU is probably mediated by the NMU-R2, considering that this receptor is expressed in the rat hypothalamus (mostly in the PVN) [46], while no expression of NMU-R1 is detected in this region [58].

This finding is supported by the fact that the reductions in food intake and body weight observed after central administration of NMU in wild-type (WT) mice were not repurposed in NMU-R2 deficient mice (NMU-R2$^{-/-}$) [89]. Novak et al. also observed an increase in non-exercise activity thermogenesis (NEAT) and in spontaneous physical activity when NMU was directly injected in the PVN and ARC of rats [90]. Furthermore, in obese rats compared to lean controls, the PVN displays a decreased sensitivity to the locomotor-stimulating properties of NMU, reflecting a decreased NEAT [91].

Thus, a decreased sensitivity of the PVN to the physical activity-stimulating properties of NMU might represent an important factor in the etiology and maintenance of the obesity status. The results of these studies suggest that the potential effectiveness of NMU in the obesity management is related not only to an anorexigenic ability, but also to an increase in the energy expenditure induced by the neuropeptide at a hypothalamic level.

The potential role of NMU in obesity is as well evidenced by the fact that central infusion of this peptide elicits significant reductions in food intake and body weight in lean and obese mice, even under chronic administration regimens, without signs of adaptations or tolerance to these effects [89]. This consideration is of critical importance, since in humans the potential effectiveness of drugs used to counteract obesity might be limited by compensatory adaptations or desensitization, possibly observed with long-term pharmacological therapies.

However, future studies should continue to investigate the chronic efficacy of NMU in decreasing food intake, considering that the long-term peripheral administration of a lipidated analog of NMU in mice was associated to rapid tachyphylaxis [92].

Despite most of the literature pointed to an almost exclusive role for the NMU-R2 in mediating the anorectic effect of NMU, a study of Peier et al. revealed that even the NMU-R1 subtype might be importantly implicated. Peripheral administration of NMU was reported to reduce food intake and body weight, to increase metabolic rate and to improve glucose tolerance in lean and obese mice, but these effects were completely abolished in NMU-R1$^{-/-}$ mice [93]. Thus, it is not possible to rule out that the beneficial effects of NMU on energy homeostasis might also be dependent on an NMU-R1-mediated mechanism. However, the role of the peripherally expressed NMU-R1 in obesity and its related conditions should be carefully investigated, since elevated expression of NMU and NMU-R1 mRNA in the liver of a mouse model of non-alcoholic steatohepatitis were found. Overproduction of NMU in the liver was also associated to an acceleration in hepatic inflammation [94].

Recently, subcutaneous administration of NMU in obese mice has resulted in improved glucose tolerance, activation of brown adipose tissue (BAT), and increased BAT thermogenesis. Furthermore, NMU administration promoted white adipose tissue (WAT) beiging, increased the expression of thermogenic-related genes and restored the adipocyte hypertrophy observed in diet-induced obese (DIO) mice [95].

Collectively, these results support that the NMU system might exert beneficial effects on the obesity status in multiple ways, including via suppression of food intake, stimulation of locomotor activity and energy expenditure, improvement of glucose metabolism and increase in BAT thermogenesis.

A representation of the multiple roles played by NMU in the context of energy homeostasis is reported in Fig. 1.

2.2. Effect of nutritional status and energy balance on the endogenous NMU system

Evidence for the implication of NMU in feeding behavior and energy homeostasis was provided by studies examining alterations in NMU/NMU-Rs under different physiological conditions and nutritional status.

Initially, observing the distribution of NMU mRNA in the rat brain, Howard et al. reported a high degree of localization in the VMH and caudal brainstem [58]. Interestingly, in the VMH the expression of NMU was significantly decreased in rats fasted for 48 h [58], similarly to what observed with other anorexigenic peptides including POMC and CART, whose expression is decreased under fasting conditions [96,97]. In the same study, expression of hypothalamic NMU mRNA has been additionally investigated in a mouse model of obesity, the leptin deficient *ob/ob* mice, in which NMU expression (predominantly detected in the SCN) was found attenuated in obese versus lean mice [58].

A detailed investigation of the responsiveness of the hypothalamic NMU system has also been performed in a work of Graham et al., in which NMU mRNA in the DMH waselevated in mice food deprived for 24 h and in *ob/ob* mice, compared to ad libitum fed and lean littermates, respectively [46]. This suggests that NMU expression might be differentially modulated by the energy balance condition and the genetic background. Conversely, fasting seems not to affect the expression of the NMU-R2s [46,98].

Modulation of NMU expression by energy imbalance has been additionally observed in non-hypothalamic sites, where NMU-transcript was detected, in particular the nucleus of the solitary tract (NTS). This is a region of the caudal brainstem with an important influence in regulating satiety. Indeed, obese Zucker rats (hyperphagic and obese rats with a mutation in the leptin receptor) demonstrated a reduced expression of NMU in the NTS, and in the pars distalis and tuberalis of the pituitary [83]. Conversely, Nogueiras et al., examining the responsiveness of NMU in the pars tuberalis of Sprague-Dawley rats, observed that 48 h of fasting upregulated NMU gene expression in this brain region, thought to work as a timer in seasonal physiology [98]. However, the role of NMU in the pars tuberalis is not completely clear, and it is possible that the physiological effects regulated by NMU in this region are separate from the control of appetite, while it appears to be more important in influencing circadian rhythmicity [99].

Expression of NMU and of NMU-R2s at extra-hypothalamic sites appears modulated by reward-related processes, including alcohol

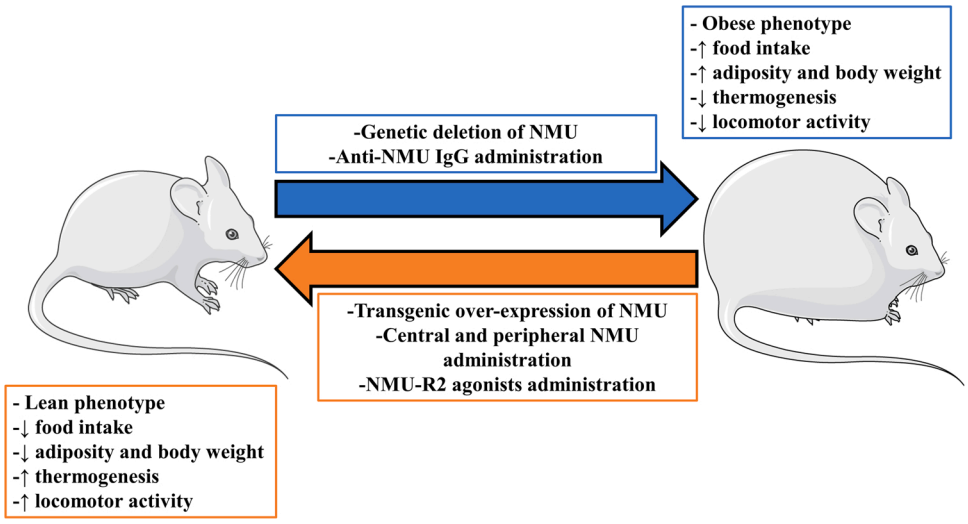

Fig. 1. Physiological features associated to the NMU system in the context of feeding behavior and energy homeostasis. The figure shows a schematic representation of the effects induced by NMU or by its deletion on feeding behavior and energy homeostasis. The deletion of NMU (NMU KO mice) or the administration of anti-NMU IgG results in an obese phenotype, with increased body weight, food intake and adiposity, and a decrease in thermogenesis and locomotor activity. Opposite physiological effects are observed with transgenic over-expression of NMU, central and peripheral NMU administration, and injection of NMU-R2 agonists, resulting in a lean phenotype. KO, knockout; NMU, neuromedin U; NMU-R2, neuromedin U receptor 2. Parts of the figure (the lean and obese mice) were drawn by using pictures from Servier Medical Art (http://smart.servier.com/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

consumption. Accordingly, rats that chronically consume high amounts of alcohol show an increase in NMU and a decrease in NMU-R2 expression in the dorsal striatum, when compared to low-alcohol consuming rats [100]. Thus, taking into account the similarities existing among drug addiction and non-homeostatic consumption of palatable foods [101–103], future studies should evaluate potential changes of the NMU system underlying pathological overeating conditions, including binge eating, which seems to be particularly influenced by NMU. Indeed, a differential modulation of the synaptosomal NMU-R2 protein expression in the VTA and NAc has been highlighted in a binge-like eating model, supporting a role for the endogenous NMU, via the NMU-R2, in regulating compulsive over-eating with an inter-brain region variability [104].

2.3. Role of NMU in energy homeostasis: evidence from genetically modified animals

Considering the effects of NMU administration, and the responsiveness of the endogenous NMU system to changes in the nutritional status, the anorectic properties of NMU were additionally investigated through transgenic animals lacking or overexpressing NMU.

The first report was performed by Hanada et al. in 2004 in which mutant mice lacking the NMU peptide ($Nmu^{-/-}$) were compared to WT mice in several metabolic parameters. The $Nmu^{-/-}$ mice revealed a severe-obesity phenotype, with hyperphagia and decreased energy expenditure, coupled with hyperinsulinemia, late-onset hyperglycemia, hyperleptinemia, and hyperlipidemia [105], effects possibly independent from leptin signaling pathway[105].

Recently, a double genetic deletion of the NMU and NMS gene in mice has been performed to investigate the influence of the NMU system on insulin secretion and glucose homeostasis. Under fasting conditions, no significant differences were found in plasma insulin and glucose among WT and double NMU/NMS knockout (KO) mice [106].

In contrast, when mice were fed a high fat diet (HFD) for 3 weeks, body weight and plasma leptin and insulin levels were significantly increased in all animals, but these effects were exacerbated in KO mice compared to WT [106]. The elevated hyperinsulinemia observed in double KO mice fed a HFD has been hypothesized as a consequence of decreased sympathetic tone. Indeed, NMU stimulates the sympathetic nervous system [107], which consequently attenuates insulin secretion [108]. Therefore, the higher level of plasma insulin in NMU/NMS KO mice could represent an indirect central effect mediated by the absence of NMU [106].

Even NMU KO rats were recently generated, using a clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system and genome editing via the oviductal nucleic acid delivery method. Compared to WT rats, the KO littermates did not reveal hyperphagia, hypertrophy, or higher fat deposition [109]. These results contrasted with those obtained in mice, failing to evidence NMU as a central regulator of food intake, even though the presence of species-specific differences and/or compensatory mechanisms for NMU deficiency cannot be excluded [109].

Differently from NMU KO [105], NMU-R2 KO mice did not develop obesity and did not increase standard food intake [89,110,111]. They also failed to evidence changes in metabolic parameters, including leptin, insulin, glucagon, glucose, or triglycerides [111].

On the other hand, the suppressive effect elicited by acute central administration of NMU is observed in WT but not in NMU-R2 KO mice. This suggests that NMU-R2 is required by NMU to influence feeding behavior, while the long-term effect on energy homeostasis could involve different receptors' mechanisms [111]. In 2009, Peier et al. further observed that male NMU-$R2^{-/-}$ mice were modestly resistant to weight gain under both regular chow and HFD [89], in contrast to what was observed with pharmacological administration of NMU.

However, the decrease in food intake after acute NMU and NMS administration in WT mice was completely absent in NMU-$R2^{-/-}$ mice. In addition, chronic central infusion of NMU and NMS decreased food intake and body weight in both lean and obese mice, and to increase core body temperature, effects abolished in NMU-$R2^{-/-}$ animals [89].

Overall, the presence of the NMU-R2 seems essential for the anorexigenic ability of NMU, given that mice deficient in the NMU-R1 did not show resistance to the suppressive effect on food intake and body weight after acute and chronic NMU administration [89], differently from NMU-R2 deficient mice [89,111].

Experiments using NMU-R2 null mice evidenced that the role of central NMU signaling, via the NMU-R2, might be influenced by caloric intake and by sex differences. Egecioglu et al. reported that NMU-$R2^{-/-}$ mice do not differ in body weight and food intake under standard diet feeding. Conversely, when fed HFD, only female mice develop an obese phenotype. Long-term central NMU administration was effective in decreasing body weight and energy intake in DIO male mice independently from the genotype (WT versus NMU-$R2^{-/-}$), while in females this only occurred in WT animals [110]. Thus, sex-dependent mechanisms might underlie the anti-obesity and anorectic ability of NMU.

Kowalski et al. in 2005 investigated whether transgenic over-expression of NMU in mice might influence feeding behavior and body

weight, revealing a lean and hypophagic phenotype in these animals when maintained on chow diet. Transgenic mice had smaller epididymal, inguinal, retroperitoneal, and mesenteric adipose depots, lower plasma leptin levels and improved insulin sensitivity when compared to WT mice. When exposed to HFD, they were not refractory to develop weight gain, but displayed an improved glucose tolerance [112].

Altogether, the results of this work resembled those obtained after central administration of NMU. The overexpression of NMU also increased expression of hypothalamic neuropeptides, such as the orexigenic NPY and MCH, and the anorexigenic POMC [112].

3. Neuronal mechanisms and endocrine signals implicated in the anorectic effect of NMU

3.1. The role of the NTS in mediating NMU-induced satiety responses

The influence of NMU on feeding behavior involves multiple brain regions and different population of neurons, highlighting the complexity of the NMU system in regulating food intake.

First of all, ICV administration of NMU promotes neuronal activation in several brain areas (assessed by c-Fos immunohistochemistry, marker of neuronal activation), including the hypothalamic feeding centers PVN, ARC, DMH and LHA, the supraoptic nucleus of the hypothalamus, and extra-hypothalamic sites, such as the central amygdala, parabrachial nucleus of the brainstem, NTS and ventrolateral medulla [83,87,113]. Robust NMU-related c-Fos induction was reported in the rat PVN [83], which is consistent with the dense distribution of NMU-immunoreactive fibers and expression of NMU-R2 in this hypothalamic nucleus [43,58]. Interestingly, a significant proportion of the c-Fos-positive cells (activated neurons) in the ARC, NTS and ventrolateral medulla, was reported to be catecholaminergic, co-expressing the enzyme tyrosine hydroxylase (TH) [83].

Accordingly, in the rat caudal brainstem, the NTS seems an important brain region in mediating the anorexigenic effect of NMU. *In situ* hybridization studies revealed the presence of NMU-expressing neurons in the NTS, among which a high degree of co-localization with TH was detected, corresponding to the α_2 adrenergic neurons [114]. These neurons send afferent projections to the PVN [115], where NMU-R2 is highly expressed in the rat brain. It is possible that NMU produced in the NTS is released in the PVN, activating the NMU-R2, via neuronal projections arising from NMU-expressing noradrenergic neurons [114]. Interestingly, NMU-expressing neurons of the NTS appear responsive to the anorexigenic gut-derived peptide CCK, significantly increasing c-Fos expression after CCK administration [114]. Thus, it is possible that NMU mediates the anorexigenic effect of CCK.

The NTS is also engaged by the peripheral NMU system in delaying gastric emptying [92,116]. Indeed, NMU has been demonstrated by Jarry et al. to block gastric emptying via two possible mechanisms: the first involves a direct contraction of the pylorus, which expresses the NMU-R1 and NMU-R2, and the second involves the vagal neuronal fibers projecting to the NTS [116]. The vagus nerve is composed of afferent sensory fibers directed to the NTS and by efferent motor fibers [117]. Peripheral administration of NMU induced c-Fos expression in the NTS of mice, which is considered a marker of vagal afferent neurons activation, effect completely abrogated in vagotomized mice [116]. Therefore, it seems likely that the reduction in food intake observed after NMU administration is at least partially mediated by the inhibition of gastric emptying [92], through a mechanism involving vagal afferents directed to the NTS [116]. The role of the NTS in mediating the anorexigenic effect of NMU is schematically represented in Fig. 2.

3.2. NMU and leptin signaling pathways

Leptin, produced by the obese (ob) gene, is a 167 amino acids peptide primarily released by the WAT, that acts by binding leptin receptors (LEP-Rs), whose activation results in food intake inhibition and increase in energy expenditure [118]. At the hypothalamic level, leptin inhibits orexigenic neuronal pathways (including NPY and AgRP neurotransmission) and activates pathways associated to anorexigenic neuropeptides (such as the melanocortin pathways, including POMC and α-MSH) [119,120].

Specifically, leptin positively influences POMC transcription in the ARC, increasing the release of α-MSH [121] which then activates the melanocortin 4 receptor (MC4R), suppressing appetite [122]. In light of the neuroanatomical localization of NMU and NMU-R2 in the hypothalamus, and their role in regulating food intake, a putative interaction of leptin and NMU in the control of feeding behavior has been investigated. Considering the decrease in hypothalamic NMU expression in

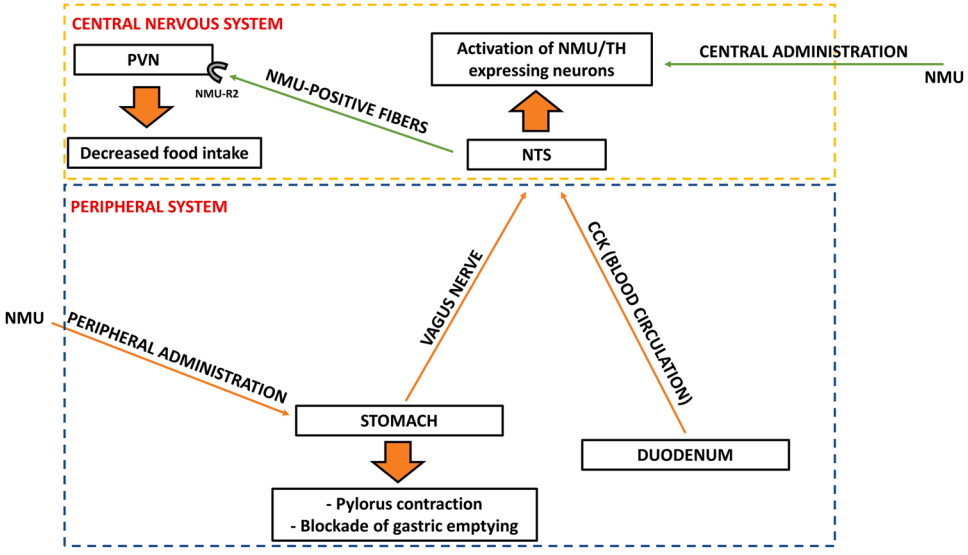

Fig. 2. Schematic representation of the role of the NTS in the anorexigenic effect of NMU. Superior panel: central administration of NMU has been reported to increase c-Fos immunoreactivity in neurons of the NTS. These neurons are catecholaminergic, co-expressing the enzyme TH, and corresponded to the α_2 noradrenergic neurons. Also, co-expression of NMU and TH has been demonstrated in the NTS. Considering that these catecholaminergic neurons send projections to the PVN, it is possible that NMU produced in the NTS is released in the PVN, activating the NMU-R2, via neuronal projections arising from NMU-expressing noradrenergic neurons. The activation of the NMU-R2 in the PVN consequently results in a decrease in food intake. Inferior panel: peripheral NMU administration can directly promote pylorus contraction and delay gastric emptying. In addition, peripherally administered NMU can lead to the activation of vagal afferent neurons of the NTS. Both these effects are supposed to participate in the anorexigenic potential of NMU. Finally, the NMU-producing neurons of the NTS appear to be responsive to the anorexigenic gut-derived peptide CCK, implying an interaction of this hormone and NMU in the regulation of food intake. CCK, cholecystokinin; NMU, neuromedin U; NMU-R2, neuromedin U receptor 2; NTS, nucleus of the solitary tract; PVN, paraventricular nucleus of the hypothalamus; TH, tyrosine-hydroxylase.

fasted rats and in the obese *ob/ob* mice (both conditions characterized by low-circulating leptin) [58], Wren et al. examined the effect of leptin application on NMU release from hypothalamic explants [88]. Leptin stimulated the release of NMU, suggesting that NMU neurotransmission might be influenced by the presence or absence of the satiety hormone leptin [88]. Subsequently, it was demonstrated that anorexia induced by both central and peripheral leptin administration was partially attenuated by ICV injection of an anti-NMU IgG antibody [84]. This potential interaction of NMU and leptin might have implications in regulating the activity of the hypothalamic-pituitary adrenal (HPA) axis, influencing CRF neurotransmission, and consequently the stress response. Indeed, anti-NMU IgG and leptin coadministration attenuates leptin-induced release of CRF from *ex vivo* hypothalamic explants [123].

ICV administration of the anti-NMU IgG also partially attenuates the rise in plasma adrenocorticotropic hormone (ACTH) and corticosterone observed with leptin injection [123].

From these results, it emerges that NMU might mediate some of the effects of leptin on the HPA axis. It is possible that circulating leptin promotes the release of NMU from neurons in the ARC, which then communicate with the PVN leading to the activation of the HPA axis and CRF release [123].

A study of Nogueiras et al. additionally reported that short- and long-term ICV leptin injection led to a decrease in NMU expression in the rat pars tuberalis [98], even though the authors failed to evidence the presence of LEP-Rs in this brain region, suggesting that the influence of leptin on NMU gene expression in the pars tuberalis might be indirect [98].

However, some studies evidenced that NMU signaling might not be required by leptin to exert its anorexigenic effects. ICV or peripheral administration of leptin does not change hypothalamic NMU mRNA expression [46,105,123] and does not induce c-Fos expression on NMU neurons of the ARC [105].

Furthermore, no colocalization of NMU and LEP-Rs was detected in the ARC by Hanada et al. [105]. In addition, leptin can reduce body weight and food intake in both WT and in obese $NMU^{-/-}$ mice, and NMU maintains its anorexigenic effect even in obese animals lacking functional leptin signaling (*ob/ob* mice, *db/db* mice and Zucker fatty (*fa/fa*) rats) [105]. Further investigation is needed to better understand whether NMU and leptin signaling pathways might interact or not in the regulation of food intake and energy balance.

3.3. Interaction of NMU and CRF neurotransmissions in mediating satiety signals

A functional and anatomical interaction of NMU and CRF in the physiological regulation of stress response has been demonstrated [82,124–127].

CRF, the principal mediator of stress response, is mainly synthesized in the PVN [128–130], where NMU-immunoreactive fibers project and where NMU-R2 is abundantly expressed [46,58]. Centrally-administered NMU promotes activation of CRF-containing neurons in the parvocellular division of the PVN [113,124,131] and increases plasma levels of ACTH and corticosterone, indicating activation of the HPA axis [113].

Stimulation of CRF release from hypothalamic explants has been observed in vitro, following incubation with NMU [88]. From a behavioral perspective, ICV administration of NMU stimulates locomotor activity, grooming and face washing behavior, effects blocked by pretreatment with an anti-CRF IgG and by the CRF receptor (CRF-R) antagonist α-helical CRF(9−41) [125].

NMU also increases locomotor activity of WT, but not of CRF KO mice [125]. Given that CRF is known to suppress appetite and food intake, and to be implicated in the development of obesity [74,132], studies have been performed to investigate whether CRF participates in the anorectic action of NMU.

Similarly to what observed for stress response [125], the reduction in food intake in sated and fasted WT mice, and the increase in heat production and body temperature induced by ICV NMU administration were completely abolished in CRF KO mice [82]. This suggests a direct participation of CRF neurotransmission in mediating the influence of NMU on energy homeostasis. This is also supported by the fact that ICV injection of the synthetic goldfish NMU isoform (NMU-21) can potently inhibit food intake in a goldfish model, while increasing the expression level of CRF in the hypothalamus [133].

The anorexigenic effect of NMU-21 can be prevented by prior administration of α-helical CRF(9−41) [133], as similarly observed in rodents [125]. Considering that even in chicks, central administration of NMU suppressed food intake and concomitantly upregulated the expression of hypothalamic CRF mRNA [134], the CRF influence on NMU-induced anorexia is further emphasized, and it can be repurposed and studied in different animal species.

The modulation of the HPA axis activity by NMU might also involve leptin, as previously reported in Section 3.2. The coadministration of an anti-NMU IgG antibody with leptin is able to block the stimulatory effect of leptin on CRF release from hypothalamic explants. Also, ICV-injected anti-NMU IgG partially suppresses leptin-associated increase in plasma ACTH and corticosterone [123].

Altogether, these studies support the importance of the NMU/CRF signaling pathways in suppressing appetite and food intake.

By contrast, a study by Thompson et al. observed that NMU in the PVN might differentially act in stimulating the HPA axis activity and in attenuating feeding behavior. Chronic intra-PVN injections (twice daily for seven days) of NMU elevated plasma corticosterone and grooming behavior in rats, even though no effect on food intake and body weight was observed under satiety conditions [135]. However, the dose used in this work (0.3 nmol) was previously reported by others [88] to inhibit food intake in fasted rats following intra-PVN administration.

Collectively, a clear functional interaction of NMU and CRF has been demonstrated with NMU administration being able to activate CRF-expressing neurons in the PVN, to promote CRF release and to increase CRF mRNA expression in different animal species.

Prior administration of CRF receptor antagonists, or genetic deletion of CRF, can prevent the reduction in food intake following injection of NMU, supporting the implication of CRF in the anorectic effect of NMU. However, future studies are needed to better elucidate whether the interaction of CRF and NMU in satiety responses is context-dependent and might be influenced by the nutritional status of the animals.

4. NMU and the non-homeostatic aspect of food intake: implications for binge eating behavior and food motivation

4.1. NMU and binge eating behavior

Experimental evidence supports a role for NMU in the non-homeostatic aspect of food intake.

Recent findings also imply the participation of NMU in the neurobiological processes underlying alcohol and drug abuse [100,136–138]. Taking into account the similarities existing between the rewarding mechanisms driving drug addiction and compulsive consumption of HPF [101,102,139,140], the NMU system could represent an important target for the development of pharmacological strategies to treat individuals with difficulties in controlling overeating.

Specific neuronal circuitries involving the NMU-R2 have been demonstrated to influence the consumption of HPF, with a potential neurobiological role in binge eating behavior [104,141–143].

In particular, the PVN represents an important brain region in which the NMU-R2 regulates food consumption, food preference and binge-like eating [141–143]. To deeply elucidate the role of the PVN NMU-R2, Benzon et al. performed a conditional knockdown of the NMU-R2 in the PVN of adult rats, using an adeno-associated virus mediated RNAi to knockdown the NMU-R2. Behavioral analysis revealed that the knockdown of PVN NMU-R2 increased intake of HFD with a consequent

increase in weight gain and enhanced the preference for fat but not for sucrose [141].

In contrast, no difference in consumption of standard chow and related body weight gain was observed with the conditional knockdown [141]. NMU-R2 knockdown in the PVN also exacerbates binge-type eating behavior relative to non-NMU-R2-depleted rats [141], using a preclinical model of binge eating in which limited intermittent access to HFD significantly increases HPF intake [144,145]. These results suggest that a decreased NMU-R2 signaling in the PVN promotes obesity via overconsumption of HPF, resulting in compulsive-like eating for obesogenic food [141].

Interest about the role of NMU in binge eating behavior also derives from the presence of the NMU-R2 in brain regions associated to reinforcement processes, such as the NAc and the VTA, where the NMU-R2 is pre-synaptically located [104,137]. Indeed, Smith et al. reported a strong negative correlation of VTA synaptosomal NMU-R2 expression and binge consumption of an extreme HFD,while a strong positive correlation was observed between binge intake of a low fat diet with the expression of NMU-R2 in the NAc [104].

Thus, the endogenous NMU-R2 might represent a driver of compulsive-consumption of HPF, depending on individual differences in its expression on key reward-related brain regions [104].

Overall, preclinical evidence supports a therapeutic potential of the NMU system in the treatment of obesity and binge eating. In this context, the NMU-R2 represents a druggable target considering its expression in brain regions involved in the pathophysiology of binge eating.

Future studies should explore in depth the contribution of the NMU-R2 to altered eating behaviors, using well-validated animal models of binge eating.

4.2. NMU and motivation for palatable food

The NMU-R2 in the PVN was hypothesized to play a role in the incubation of feeding behavior after forced abstinence, condition known to elevate motivation to consume food and frequently associated to failure in dieting regimen maintenance in obese individuals [146,147]. In this context, the knockdown of the NMU-R2 in the PVN blunted the increase in fixed and progressive ratio operant responding for palatable food observed in control rats after 30 days of abstinence from HPF, and attenuated food-associated cues reactivity [143]. This result appears to contrast that of Benzon et al. [141]. However, considering that the neuronal circuitries implicated in incubation of feeding behavior and those involved in binge eating are different, it is possible that PVN NMU-R2s are differentially engaged and exert divergent effects depending on the experimental conditions and paradigms used [141].

Peripherally injected NMU proved to be effective in decreasing the motivation for high fat food pellets under progressive ratio schedule of reinforcement [142], a behavioral effect accompanied by a decrease in c-Fos immunoreactivity in the PVN and DRN of treated rats [142]. These brain regions express the NMU-R2 [46,142,148], and site-specific injections of NMU in the PVN and DRN decreased consumption of standard diet and HFD, and progressive ratio responding for high fat pellets [142]. Given that NMU-neuronal projections arising from the LH and directed to the PVN and DRN have been highlighted [142], these pathways appear of critical importance in regulating consumption and motivation for HPF. Thus, the characterization of the DRN as a potential site in which NMU, via NMU-R2, might regulate food-associated behaviors, is particularly interesting.

Previously, NMU was found to affect serotonin receptors function in the brain, when centrally injected [149], and a GABAergic pathway originating in the DRN and targeting the NAc was demonstrated to be modulated by the NMU-R2 [137]. Therefore, the role of DRN in the NMU influence on HPF intake and the interaction with serotonin neurotransmission should be deeply investigated.

5. Clinical evidence for the implication of NMU in obesity

The NMU system, with its anorexigenic action, has also been studied in human obesity, investigating how possible gene variants can impact this condition.

There was more focus on the NMU-R2, for its function and localization in the CNS.

NMU-2R gene was studied in a cohort of 94 children with severe early-onset obesity, finding six single nucleotide polymorphisms (SNPs) [150]. Two of them were silent mutations, one a conservative change, three are non-conservative changes, but no one of these variations were associated with human obesity-related traits. However, the study for further confirmation would need a larger population [150].

In addition, there was evidence of an unusual recurrent haplotype involving four nucleotide changes spread over two exons, causing modification in amino acid sequence. This haplotype allowed to identify two highly prevalent ancestral forms of NMU-2R, and it might ultimately reveal altered functional properties. Even in this case, more studies with larger samples would be required [150].

A subsequent study, analyzing 289 children and adolescents with early-onset obesity and 84 obese adults of both sexes, focused on a new NMU variant. The Ala19Glu polymorphism carriers had an increased BMI, waist, and hip circumference. All these effects were observed almost exclusively in middle-aged white men and there was not association with BED. The replacement of the Ala-allele with the Glu-allele predicts a protein structure modification that could facilitate the obese status [151]. Subsequently, through a study in European children, among several haplotypes, the CCT haplotype was associated with adiposity regulation in girls, showing lower BMI and reduction in fat mass as well as in hip and arm circumferences (calculated by inelastic tape and biological impedance measurements) [152].

Thus, the CCT haplotype indicated a decreased risk of overweight/obesity. Moreover, the carriers of this haplotype showed a preference for the umami food, compared to the most frequent haplotype (TTC) [153]. Umami food preference appears to have a protective effect and is associated with low values of BMI, arm circumferences, skinfolds, and fat mass [153,154].

6. NMU analogs and NMU-Rs agonists: strategies to improve the pharmacokinetics profile of NMU and effect on food intake and energy homeostasis

In light of the anorexigenic effects observed with peripheral and central NMU administration, efforts were made to synthesize NMU analogs, examining whether they could exert more potent effects in reducing food intake.

The synthesis of NMU analogs is of high importance from a clinical point of view, considering the potential use of NMU being limited by its short half-life and rapid in vivo proteolytic degradation [155].

The development of NMU analogs with improved pharmacokinetics profile and stability compared to the original peptide is strongly necessary. The aim of this section is to summarize the approaches used to increase the anorectic potential of NMU and to improve the pharmacokinetic parameters of this peptide. These approaches are represented in Fig. 3.

6.1. Development of NMU-analogs by changes in the electronic density of aromatic residues

In 2003, a study of Abiko and Takamura reported the synthesis of two aromatic ring substituted analogues of NMU-23, named [Phe(4 F)^{16,17,19}]NMU-23 and [Tyr(Me)^{1,6}]NMU-23 [156], in which the Phe residues at positions 16, 17 and 19 were replaced by Phe(4 F) residues, while the Tyr residues at 1 and 6 were replaced by Tyr(Me) residues, respectively. The effects of these two analogs were analyzed in reducing food intake after ICV administration in rats and compared to NMU-23.

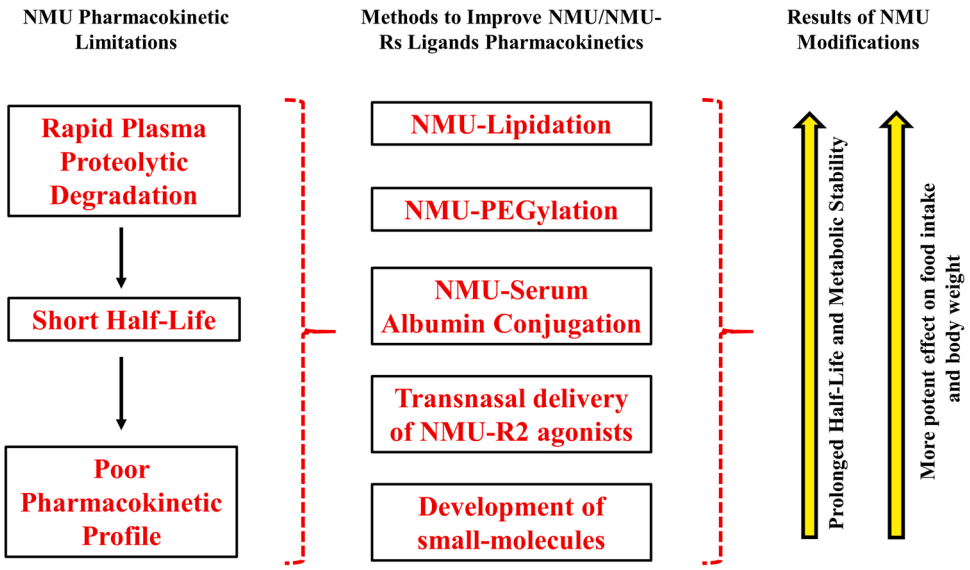

Fig. 3. Approaches evaluated to improve the pharmacokinetic profile of NMU to exert more powerful and long-lasting effects on feeding behavior. The potential use of NMU in the pharmacotherapy of obesity is limited by its poor pharmacokinetic profile. Indeed, NMU in the plasma is rapidly degraded, showing a short half-life that limits its anorectic effect. Therefore, different strategies have been tested to improve the pharmacokinetic profile of NMU, protecting this peptide from the plasma proteolytic degradation. These includes NMU-lipidation, NMU-PEGylation and serum albumin conjugation. In addition, the transnasal delivery of NMU-R2 agonists and the development of small non-peptide agonists of the NMU-R2 proved to be promising approaches to target the NMU system in the treatment of obesity. NMU, neuromedin U; NMU-Rs, neuromedin receptors NMU-R2, neuromedin U receptor 2.

[Phe(4 F)^{16,17,19}]NMU-23 demonstrated a more potent reducing activity on food intake compared to NMU-23, while the analog [Tyr(Me)^{1,6}] NMU-23 was ineffective [156]. These results suggested that Phe residues at positions 16, 17 and 19 of NMU-23 and Tyr residues at positions 1 and 6 of NMU-23 are important for the influence of feeding behavior, and that the electronic density of these aromatic residues is crucial to develop NMU-23 analogs with anorexigenic activity [156].

6.2. Lipidation of NMU

One approach to protect NMU from the proteolytic degradation and to increase the plasma half-life consists in the lipidation of the peptide. Accordingly, Dalbøge et al. developed a series of novel lipidated NMU analogs which were evaluated for their in vitro potency at NMU-Rs, plasma stability and in vivo effect on food intake. The lipidated analogs maintained the full agonist activity on both NMU-Rs, prolonged plasma half-life compared to the naturally-occurring NMU, and decreased food intake in mice after acute subcutaneous administration, in a dose dependent manner and with more potency compared to NMU [155]. Probably, the more pronounced effect on food intake was related to the improved pharmacokinetic stability of the lipidated analogs, which were more resistant to the proteolytic degradation [155].

Among these lipidated NMU analogs, one of the most efficacious was GUB07–007, subsequently reported by the same group to potently inhibit food intake and gastric emptying, and to improve glycemic control in mice, even though with a short-lasting efficacy and rapid onset of tachyphylaxis [92].

Interestingly, another laboratory described the synthesis of a series of short lipidated peptide analogs derived from NMU, among which three potent truncated-lipidated NMU-Rs agonists were obtained: NM4, NM4A and NM4-C_{16} [157]. These compounds were physiologically active and proved to exert their weight-loss efficacy in a DIO mouse model not only under acute, but also under chronic administration regimen, after subcutaneous administration [157].

6.3. NMU PEGylation

Conjugation of NMU with poly(ethylene) glycol (PEG) represents an additional possibility to overcome its poor pharmacokinetics profile, increasing the metabolic stability and the duration of the anorexigenic effect in vivo.

In 2012, Ingallinella et al. reported the synthesis of a metabolically stable analog of the human NMU-25, conjugated with 40 kDa PEG at the N-terminus, termed PEG40-NMU [158]. Differently from the half-life of the WT NMU (less than 5 min), PEG40-NMU showed an excellent pharmacokinetic profile, with a half-life of 25 h.

Subcutaneous administration of PEG40-NMU decreased food intake in mice with a prolonged efficacy compared to NMU, effect observed even with repeated administration [158]. Interestingly, PEG40-NMU crosses the blood brain barrier andconsequently interacts with the centrally-expressed NMU-R2 after subcutaneous administration, since it was able to significantly decrease body weight and food intake in NMU-R1$^{-/-}$ mice, whereas only a short-lasting anorexigenic effect was observed in NMU-R2$^{-/-}$ [158].

Therefore, pegylation seems to promote brain penetration of NMU, protecting this peptide from its fast metabolism and allowing the interaction with the NMU-R2 in the CNS. Pegylation of the short-length NMU-8 also proved to be effective in maintaining biological activity towards the NMU-Rs and the in vivo anorectic effects.

Peripherally-injected NMU-0002, a 20 kDa PEG conjugated derivative of NMU-8, showed a long-lasting reduction in food intake and powerful anti-obesity properties in mice, comparable to those exerted by exendin (a GLP-1R agonist) and with a slow clearance rate [159].

The pegylation appears to confer to NMU-8 a robust and long-lasting activity, which is not observed with the unconjugated peptide. Masuda et al. additionally found that PEG-conjugated-NMU-8 exerted a longer anorectic activity compared to the conjugated NMU-23 and NMS [160].

Considering the predominant distribution of the NMU-R2 in the CNS compared to the peripherally expressed NMU-R1, subtype-selective NMU-R2 agonists with a potential anti-obesity and appetite-suppressive efficacy and modified with a PEG moiety were designated and synthesized.

Amino-acid substitution at position 19 combined with PEGylation with 20 kDa PEG led to the synthesis of a selective NMU-R2 agonist with a good subcutaneous penetration profile, slow clearance rate, and once-daily dosing potential [161]. This PEGylated NMU-8 analog exerted dose-dependent inhibitory effects on food intake and body weight in DIO mice, with repeated subcutaneous administration over a two-weeks period, further supporting the brain penetration of PEGylated NMU analogs [161]. The NMU-R2 PEGylated selective agonist NMU-2084 also revealed a more potent body weight lowering ability with less diarrhea scores, when compared to both the non-selective NMU-Rs agonist NMU-0002 and the NMU-R1 selective agonist NMU-6014, effects accompanied by activation of POMC neurons in the ARC [162].

Thus, NMU-R2 selective agonists might represent promising anti-obesity drugs with a better safety and efficacy profile than subtype-

selective NMU-R1 ligands. However, despite the promising and positive results obtained with NMU-PEGylation, some limitations might be considered. PEG is not biodegradable, and its accumulation in tissues might have some safety concerns. Furthermore, while PEG increases the half-life of peptides preventing their proteolytic degradation, the receptor activation could decrease because of the large size of PEG-based moieties [163].

6.4. Conjugation of NMU with serum albumin

Chemical conjugation to proteins with a long-circulatory half-life, such as human serum albumin (HSA), represents an alternative strategy to improve the pharmacokinetic profile.

Albumin is the most abundant plasma protein, characterized by an extraordinary half-life (~ three weeks), and reveals a molecular size of ~7.2 nm, above the renal clearance threshold [164]. Albumin possesses several ideal drug carrier characteristics and the conjugation has been performed and evaluated for NMU.

Using a fluorescent imaging plate reader (FLIPR, a Ca^{2+} mobilization assay), to measure the functional activity in cell lines stably expressing the human or mouse NMU-Rs, HSA-conjugated NMU reported full potency at both NMU-Rs [165], differently from the reduced potency displayed by the PEG-conjugated NMU [158]. In addition, the half-life of HSA-NMU was longer than that of PEG-NMU [158,165]. Thus, the pharmacological profile of HSA-NMU appears superior to the PEGylated form.

In vivo, subcutaneous injection of HSA-NMU dose-dependently decreased food intake and body weight in DIO mice, with a minimum efficacious dose lower than that requested by PEG-NMU [158,165]. HSA-conjugated NMU also exerted glucose lowering activities [165].

Overall, both PEG- and HSA-conjugated forms of NMU seem to be efficacious in the treatment of obesity and to ameliorate the bioavailability of NMU, even though the pharmacological profile of the HSA-conjugated NMU appears to be preferrable, with a longer half-life and increased potency.

6.5. Transnasal delivery of NMU-R2 peptide agonists

The transnasal delivery was investigated to optimize the therapeutic efficacy of peptides directed to the NMU-R2. This method was used for CPN, a specific peptide agonist to NMU-R2 [166], taking into account that the direct transport from nose to the brain can provide many advantages, such as rapid onset of drug action, lack of systemic side effects, and reduction in drug doses.

Nasal application of CPN led to brain concentrations higher than those reached by intraperitoneal and intravenous administration, suggesting an efficient delivery of the drug [167]. Through this administration route, CPN was able to inhibit food intake and body weight of mice with a dose-response relationship [167]. Therefore, direct delivery of CPN to the brain, via the nasal cavity, could be hypothesized as promising approach for the treatment of obesity.

6.6. Small molecules targeting the NMU-R2

Using a high-throughput screen, Meng et al. reported the discovery of two small molecules (NY0116 and NY0128) acting as agonists of both the human NMU-R1 and NMU-R2.

When evaluated in intracellular Ca^{2+} mobilization assays, these compounds showed EC_{50} values of 27.76 μmol/L for NMUR1 and 13.61 μmol/L for NMUR2, and 29.99 μmol/L for NMUR1 and 10.30 μmol/L for NMUR2, respectively [168].

Notably, these molecules were also evaluated for their ability to modulate cAMP signaling through the NMU-R2 and were more efficacious than NMU-8 in decreasing cAMP. Thus, NY0116 and NY0128 behave as NMU-R2 agonists for G_q-mediated Ca^{2+} signaling and G_i-mediated cAMP inhibition, while showing undetectable/weak potency for β-arrestin recruitment [169].

The two NMU-R2 agonists achieved appreciable brain levels after subcutaneous administration, acutely decreased HFD intake in rats, and chronically reduced body weight, visceral adipose tissue and cholesterol levels in mice fed with HFD [169].

Collectively, these data support the pharmacotherapy use of NMU-R2 agonists for obesity, and the small molecules evaluated might represent useful lead compounds to develop more selective NMU-R2 agonists. The development of compounds that selectively target the NMU-R2 would be of critical importance, considering that they could exert appetite-suppressants effects at a central level, avoiding the systemic adverse effects, mostly mediated by the NMU-R1.

7. Conclusions

The studies discussed in this review point to an important role of NMU in the pharmacotherapy of obesity, considering the consistent anorexigenic effects observed with central and peripheral administration of NMU, NMU analogs or NMU-R2 agonists. The importance of the NMU system in the control of food intake is further emphasized by the recently hypothesized association with binge eating, the core behavioral feature of BED.

The development of NMU-R peptide agonists with an improved pharmacokinetic profile compared to the original NMU peptide, and the synthesis of more selective small molecules targeting the NMU-R2 should be encouraged to develop effective anti-obesity medications. The efficacy of these compounds should be tested in obesity and/or BED clinical studies, since, until now, only a possible association of polymorphisms/haplotypes of NMU/NMU-R2 with obesity and food preference has been investigated.

CRediT authorship contribution statement

Luca Botticelli: Conceptualization, Writing – original draft, Writing – review & editing, Visualization (contributed equally to this review). **Emanuela Micioni Di Bonaventura**: Conceptualization, Writing – original draft, Writing – review & editing, Visualization (contributed equally to this review). **Fabio Del Bello**: Writing – review & editing, Visualization. **Gianfabio Giorgioni**: Writing – review & editing, Visualization. **Alessandro Piergentili**: Writing – review & editing, Visualization. **Wilma Quaglia**: Writing – review & editing, Visualization. **Alessandro Bonifazi**: Writing – review & editing, Visualization. **Maria Vittoria Micioni Di Bonaventura**: Writing – review & editing, Supervision, Visualization. **Carlo Cifani**: Writing – review & editing, Supervision, Visualization. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

All authors declare that there are no conflicts of interest associated with the content of this paper.

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