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## Ghrelin, des-acyl ghrelin and nesfatin-1 in gastric X/A-like cells: Role as regulators of food intake and body weight

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### Abstract

Numerous peptides released from endocrine cells in the intestinal mucosa were established early on to be involved in the physiological regulation of food intake with a prominent role in termination of food ingestion when nutrients pass along the intestinal tract. Recently, peptides released from X/A-like endocrine cells of the gastric oxyntic mucosa were recognized as additional key players in the regulation of feeding and energy expenditure. Gastric X/A-like cells release the octanoylated peptide, ghrelin, the only known peripherally produced hormone stimulating food intake through interaction with growth hormone secretagogue 1a receptor (GHS-R1a). Additionally, non-octanoylated (des-acyl) ghrelin present in the circulation at higher levels than ghrelin is currently discussed as potential modulator of food intake by opposing ghrelin's action independent from GHS-R1a although the functional significance remains to be established. Obestatin, a ghrelin-associated peptide was initially reported as anorexigenic modulator of ghrelin's orexigenic action. However, subsequent reports did not support this contention. Interesting is the recent identification of nesfatin-1, a peptide derived from the nucleobindin2 gene prominently expressed in the stomach in gastric X/A-like cells in different vesicles than ghrelin. Circulating nesfatin-1 levels vary with metabolic state and peripheral or central injection inhibits dark phase feeding in rodents. Overall, these data point to an important role of X/A-like cells of the stomach in food intake regulation through the expression of the orexigenic peptide ghrelin along with des-acyl ghrelin and nesfatin-1 capable of reducing food intake upon exogenous injection although their mechanisms of action and functional significance remain to be established.

### 1. Introduction

Several enteroendocrine cells scattered within the intestinal mucosa [114] have been recognized early on to influence food intake by releasing peptide hormones in response to changes in nutritional status [57, 77, 101] while endocrine cells in the gastric mucosa were mainly implicated in the regulation of acid secretion [34]. The gastric endocrine cells encompass enterochromaffin-like cells releasing histamine (ECL, 30% in human and 65% in the rat), gastrin-producing cells (G cells), somatostatin-containing cells (D cells >20% of gastric oxyntic endocrine cells in humans and 5–10% in rats), and the less abundant

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serotonin-containing enterochromaffin (EC) cells (Fig. 1) [113, 114]. In addition, there is a distinct 5<sup>th</sup> endocrine cell type without connection to the lumen (closed-type) distributed throughout the gastric oxyntic glands that was labeled P/D<sub>1</sub> cell in humans and X/A-like cell in rats due to similarity with the rat pancreatic A-cell [21]. This cell type accounts for up to 20–30% of the oxyntic endocrine cell population and represents the second most frequent type among the gastric endocrine cells [21, 114]. Their content and function remained largely unknown until ghrelin was identified in rat X/A-like and human P/D<sub>1</sub> cells as the only peripherally produced and centrally acting peptide hormone known so far to increase food intake [84, 115]. This seminal discovery revealed that the gastric mucosa contains endocrine cells able to influence food consumption by the release of a specific peptide as previously identified in the intestinal mucosa. Of functional relevance was the subsequent demonstration of interaction between ghrelin and anorexigenic peptides produced by intestinal endocrine cells such as cholecystokinin (CCK), peptide YY (PYY), and glucagon-like peptide 1 (GLP-1) (for review see [77, 101]).

Furthermore, the last years witnessed the identification and characterization of additional gene products within the X/A-like cells also able to influence food intake. These include peptides resulting from differential post-translational modifications of pro-ghrelin (non-octanoylated ghrelin or des-acyl ghrelin and *n*-decanoyl ghrelin) [59, 63], distinct potential processing of the ghrelin gene (obestatin) [164] or the processing of a distinct gene such as nucleobindin2 (nesfatin-1) [137]. Recent functional reports indicate the reduction of food intake induced by exogenous administration of des-acyl ghrelin [9, 31] and nesfatin-1 [132] whereas the action of obestatin remains largely equivocal [49]. In the present review we will highlight gastric X/A-like cells and the actions of peptides processed in these cells on food intake and modalities pursued targeting these peptides for potential novel therapeutic venues in the treatment of obesity with emphasis on recent developments.

## 2. Ghrelin

Ghrelin (also known as acyl ghrelin or octanoylated ghrelin) was discovered in 1999 by Kojima and colleagues as the endogenous ligand of the long known growth hormone secretagogue receptor 1a isoform (GHS-R1a, Fig. 2) [84]. The 28-amino acid peptide ghrelin displays a unique feature among peptides which is an acyl group at the serine-3 residue providing increased lipophilicity essential for the binding to the GHS-R1a receptor [19]. While an *n*-octanoyl group (8-carbon chain with no double bond) is the primary acyl chain in human and rodent ghrelin [85], an additional acyl modification namely *n*-decanoyl (10-carbon chain without double bond) ghrelin is also produced in X/A-like cells contributing up to 40–60% of the circulating esterified ghrelin in mice [59]. In the human stomach, the ratio of octanoylated to decanoylated ghrelin is around 3:1 [64]. Additional studies in mice established that orally ingested medium chain fatty acids are directly incorporated to form the acyl group of ghrelin resulting in stomach concentrations of ghrelin with an acyl group that bears the signature of the corresponding carbon chain lengths (*n*-hexanoyl ghrelin, *n*-octanoyl ghrelin and *n*-decanoyl ghrelin) of medium-chain fatty acids added to the diet [105]. The enzyme responsible for acylation of ghrelin was unknown for almost 10 years and has recently been identified in mice and humans as a member of the superfamily of membrane-bound *O*-acyltransferases (MBOATs) and was termed ghrelin-*O*-acyltransferase (GOAT) [55, 160]. GOAT is highly co-expressed with ghrelin in cells of the mouse gastric mucosa as shown by *in situ* hybridization [120]. The enzyme octanoylates pro-ghrelin before its transport to the Golgi apparatus where it is cleaved by prohormone convertase (PC) to form the mature ghrelin [160]. Among seven mammalian PCs, PC1/3 has been reported to be primarily involved in the processing of the proghrelin precursor protein [167]. However, *in vitro* studies further indicate that not only PC1/3 but also both PC2 and furin could process proghrelin to the 28-amino acid ghrelin in presence of GOAT expression

in the cells as well as *n*-octanoic acid in the culture medium which are both required to produce *n*-octanoyl ghrelin [142].

## 2.1 Ghrelin release and receptor interaction

Ghrelin positive X/A-like cells distributed throughout the gastric oxyntic mucosa [38, 98] are the main source of circulating ghrelin [7] as demonstrated by the sharp decline of ghrelin levels following gastrectomy [69]. Moreover, ghrelin is produced, although in much lower amounts, in the small and large bowel [38], pancreas [40] as well as other peripheral viscera such as kidney, liver, heart, testis, adipose tissue and skin [18, 48]. These production sites could contribute to the circulating ghrelin but are also thought to respond to ghrelin through autocrine or paracrine mechanisms. Moreover, ghrelin has been detected in the central nervous system in the arcuate nucleus of the hypothalamus [95] as well as in neurons adjacent to the third ventricle [33]. Circulating ghrelin levels increase before and decline after a meal in experimental animals and humans (Fig. 3) [36]. Likewise, *n*-decanoyl ghrelin immunoreactivity is increased by fasting in the murine stomach and plasma [59]. Furthermore, total ghrelin levels inversely correlate with body mass index with increased levels in anorexic and cachectic patients and decreased levels under conditions of obesity (Fig. 3) [37, 148]. However, in the Prader-Willi syndrome, the most common hereditary cause for obesity, plasma ghrelin levels are elevated despite the increased body weight [35], likely contributing to the sustained appetite in those patients.

Ghrelin secretion is influenced by a number of hormones, neurotransmitters and cytokines. In particular, circulating ghrelin levels are decreased by peripheral administration of bombesin/gastrin releasing peptide [42], somatostatin [42, 129], CCK [22], GLP-1 [56, 94, 110], insulin [117], interleukin-1 [152] and prostacyclin [97] and increased by adrenaline, noradrenaline [42], secretin [42], central vagal stimulation [5], sham feeding [134], endothelin 1 and 3 [42, 144], and the stable analog of the endogenous cannabinoid, anandamide (Fig. 4) [162]. However, it is not known whether most of these substances act directly on ghrelin cells expressing the respective receptors or indirectly by releasing downstream signals that then influence X/A-like cells. Isolation of purified X/A-like cells will help to address the mechanisms regulating ghrelin secretion. Genetic labeling of X/A-like cells with markers such as green fluorescent protein has recently been successfully used to enrich ghrelin cells from the murine gastric mucosa [118, 120]. Further studies on enriched ghrelin cell populations will help to delineate receptor expression and underlying signaling mechanisms regulating gastric ghrelin release.

Ghrelin binds to the GHS-R1a, which is expressed in the brain as well as in the periphery at sites not only involved in food intake regulation and reward but also memory and a variety of other functions [48, 54, 65, 103]. In the brain, ghrelin receptor mRNA is expressed in the arcuate nucleus, the ventromedial nucleus, the hippocampus in areas CA2 and CA3, the dentate gyrus, substantia nigra, ventral tegmental area, dorsal and median raphe nuclei and in the pituitary [54, 65, 103, 168]. Compared to the pituitary, ghrelin receptor mRNA has been detected in lower amounts in the thyroid, pancreas, spleen, myocardium and adrenal gland [48]. Additionally, ghrelin receptor mRNA expression was described in adipose tissue, immune cells and on vagal afferents [58, 125].

The octanoyl group on the serine in the third position is essential for binding to and activating the receptor [84, 85]. Moreover, structure function studies established that the active core of the ghrelin receptor-ligand interaction encompasses the first five N-terminal amino acids coupled to the hydrophobic residue [19]. Recent studies show a modulation of ghrelin signaling linked with homo- and heterodimerization of the GHS-R1a [125]. Homodimerization of the GHS-R1a seems to contribute to the synergistic interaction between growth hormone-releasing hormone and ghrelin on growth hormone release [125].

In contrast, heterodimerization of GHS-R1a and GHS-R1b decreases ghrelin responsiveness whereas heterodimerization between GHSR-1a and cannabinoid 1 (CB<sub>1</sub>) receptors is likely to play a role in the food intake stimulatory interaction between ghrelin and endocannabinoids [125]. It is noteworthy to mention that GHS-R1a is among the few G-coupled protein receptors which are constitutively active, resulting in signaling even in the absence of the ligand [62].

## 2.2 Stimulatory effect of ghrelin on food intake

Ghrelin is well established to stimulate food consumption in both lean and obese humans [44] and food intake upon peripheral and brain injection in various naïve animal species (Fig. 3) [157]. In addition, exogenous injection of ghrelin is able to restore food intake suppressed by endotoxin [152] or in experimental conditions mimicking chronic renal failure by subtotal nephrectomy in rats [3]. Recent studies to assess the biological action of other acylated forms of ghrelin produced in the stomach show that *n*-decanoyl ghrelin displays similar orexigenic effects as ghrelin in mice [59]. Ghrelin's orexigenic action is GHS-R1a-mediated as characterized by the suppression of exogenous and endogenous (fasting) ghrelin-induced food intake and activation of hypothalamic ghrelin responsive cells by several developed GHS-R1a antagonists (e.g. JMV 3002, 2959 and 2810) [121]. In addition, GHS-R1a knockout mice do not increase food intake after a single ghrelin injection [141, 169].

Ghrelin can reach hypothalamic food intake regulatory centers directly after passage of the blood-brain barrier (Fig. 3) [17, 109]. In addition, the presence of ghrelin receptors on vagal afferents and neurons in the rat nodose ganglion provides anatomical support for ghrelin signaling to the brain *via* the vagus nerve (Fig. 3) [39, 119]. This is further supported by the demonstration that vagotomy abolishes the ghrelin-induced food intake [39]. Moreover, exogenous ghrelin failed to stimulate food intake in patients after surgical procedures involving vagotomy [92]. However there is also a report showing that intraperitoneal injection of ghrelin can stimulate food intake in rats with selective subdiaphragmatic vagal deafferentation [8] indicating that peripheral ghrelin-induced stimulation of food intake may be mediated *via* the afferent vagus nerve along with direct access to the brain.

## 2.3 Effects of ghrelin on fat mass, energy expenditure and body weight

Besides regulating food consumption, ghrelin is also involved in body weight modulation. Chronic delivery of the peptide leads to body weight gain in rodents not only through increasing appetite but more prominently by promoting fat storage in white adipose tissue [41, 148]. Particularly, adipose tissues such as retroperitoneal and inguinal fat depots are enlarged whereas superficial adipose fat in subcutaneous depots is not altered and such lipogenic effect is growth hormone-independent [41, 123]. Other studies indicate that reduced lipid mobilization reflected by an increased respiratory exchange ratio may underlie ghrelin's adipose tissue promoting effect [41] independently of its orexigenic properties (Fig. 3) [145]. Genetic models directed to knockdown or over-express the ghrelin signaling pathways result in a phenotype that is not as clear as that induced by pharmacological approaches. Ghrelin over-expressing mice resulting in increased circulating levels of ghrelin and des-acyl ghrelin have no alterations of food intake or body weight, however, they have an impaired glucose tolerance most likely due to suppressed insulin secretion [68]. Mice lacking both ghrelin and the ghrelin GHS-R1a however show increased energy expenditure and consecutively decreased body weight when fed a normal diet whereas single knockout (KO) mice (for either ghrelin or its receptor) do not show this phenotype [112]. When fed a high fat diet, ghrelin null mice exhibited a reduced respiratory quotient indicating increased fat utilization [156]. Moreover, GHS-Ra knockout (KO) mice were shown to be resistant to diet-induced obesity, which further supports the role of ghrelin in adipogenesis [169]. In

contrast, another research group showed in GHS-Ra KO mice that ghrelin is involved in insulin sensitivity and glucose regulation while these mice were not resistant to diet-induced obesity [140]. This discrepancy has been suggested to be related to difference in the genetic background of the KO mice colonies. Taken together, these studies indicate that chronic alterations of ghrelin signaling pathways more prominently impact on energy expenditure than food intake although adaptive and compensatory regulatory mechanisms may also take place under conditions of chronically altered ghrelin signaling by genetic modifications.

Recently the role of GOAT and ghrelin in the control of energy balance and adiposity was investigated in mice lacking the GOAT gene (*Mboat4*) or over-expressing human ghrelin and GOAT [79]. GOAT knockout mice did not show alterations in body weight when fed a standard rodent diet but displayed a decreased body weight when kept on a high fat diet compared to their wildtype littermates [79]. Moreover, when mice are fed with a diet enriched in medium-chain triglycerides, GOAT deficient mice have a lower body weight and reduced fat mass compared to wildtype [79]. These findings provide new insight to the ghrelin-GOAT system as a lipid sensor not only in the absence of food but also when high caloric food is available [79, 135]. Moreover, a recent study described that GOAT prefers n-hexanoyl-CoA over n-octanoyl-CoA as the acyl donor *in vitro* [108]. However, the concentration of n-hexanoyl ghrelin in the murine stomach is markedly lower than n-octanoyl ghrelin [108] probably due to the lower amount of this acyl donor *in vivo*.

#### 2.4 Mediation of ghrelin's orexigenic effect by appetite-regulating neuropeptides in the hypothalamus

As described above ghrelin is also produced centrally in the arcuate nucleus of the hypothalamus [95] and in neurons adjacent to the third ventricle [33]. The arcuate nucleus is strongly implicated in the regulation of food intake [126]. Ghrelin-containing neurons in the arcuate nucleus send projections to neuropeptide Y (NPY) and agouti-related peptide (AgRP) positive neurons [33, 53]. NPY and AgRP are orexigenic neuropeptides [1] and regulated by ghrelin. Peripheral injection of ghrelin selectively activates NPY-containing neurons of the arcuate nucleus in mice [153]. Likewise, intracerebroventricular (icv) administration of ghrelin activates NPY/AgRP-expressing neurons and stimulates the expression of NPY and AgRP mRNA in the arcuate nucleus (Fig. 3) [75]. Conversely, the orexigenic effect of icv ghrelin is blocked by the co-injection of an anti-NPY or anti-AgRP antibody [103]. Studies using double NPY and AgRP KO mice corroborated these findings with peripherally injected ghrelin. Whereas single deletion of NPY or AgRP was not effective in blocking the food intake stimulatory action of intraperitoneally administered ghrelin suggesting compensatory actions, deletion of both, NPY and AgRP genes resulted in a complete suppression of ghrelin's orexigenic action [32]. Thus, there is convincing evidence that the central mediation of ghrelin's orexigenic effect involves hypothalamic NPY and AgRP.

#### 2.5 Interaction between ghrelin and gut peptides or brain transmitters

The interplay between gastric and intestinal peptides is of importance since they are often released simultaneously in response to changes in metabolic conditions and nutritional status. The orexigenic action of ghrelin and related Fos expression in specific hypothalamic nuclei regulating food intake are completely abolished by intraperitoneal co-injection with CCK-8 in rats (Fig. 4) [82]. Likewise, peripheral injection of bombesin, unlike amylin, abolishes ghrelin's orexigenic effect in rats (Fig. 4) [80]. Moreover, GLP-1<sub>7-36</sub> amide inhibits ghrelin secretion in the isolated rat stomach [94]. Likewise, exendin-4, a GLP-1 receptor agonist, reduces circulating ghrelin levels by up to 70% after peripheral as well as central injection in rats [110]. Therefore, GLP-1 may contribute to the postprandial decline



in ghrelin levels. Conversely, the anorexigenic effect of PYY<sub>3-36</sub> or GLP-1 is attenuated by ghrelin by up to 64% [30].

The anorexigenic hormone leptin is a 16 kDa protein mainly derived from the white adipose tissue [46] but also expressed in the gastric mucosa [14]. Gastric ghrelin mRNA levels are decreased by peripheral administration of leptin (Fig. 4) and increased in leptin-deficient *ob/ob* mice [11] indicative of a negative regulation of gastric ghrelin gene expression by leptin. In the brain, central injection of ghrelin reverses the central leptin-induced inhibition of food intake in rats [103] possibly due to restoring the leptin-induced decrease in hypothalamic NPY mRNA levels [133]. These data indicate an interaction between leptin and ghrelin in the hypothalamus on neuronal circuits regulating food intake. Whether endogenous ghrelin released into the circulation and crossing the blood-brain barrier [17, 109] or locally produced ghrelin in the brain [33, 84, 95] interact with leptin remains to be investigated.

The mesocorticolimbic dopamine system is involved in the hedonic aspects of food [124]. Several lines of evidence highlight the impact of ghrelin on reward mechanisms [73]. Central as well as peripheral administration of ghrelin induces dopamine overflow in the nucleus accumbens of mice (Fig. 4) [70, 71]. Furthermore, microinjection of ghrelin into the ventral tegmental area and latero-dorsal tegmental area increased the extracellular concentration of accumbal dopamine [72]. A single intra-tegmental infusion of ghrelin significantly increases food intake [2]. Moreover, ghrelin directly influences the electrophysiological responses in most dopamine-positive neurons in mice and rats, increases dopamine turnover and thus enhances the activity of dopaminergic cells in the nucleus accumbens of the ventral tegmental area as shown by whole-cell patch clamp recordings [2]. At the cellular level, activation of GHS-R1a by ghrelin also amplifies dopamine/dopamine<sub>1</sub> receptor-induced cAMP accumulation with a mechanism consistent with agonist-dependent formation of GHS-R1a/dopamine<sub>1</sub> receptor heterodimers [74]. These direct actions of ghrelin on dopamine-producing cells could underlie the effects of ghrelin on reward.

Cannabinoids (CB) are lipids that stimulate food intake and body weight gain similarly to ghrelin and could therefore share common pathways [125]. This is supported by the demonstration that central injection of ghrelin is unable to increase food intake in CB<sub>1</sub> receptor knockout mice [86] indicating that the endocannabinoid signaling system is necessary for ghrelin's orexigenic effect. Anandamide, an endocannabinoid, injected peripherally increases circulating ghrelin levels [162]. Conversely, rimonabant, a CB<sub>1</sub> receptor antagonist, reduces systemic ghrelin levels (Fig. 4) [25]. The crosstalk between ghrelin and endocannabinoids could take place at the receptor level based on the observation that the GHS-R1a and CB<sub>1</sub> are expressed in the hypothalamus with high expression levels in the arcuate nucleus and the assumption of the formation of heterodimers which warrants further characterization [125]. Evidence so far indicates a complex interplay between ghrelin and other peripheral and central mechanisms modulating food intake.

## 2.6 Ghrelin in the clinical setting

Much attention has been given to ghrelin due to its unique orexigenic action in animals and humans. Ghrelin is able to stimulate food intake under conditions of impaired appetite as shown in two randomized placebo-controlled trials with patients suffering from end-stage kidney disease (Table 1) [12, 158]. Moreover, daily oral application of a formula rich in octanoic acids over a period of two weeks increases plasma ghrelin levels as well as nutritional status in cachectic patients with chronic respiratory disease (Table 1) [13].

The blocking of endogenous or exogenous ghrelin was actively investigated using various approaches in order to reduce food intake and body weight gain for potential new

therapeutic venues. Anti-ghrelin vaccination gave very promising results by curtailing body weight gain in pre-clinical studies in pigs and rats (Table 1) [151, 171]. However, in mice fed a high fat diet anti-ghrelin vaccination failed to decrease body weight (Table 1) although acutely reducing food intake [78]. One difference between those studies, possibly contributing to the divergent outcome, is the adjuvant used. In pigs, the ghrelin peptide was linked to bovine serum albumin [151] and in rats to the carrier protein, keyhole limpet hemocyanin [171], while in mice Pan-DR epitope peptides were used [78]. However, the exact underlying mechanism remains to be determined. Likewise, another study using a ghrelin-neutralizing antibody failed to demonstrate a reduction in body weight in mice kept on a high fat diet (Table 1) [96]. Both studies in mice showed an increase in circulating ghrelin levels [78, 96] that may account for the lack of effect under conditions of already reduced ghrelin sensitivity [111]. Furthermore, species differences could also contribute to the different outcomes. Likewise, in obese human subjects ghrelin vaccination failed to induce body weight loss (Table 1) [20] suggesting compensatory mechanisms. Another experimental approach was the use of synthetic L-isomer oligonucleotides which are able to specifically bind to ghrelin with high affinity and to be resistant to endogenous nucleases [81]. This anti-ghrelin Spiegelmer (German Spiegel = mirror) NOX-B11 acutely blocks the exogenous ghrelin-induced food intake and neuronal activation [81]. A more advanced version (NOX-B11-2) also reduced body weight gain in high fat diet-induced obese mice upon chronic subcutaneous administration (Table 1) [128]. Interfering with ghrelin receptors, GHS-R1a antagonists that can cross the blood-brain barrier also reduce energy intake in lean and obese rodents (Table 1) [10].

Until now, no anti-obesity drug targeting ghrelin or the GHS-R1a has reached clinical efficacy as shown by the lack of sustained body weight loss in one clinical study [20]. This is most likely related to associated compensatory mechanisms [125] which may occur after chronic treatment design in human studies (e.g. 24 weeks) [20] when compared to animal studies (e.g. 12 weeks) [171], as like in humans, ghrelin KO mice also displayed normal growth and a similar food intake pattern as wildtype [156]. The existence of additional ghrelin receptor subtypes that have yet to be identified [125] may also contribute to the lack of efficacy of the current drugs, thereby bypassing the effect of selective antagonists.

Bariatric surgery is the most effective treatment for long-term weight loss in morbidly obese patients as shown by reports with the commonly performed Roux-en-Y gastric bypass surgery (RYGB) [143]. In those patients circulating ghrelin levels are low postoperatively and no meal-related ghrelin changes can be observed which has been proposed to underlie, or at least contribute, to the efficacy of bariatric surgery (Table 2) [37]. Likewise, sleeve gastrectomy, a technique that involves removal of the gastric fundus, reduces body weight in animal models [154] as well as obese patients (Table 2) [76]. Ghrelin levels decrease after this procedure [90], consistent with the stomach being the major production site of circulating ghrelin (Table 2). This long-lasting decrease is thought to play a role in the beneficial effect of sleeve gastrectomy on body weight [45] and also the observed improvement of glucose control [93]. This decrease contrasts with the increased levels of circulating ghrelin seen after diet-induced weight loss in obese patients (Table 2) [87]. Similarly, after gastric banding that maintains the normal food passage as well as keeps the gastric fundus intact but decreases the volume of the proximal gastric reservoir, circulating ghrelin levels increase (Table 2) [87, 90]. One proposed interpretation relates to the ghrelin response as reflecting a state of starvation to restore the previous obese “normal” state since the homeostatic set-point is changed in obese patients and consecutively the obese state is perceived as “normal” [155].

### 3. Des-acyl ghrelin

Initial reports indicated that the vast majority of the circulating ghrelin form is represented by des-acyl ghrelin with an acyl/total ghrelin ratio of 1:55 [63]. However, we have recently shown that higher yields of the labile acylated ghrelin form can be obtained when a novel method is applied to blood processing [139] compared with the commonly used standard method using mainly EDTA blood on ice [15]. We found in *ad libitum* fed rats that the acyl/total ghrelin ratio is 1:5 with the new blood processing method compared to 1:19 with the standard method. In fasted rats the acyl/total ghrelin ratio remains 1:5 with the new method and increased to 1:41 with the standard method of blood processing [139]. The novel RAPID method encompasses *Reduced* temperatures, *Acidification*, *Protease inhibition*, *Isotopic exogenous controls* and *Dilution* as recently detailed [139]. Such a procedure of blood treatment immediately after collection results in an 80% higher recovery of circulating acyl ghrelin levels and therefore represents a valuable advance to detect circulating concentrations of orexigenic ghrelin form [139]. Recent studies in the vascularly perfused rat stomach indicate that des-acyl ghrelin release is enhanced under conditions of low pH while that of acyl ghrelin is not [98]. Moreover, while des-acyl ghrelin co-localized with acyl ghrelin in round closed-type cells of the oxyntic mucosa, open-type cells were positive for des-acyl but not acyl ghrelin [98]. Furthermore a portion of des-acyl ghrelin-positive open-type cells contained somatostatin [98]. Taken together, these recent findings indicate a differential regulation in the post-translational processing of proghrelin leading to ghrelin and des-acyl ghrelin.

#### 3.1 Des-acyl ghrelin and receptor interaction

In contrast to ghrelin, des-acyl ghrelin does not bind to the GHS-R1a due to the lack of the fatty acid group and therefore has been initially categorized as a non-active peptide (Fig. 2) [84]. However, since both ghrelin and des-acyl ghrelin affect cells that do not bear the GHS-R1a, the existence of yet unknown additional ghrelin receptors is strongly assumed [125, 136]. This is supported by the observations that des-acyl ghrelin stimulates insulin release from INS-1E cells independently of the GHS-R1a [47] and inhibits cell proliferation in human breast cancer [28] and prostate cancer [27] cell lines which do not express the GHS-R1a.

#### 3.2 Des-acyl ghrelin: effects on food intake

Compared to ghrelin the effects of des-acyl ghrelin on food intake are less well characterized [67]. However, it is currently discussed as a possible anorexigenic hormone and modulator of ghrelin-induced food intake although some findings are still equivocal [67] (Table 3). Initial studies showed a reduction of food intake following intraperitoneal injection of des-acyl ghrelin in fasted rats during the light phase or *ad libitum* fed rats during the dark phase [31] and intracerebroventricular or intraperitoneal injection in fasted mice during the light phase [9]. Underlying mechanisms of the des-acyl ghrelin-induced anorexigenic effect following peripheral injection have been reported to be capsaicin-insensitive and associated with the activation of neurons in the paraventricular nucleus of the hypothalamus along with the disruption of motor activity in the stomach [31]. Whether the peptide action is secondary to induction of food aversion or altered behavior including the experience of sickness has not yet been investigated. However, subsequent studies failed to reproduce the anorexigenic effect following peripheral injection of des-acyl ghrelin in fasted rats [66] or mice [104]. In addition, one report shows a stimulation of food intake after intracerebroventricular injection of a low dose (1.9 µg/kg) of the peptide in rats [146]. Whether this reflects a direct mechanism of the peptide action or an effect after acylation remains to be elucidated. Recently, we demonstrated that des-acyl ghrelin, which does not influence food intake during the light as well as dark phase when injected alone, blocked the



ghrelin-induced increase in food intake following simultaneous intraperitoneal injection in rats [66] (Table 3) suggesting a possible interaction between the two peptide forms depending upon the ratio released. The identification of des-acyl ghrelin receptors mediating these effects will increase our knowledge on mechanisms and site of action.

### 3.3 Effect of des-acyl ghrelin on fat mass and energy expenditure

Transgenic mice that over-express des-acyl ghrelin display a reduced body size accompanied by a decreased body weight compared to their wildtype littermates [6]. These changes were observed in the absence of altered food intake suggesting that undernutrition does not contribute to the observed effects on body weight. However, it has to be noted that these transgenic mice had 10 to 50-fold higher, and therefore supraphysiological, des-acyl ghrelin levels than their wildtype littermates. When des-acyl ghrelin expression was increased by the fatty acid-binding protein-4 (FABP4) promoter, circulating des-acyl ghrelin levels were markedly increased whereas ghrelin or obestatin levels were not altered [165]. Epididymal and perirenal fat depots were reduced in those transgenic mice compared to their wildtype littermates whereas brown adipose tissue was not changed [165]. Furthermore, these mice also display increased insulin sensitivity [165] suggesting that des-acyl ghrelin may play a role not only to reduce food intake and control body weight but also in glucose homeostasis. However, des-acyl ghrelin stimulates intracellular lipid accumulation in human adipocytes *in vitro* that were obtained from obese subjects [116] which may contribute to the fat accumulation in those patients. Whether this does reflect species differences or is attributable to divergent responses under *in vivo* and *in vitro* conditions or obesity status requires further investigations.

### 3.4 Possible use of targeting the ghrelin acylation process in the treatment of obesity

The recently identified enzyme that acetylates ghrelin, GOAT, attracted much attention as a potential target in the drug treatment of obesity. One study reported the first pentapeptide representing the octanoylated N-terminus of ghrelin able to inhibit GOAT [161]. However, further studies will be required to develop new inhibitors without receptor-signaling activity. This strategy may provide a mean to selectively reduce circulating ghrelin and concomitantly increase des-acyl ghrelin.

## 4. The ghrelin-associated peptide, obestatin

Obestatin or proghrelin<sub>(53-75)</sub> is a proposed 23 amino acid residue amidated peptide resulting from alternative splicing and posttranslational modification from a computer-based predicted cleavage site of C-terminal proghrelin that was initially reported to have anorexigenic properties (Fig. 2) [136, 164]. Obestatin immunoreactivity can be detected together with ghrelin in the vesicles of the same cells in normal human gastric mucosa as well as gastric endocrine tumors [52, 149]. The co-localization between obestatin and proghrelin was also shown in the rat gastric oxyntic mucosa, however less co-localization (60%) was shown with mature ghrelin pointing towards differential post-translational expression [166].

### 4.1 Release of obestatin and receptor interaction

In contrast to the fasting-induced increased expression of ghrelin both at a cellular and a systemic level, obestatin immunoreactivity in the gastric mucosa does not change during food deprivation [166]. Furthermore, circulating obestatin levels are below detection threshold [16, 100] and not affected by fasting or feeding [164] or in presence of a gastric neuroendocrine tumor [149]. However, one study showed a significant reduction of circulating obestatin after fasting [170]. Taken together, these data suggest differential

mechanisms regulating post-translational modifications/cleavage leading to obestatin and ghrelin in X/A-like cells under conditions of changes in metabolic status.

When obestatin was described in 2005 it was thought to be the endogenous ligand of the seven transmembrane domain G protein-coupled receptor, GPR39 [164]. This receptor, which displays high constitutive activity, was an orphan member of the ghrelin receptor family [61]. However, all subsequent studies performed by several independent groups [29, 60, 91, 147] and also the original investigators [163] could reproduce neither the binding of obestatin to tissue homogenates and to cells transiently transfected with recombinant GPR39 nor the activation of the receptor by obestatin. Therefore, the endogenous ligand of GPR39 is still unknown as well as the cognate receptor for obestatin.

#### 4.2 Controversial effects of obestatin on food intake

The original report on obestatin in 2005 suggested that the peptide suppresses food intake, decreases body weight gain and is able to counteract ghrelin's orexigenic action [164]. However, only a few studies were able to partially reproduce these data when using very specific conditions [23, 26, 88, 102] and some of those have been recently retracted [89]. So far the vast majority of studies conducted by several independent groups have not been able to confirm the inhibitory effect of obestatin on food intake, body weight gain or its interaction with ghrelin [4, 24, 43, 50, 60, 83, 88, 99, 100, 106, 122, 127, 147, 150, 159, 170] as recently exhaustively reviewed [49]. Taken together, these data do not support the assumption of obestatin as a physiologically relevant peptide in the regulation of food intake. Taken together, obestatin does not appear to be a "statin" for obesity and is proposed to be renamed ghrelin-associated peptide (GAP) [51].

### 5. Nesfatin-1

Nesfatin-1 was identified by Oh-I et al. in the rat hypothalamus and reported to decrease food intake upon 3<sup>rd</sup> ventricle injection and named nesfatin for *NUCB2* encoded satiety and *fat*-influencing protein [107]. Posttranslational processing results in nesfatin-1, nesfatin-2 and nesfatin-3 (Table 4), however, only nesfatin-1 exhibits a food intake-reducing effect [107]. We could recently establish that the nesfatin-1 precursor *NUCB2* is more prominently expressed at the mRNA and protein level in the rat gastric oxyntic mucosa as shown by microarray analysis substantiated by RT-qPCR and Western Blot (Fig. 2) than in other viscera such as the heart and even the brain (Fig. 5) [137]. Moreover, *NUCB2* mRNA expression is significantly enriched in a population of gastric oxyntic small endocrine cells [137]. Co-localization of ghrelin and nesfatin-1 immunoreactivity in the X/A-like cells could be demonstrated by confocal microscopy where both peptides reside in different pools of vesicles (Fig. 6) [137]. This co-localization is further supported by the occurrence of PC1/3 in X/A-like cells [160] as PC1/3 is involved in the processing of *NUCB2* to nesfatin-1 in the brain [131]. Moreover, nesfatin-1 immunoreactivity was also detected in the pancreas, testis and pituitary gland [137]. However, the function at these production sites remains unclear but may point towards an action in an autocrine or paracrine fashion.

#### 5.1 Release of nesfatin-1

We recently reported that fasting for 24 h decreases *NUCB2* mRNA expression in a pool of enriched small gastric endocrine cells [137] and significantly reduces nesfatin-1 plasma levels in rats [138]. These data indicate that *NUCB2*/nesfatin-1 expression and release are regulated by nutritional status. Nesfatin-1 has also been detected in human serum by Western blot analysis and possible changes in circulating levels in various nutritional conditions and relationship to body mass index are currently under investigation [131]. Further studies are warranted to elucidate the differential regulation of nesfatin-1 and ghrelin

within the same X/A-like cells along with the receptor for nesfatin-1 to characterize its action sites.

## 5.2 Inhibitory effect of nesfatin-1 on food intake

Recently, Shimizu and colleagues showed that the intraperitoneal injection of nesfatin-1 reduces the dark phase food intake in mice without causing taste aversion [130]. Furthermore, repeated intraperitoneal injections over a period of 6 days inhibit body weight gain [131]. The mid segment (aa 24–53) of the 82 aa nesfatin-1 is also biologically active to reduce food intake after peripheral injection, whereas the N-23 or C-29 amino acid terminal fragments are inactive [130]. The anorexigenic action of nesfatin-1 was observed in lean, *ob/ob* and high fat diet-induced obese mice providing evidence for a leptin-independent mechanism of action [130]. Furthermore, preliminary data suggest that nesfatin-1 also induces a long lasting inhibition of food intake in mice when injected subcutaneously (over 14 h) as well as after intranasal administration in rats (6 h) [131]. The observation that a nesfatin-1 mid fragment is not able to suppress food intake in capsaicin pretreated mice [132] points towards a vagal transmission of peripheral nesfatin-1 signals to the brain.

Injection of nesfatin-1 in pmol amounts into the lateral, or 4<sup>th</sup> ventricle and cisterna magna also decreases dark phase food intake for 6 h whereas the 24 h cumulative food intake is not altered [138]. However, the body weight of rats was significantly reduced 24 h following an acute intracerebroventricular injection of nesfatin-1 [138] suggesting increased energy expenditure. Further studies investigating respiratory quotient and uncoupling protein levels in brown adipose tissue are needed to corroborate this assumption.

## 5.3 Possible use of nesfatin-1 in the drug treatment of obesity

Peripheral nesfatin-1 exerts an anorexigenic action that is still retained in diet-induced obese and *ob/ob* mice [130] pointing towards a leptin-independent action. Since obese subjects commonly display leptin resistance or at least decreased leptin sensitivity this finding could make nesfatin-1 a promising target in the drug treatment of obesity as recently suggested [131].

## 6. Conclusions

Until now, the gastric endocrine X/A-like cells were thought to be restricted to the stimulation of food intake and adiposity linked with the synthesis and release of the orexigenic peptide, ghrelin. However, recent developments including the characterization of the possible modulatory effect of des-acyl ghrelin as well as the identification of nesfatin-1 in X/A-like cells highlight the importance of this cell type as regulator of food intake able to stimulate and also inhibit food consumption and influence body weight. Ghrelin's orexigenic action can be modulated by various other food intake regulatory gut peptides (e.g. CCK, bombesin, GLP-1 and PYY), proteins (e.g. leptin) and lipids (e.g. endocannabinoids) originating from the stomach and the intestine. The receptors involved in des-acyl ghrelin's and nesfatin-1's biological actions are still unknown and their identification along with the classification of possible additional ghrelin receptor subtypes will markedly enhance our understanding of these peptides' and the X/A-like cells' physiology.

The incidence and prevalence of overweight, obesity and associated diseases are rising in the developed countries throughout the world which represents an important socioeconomic burden. Drug treatment options are very limited and peptide products of the X/Alike cell have been tackled as potential promising candidates. Ghrelin signaling pathways especially have been in the spotlight not only as a mean to curtail food consumption and body weight

gain by blocking GHS-R1a mediated actions but also to stimulate these pathways to promote food consumption in pathological states linked with reduced appetite. While preclinical as well as clinical data look promising for the stimulation of food intake and consecutive improvement of the nutritional status using ghrelin analogs, the preclinical data showing a decrease in food intake and body weight by applying various strategies to block ghrelin signaling have not been successfully translated into the clinical setting. This suggests the existence of other factors compensating ghrelin's effect as well as additional ghrelin receptor subtypes yet to be identified which may be subject to species differences. A deeper understanding how different peptides are individually regulated and secreted from the same cell will require detailed characterization at the cellular and subcellular level in order to unravel the regulation and physiological function of the gastric X/A-like endocrine cell.

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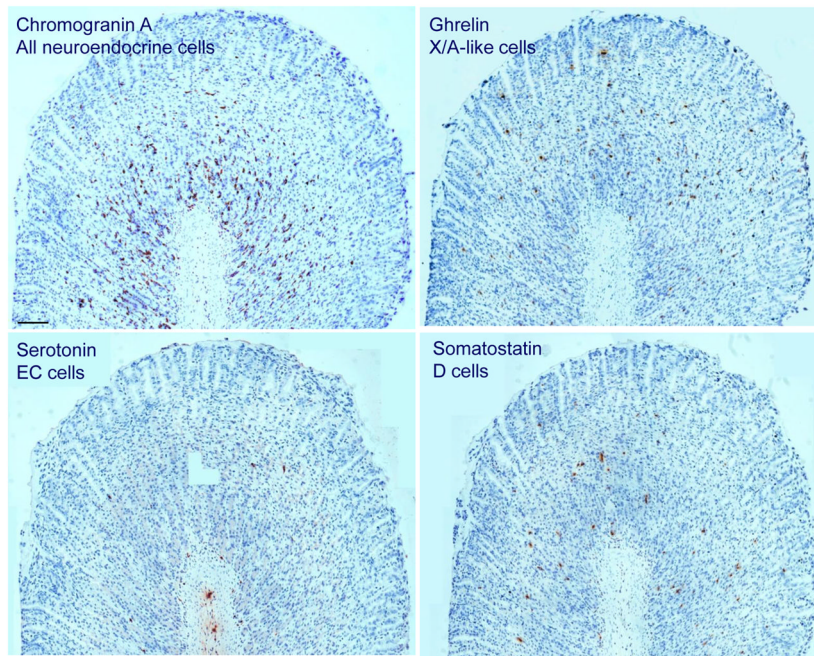
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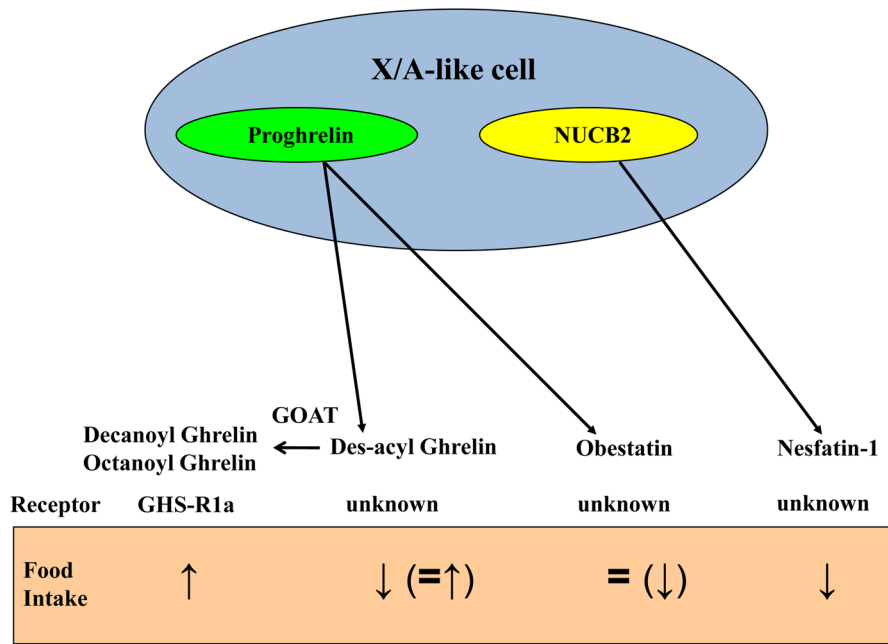
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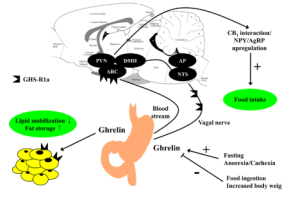


**Fig. 1.** Immunohistochemical picture of neuroendocrine cells in the rat gastric oxyntic mucosa of *ad libitum* fed male rats. While the majority of neuroendocrine cells reside in the lower part of gastric glands, ghrelin positive X/A-like cells are evenly distributed throughout the entire length of the glands. Somatostatin-positive D cells are mostly localized in the lower half of the oxyntic glands. Serotonin-positive EC cells are rare. Scale bar represents 100  $\mu\text{m}$ .

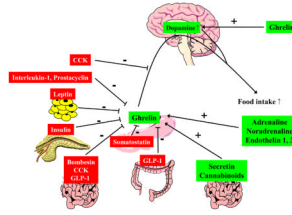


**Fig. 2.** Peptide products of the rat gastric X/A-like cell and their effects on food intake in rodents. ↑, stimulation; ↓, inhibition; =, no effect; GHS-R1a, growth hormone secretagogue receptor 1a; GOAT, ghrelin-*O*-acyltransferase; NUCB2, nucleobindin2

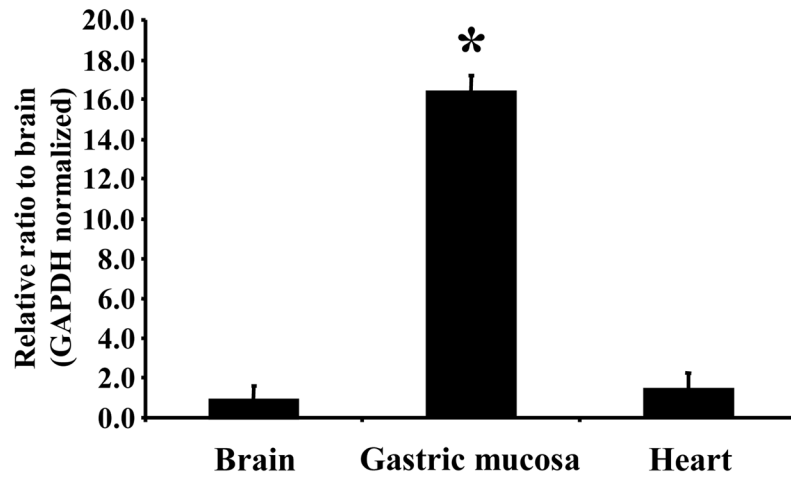




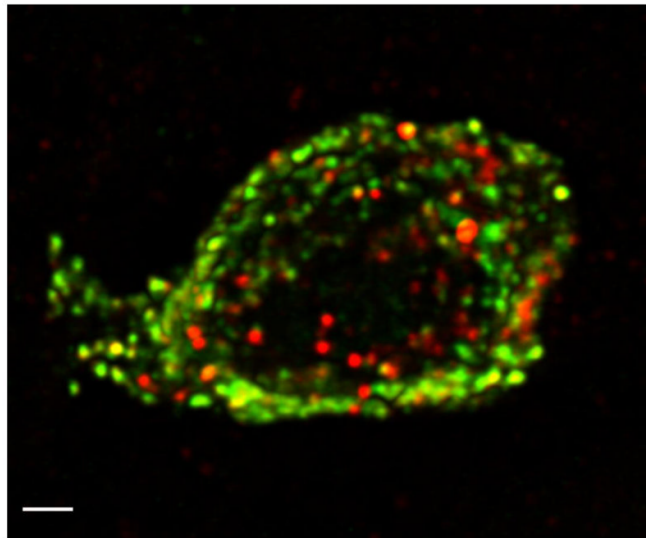
**Fig. 3.** Schematic representation of factors influencing ghrelin release and mediating ghrelin's effects on food intake and fat storage. ↑, increase; ↓, decrease; +, stimulation; −, inhibition, AgRP, agouti-related peptide; AP, area postrema; ARC, arcuate nucleus; DMH, dorsomedial nucleus of the hypothalamus; GHS-R1a, growth hormone secretagogue receptor 1a; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; PVN, paraventricular nucleus of the hypothalamus.



**Fig. 4.** Schematic illustration of the interaction of ghrelin with other gut peptides and transmitters. ↑, increase; +, stimulation; -, inhibition.



**Fig. 5.** NUCB2 mRNA is significantly higher expressed in the gastric oxyntic mucosa than in brain and heart. Data are expressed as mean  $\pm$  SD, \*  $p < 0.001$  vs. brain and heart. (Reproduced with permission from reference [137]; Copyright 2009, The Endocrine Society).



**Fig. 6.** High resolution confocal image of a single gastric mucosal endocrine cell co-expressing ghrelin (green) and nesfatin-1 (red) immunoreactivity. Confocal microscopy was performed using a 100 × objective and digital zoom, 8 images in z-axis (stack size 24 × 24 × 7 μm) with dual laser excitation at 488 and 543 nm. Images were collected in two optical channels and photomultiplier tubes using FITC and TRITC filter sets, de-convoluted and reconstructed into a 3-dimensional image. The scale bar represents 1 μm. (Reproduced with permission from reference [137]; Copyright 2009, The Endocrine Society).

**Table 1**  
Effects of targeting ghrelin in pre-clinical *versus* clinical studies to modulate food intake and/or body weight.

| Goal | Compound                       | Species and conditions                              | Route | Duration | Effect  | Reference |
|------|--------------------------------|---|-------|----------|---|-----------|
|      | <b>Pre-clinical</b>            |   |       |          |   |           |
|      | ghrelin                        | rats with chronic renal failure                     | ip    | 7 days   | food intake ↑, body weight =                                | [3]       |
|      | ghrelin                        | rats after lipopolysaccharide injection             | iv    | acute    | food intake ↑   | [152]     |
|      | <b>Clinical</b>                |   |       |          |   |           |
|      | ghrelin                        | peritoneal dialysis patients with malnutrition      | sc    | acute    | food intake ↑   | [158]     |
|      | ghrelin                        | dialysis patients with malnutrition                 | sc    | 7 days   | appetite ↑, energy intake ↑                                 | [12]      |
|      | formula rich in octanoic acids | cachectic patients with chronic respiratory disease | po    | 14 days  | body weight ↑   | [13]      |
|      | <b>Pre-clinical</b>            |   |       |          |   |           |
|      | anti-ghrelin vaccine           | rats  | ip    | 13 weeks | body weight gain ↓  | [171]     |
|      | anti-ghrelin vaccine           | pigs  | sc    | 40 days  | food intake ↓, body weight ↓                                | [151]     |
|      | anti-ghrelin vaccine           | mice on high fat diet                               | sc    | 22 weeks | body weight gain =  | [78]      |
|      | anti-ghrelin antibody          | mice with diet-induced obesity                      | ip    | 4 weeks  | acute: food intake ↓, chronic: food intake =, body weight = | [96]      |
|      | anti-ghrelin oligonucleotide   | rats  | ip    | acute    | food intake ↓, neuronal activation ↓                        | [81]      |
|      | anti-ghrelin oligonucleotide   | mice with diet-induced obesity                      | sc    | 13 days  | food intake ↓, body weight ↓                                | [128]     |
|      | GHS-R antagonist               | lean and diet-induced obese mice                    | ip    | 5 days   | food intake ↓, body weight gain ↓                           | [10]      |
|      | <b>Clinical</b>                |   |       |          |   |           |
|      | anti-ghrelin vaccine           | obese patients                                      | ns    | 24 weeks | body weight =   | [20]      |

↑, increase; ↓, decrease; =, no effect; ip, intraperitoneal; iv, intravenous; ns, not specified; po, *per os*; sc, subcutaneous



**Table 2**

Effects of three common surgical procedures compared to a dietary approach on body mass index (BMI) and circulating ghrelin levels.

| <b>Intervention/Technique</b> | <b>Duration</b> | <b>Effects on BMI</b> | <b>Effects on circulating ghrelin</b>                            | <b>Reference</b> |
|-------------------------------|-----------------|-----------------------|--|------------------|
| Roux-en-Y gastric bypass      | 9 – 31 months   | 36% reduction of BMI  | 77% reduction of the 24 h ghrelin profile (area under the curve) | [37]             |
| Sleeve gastrectomy            | 12 months       | 36% reduction of BMI  | 34% reduction of fasting total ghrelin plasma levels             | [76]             |
| Gastric banding               | 18 months       | 38% reduction of BMI  | 65% increase of fasting total ghrelin plasma levels              | [87]             |
| Diet                          | 6 months        | 9% reduction of BMI   | 110% increase of fasting total ghrelin plasma levels             | [87]             |

Table 3

Effect of des-acyl ghrelin on food intake in rodents.

| Site of administration | Dose ( $\mu\text{g}/\text{kg}$ ) | Feeding condition | Circadian phase | Species | Effect on food intake | Reference |
|------------------------|----------------------------------|-------------------|-----------------|---------|-----------------------|-----------|
| icv                    | 1.9                              | ad lib            | light, dark     | rats    | ↑                     | [146]     |
| icv                    | 9.1–91                           | ad lib            | light           | mice    | =                     | [9]       |
| icv                    | 91                               | fasted            | light           | mice    | ↓                     | [9]       |
| iv                     | 14.4–48                          | ad lib            | light           | rats    | =                     | [146]     |
| ip                     | 128                              | ad lib            | light           | mice    | =                     | [146]     |
| ip                     | 274                              | fasted            | light           | mice    | ↓                     | [9]       |
| ip                     | 64                               | ad lib            | light           | rats    | =                     | [31]      |
| ip                     | 64                               | fasted            | light           | rats    | ↓                     | [31]      |
| ip                     | 64                               | ad lib            | dark            | rats    | ↓                     | [31]      |
| ip                     | 960                              | ad lib, fasted    | ns              | mice    | =                     | [104]     |
| ip                     | 64–125                           | ad lib, fasted    | light           | rats    | =                     | [66]      |

icv: intracerebroventricular, iv: intravenous, ip: intraperitoneal, ad lib: *ad libitum* fed, fas: fasted, ns: not specified

**Table 4**

Amino acid sequences of rat nesfatin-1, 2 and 3.

|                                |   |
|--------------------------------|---|
| <b>Nesfatin-1 (aa 1–82)</b>    | VPIDV DKTKV HNVEP VESAR IEPPD TGLYY DEYLK QVIEV<br>LETDP HFREK LQKAD IEEIR SGRLS QELDL VSHKV RTRLD EL   |
| <b>Nesfatin-2 (aa 85–163)</b>  | QEVGR LRMLI KAKLD ALQDT GMNHH LLLKQ FEHLN HQNPD<br>TFESK DLDML IKAAT ADLEQ YDRTR HEEFK KYEMM KEHE<br>YLKTL SEEKR KEEEA KFAEM KRKHE DHPKV NHPGS KDQLK<br>EYWEE TDGLD PNDFD PKTFF KLHDV NNDGF LDEQE LEALF |
| <b>Nesfatin-3 (aa 166–396)</b> | TKELD KVYNP QNAED DMIEM EEERL RMREH VMNEI DNNKD<br>RLVTL EEFLR ATEKK EFLEP DSWET LDQQQ LFTEE ELKEY<br>ESIIA IQESE LKKKA DELQK QKEEL QRQHD HLEAQ KQEYQ<br>QAVQQ LEQKK FQQGI APSGP AGELK FEPHT            |