

Nesfatin-1: functions and physiology of a novel regulatory peptide

Riccardo Dore, Luka Levata, Hendrik Lehnert and Carla Schulz

Department of Internal Medicine I, Center of Brain, Behavior and Metabolism (CBBM), University of Lübeck, Lübeck, Germany

Correspondence
should be addressed
to C Schulz

Email
carla.schulz@uksh.de

Abstract

Nesfatin-1 was identified in 2006 as a potent anorexigenic peptide involved in the regulation of homeostatic feeding. It is processed from the precursor-peptide NEFA/nucleobindin 2 (NUCB2), which is expressed both in the central nervous system as well as in the periphery, from where it can access the brain via non-saturable transmembrane diffusion. In hypothalamus and brainstem, nesfatin-1 recruits the oxytocin, the melanocortin and other systems to relay its anorexigenic properties. NUCB2/nesfatin-1 peptide expression in reward-related areas suggests that nesfatin-1 might also be involved in hedonic feeding. Besides its initially discovered anorexigenic properties, over the last years, other important functions of nesfatin-1 have been discovered, many of them related to energy homeostasis, e.g. energy expenditure and glucose homeostasis. Nesfatin-1 is not only affecting these physiological processes but also the alterations of the metabolic state (e.g. fat mass, glycemic state) have an impact on the synthesis and release of NUCB2 and/or nesfatin-1. Furthermore, nesfatin-1 exerts pleiotropic actions at the level of cardiovascular and digestive systems, as well as plays a role in stress response, behavior, sleep and reproduction. Despite the recent advances in nesfatin-1 research, a putative receptor has not been identified and furthermore potentially distinct functions of nesfatin-1 and its precursor NUCB2 have not been dissected yet. To tackle these open questions will be the major objectives of future research to broaden our knowledge on NUCB2/nesfatin-1.

Key Words

- ▶ NUCB2
- ▶ nesfatin-1
- ▶ energy homeostasis
- ▶ glucose metabolism
- ▶ cardiovascular

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Introduction

In quest of novel appetite regulating molecules, in 2006, Oh-I and coworkers re-discovered NEFA/nucleobindin2 (*Nucb2*), a peroxisome proliferator γ receptor (PPARG)-activated gene in immortalized cell lines (Oh-I *et al.* 2006) and later in the hypothalamus of rodents (Oh-I *et al.* 2006). Its gene product NUCB2, a 396 amino acid (AA) peptide, which was originally described as a secreted protein of unknown function (Miura *et al.* 1992, Barnikol-Watanabe *et al.* 1994), possesses a number of putative cleavage sites, suggesting further processing (Oh-I *et al.* 2006). The N-terminal fragment, named nesfatin-1, was subsequently identified in rat cerebrospinal fluid

(CSF) and the hypothalamic paraventricular nucleus (PVN), and it was found to dose-dependently reduce food intake when administered intracerebroventricularly (i.c.v.) in rodents (Oh-I *et al.* 2006). Since the seminal work by Oh-I *et al.* (2006), numerous research groups have aimed at elucidating the role of nesfatin-1 not only in the regulation of food intake but also in other physiological functions. With this review, we aim at summarizing the current knowledge in the field with a particular focus on energy homeostasis. Furthermore, we provide comprehensive tables on nesfatin-1 administration, dosages and physiological effects.

NUCB2 and nesfatin-1: expression, processing and release

Nucb2 mRNA is mainly expressed in gastric mucosa and white adipose tissue and also, but to a minor extent, in other peripheral organs, e.g. pancreas and testis (Gonzalez *et al.* 2009, Shimizu *et al.* 2009b, Stengel *et al.* 2009b, Ramanjaneya *et al.* 2010, Kim *et al.* 2014). Within the central nervous system (CNS), *Nucb2* mRNA is present, for example, in the hypothalamus and brainstem (Oh-I *et al.* 2006, Brailoiu *et al.* 2007) (for more details we refer to the respective sections).

The product of the *Nucb2* gene is a 420 AA peptide consisting of a 24 AA signal peptide and 396 AA peptide termed NUCB2. The latter possesses putative cleaving sites for protein convertases (PC) 1/3 and PC2, suggesting further processing into three alleged fragments: nesfatin-1 (AA 1–82), nesfatin-2 (AA 85–163) and nesfatin-3 (AA 166–396) (Oh-I *et al.* 2006). NUCB2 is co-localized with these enzymes in the cytoplasm (Steiner *et al.* 1992, Oh-I *et al.* 2006, Mohan *et al.* 2014, 2016), suggesting that processing of NUCB2 may take place physiologically. However, to our knowledge, this has not been proven so far by *in vivo* and in *in vitro* studies; Chinese hamster ovary (CHO) cells overexpressing the relevant genes (*Nucb2*, *Pc1/3*, *Pc2*), failed to produce nesfatin-1 (Oh-I *et al.* 2013). Alternatively, other enzymes, for example, the cell surface membrane-bound protein furin, which cleaves at the same general motif as the PCs, might be involved in NUCB2 processing (Seidah & Prat 2002). In most rodent tissues studied, for example, gastric mucosa, pancreas, pituitary gland and testis, either only the precursor NUCB2 or indistinguishable NUCB2/nesfatin-1 was detected (Stengel *et al.* 2009b, Kim *et al.* 2014). However, nesfatin-1, the amino terminal putative cleavage product of NUCB2, was unambiguously identified in the hypothalamus and CSF of rodents (Oh-I *et al.* 2006) and also in human plasma (Tsuchiya *et al.* 2010).

Although this clearly proves the existence of nesfatin-1 *in vivo*, it is still unknown whether the putative cleavage products nesfatin-2 and -3 exist and are secreted *in vivo*. The administration of neither nesfatin-2 nor -3 was effective in terms of food intake (Oh-I *et al.* 2006). For the biological action, the mid-segment of nesfatin-1, which corresponds to AA 24–53 of NUCB2 and nesfatin-1, is of particular relevance (Shimizu *et al.* 2009a, Stengel *et al.* 2012, Prinz *et al.* 2015).

On the cellular level, at least in the PVN, nesfatin-1 is located mainly in secretory vesicles in perikarya near the Golgi apparatus, but not in axon terminals, suggesting

a dendritic release and thus eventually autocrine or paracrine actions (Maejima *et al.* 2009). Likewise, in the periphery, NUCB2/nesfatin-1 immunoreactivity was detected in intracellular vesicles of gastric oxyntic mucosa and endocrine cells of pancreatic islets and the anterior pituitary (Stengel *et al.* 2009b).

As mentioned previously, NUCB2 is expressed both in the CNS as well as in peripheral tissues, and it has been shown that nesfatin-1 can also cross the blood–brain barrier (BBB) by non-saturable transmembrane diffusion, consistent with its low lipophilicity (Pan *et al.* 2007, Price *et al.* 2007). This finding suggests that peripheral nesfatin-1 (either endogenous or exogenous) may access the CNS to exert biological actions.

Of note, many publications state that nesfatin-1 was detected by antibody (Ab)-based methods (e.g. immunohistochemistry, immunoassays). However, in most cases, this statement is not justified, since with rare exceptions (e.g. Oh-I *et al.* 2006, Celik *et al.* 2013), the assays used could not distinguish between proteolytically cleaved nesfatin-1 and full-length NUCB2. Only in some studies, western blots were performed to distinguish between NUCB2 and nesfatin-1 based on the molecular weight (e.g. Stengel *et al.* 2009b, Kim *et al.* 2014). Throughout this review, the term ‘NUCB2/nesfatin-1’ is used whenever one or the other was not positively identified.

Nesfatin-1: signal transduction

Although the putative NUCB2/nesfatin-1 receptor has not yet been identified, specific binding sites for nesfatin-1 were detected both in the CNS (e.g. hypothalamus, cortex) and peripheral organs (e.g. gastrointestinal system, pituitary, pancreas) (Ishida *et al.* 2012, Prinz *et al.* 2016).

Some studies have investigated intracellular signaling events of nesfatin-1 not only in various cell types, e.g. in hypothalamic, *nucleus ambiguus* or dorsal root ganglia (DRG) neurons or neuronal cell lines but also in pancreatic β -cells and cardiac myocytes.

As a universal principle, in most cell types, nesfatin-1 stimulates Ca^{2+} influx either through L- (Brailoiu *et al.* 2007, Nakata *et al.* 2011, Ishida *et al.* 2012), P/Q- (Brailoiu *et al.* 2007, 2013) or N- (Iwasaki *et al.* 2009) type Ca^{2+} channels. Both neuronal depolarization (Brailoiu *et al.* 2013) as well as the increase in intracellular Ca^{2+} (Brailoiu *et al.* 2007, 2013, Ozcan *et al.* 2016) were prevented by pretreatment with pertussis toxin, indicating the involvement of a G_i -protein-coupled receptor. Accordingly, no increase

in cyclic adenosine monophosphate (cAMP) formation was observed (Ishida *et al.* 2012). Nevertheless, CREB phosphorylation was increased in a neuroblastoma cell line (Ishida *et al.* 2012), however, not *in vivo* (Tanida *et al.* 2015). The activation of protein kinase A (PKA) appears to differ across tissues and might indicate a tissue-specific recruitment of G-protein subunits (Brailoiu *et al.* 2007, Nakata *et al.* 2011, Ishida *et al.* 2012). In addition to these established G-protein-related pathways, in cardiac myocytes and DRG neurons, nesfatin-1 acts to inhibit L-type Ca²⁺ channels through protein kinase C (PKC), indicating an involvement of G_q (Ying *et al.* 2015, Ozcan *et al.* 2016). These findings, albeit unequivocal with regard to the type of G-proteins involved, suggest the existence of one or more 7-transmembrane receptor(s). Furthermore, mitogen-activated protein kinases (MAPK) (extracellular signal-regulated kinase (ERK1/2)) (Ishida *et al.* 2012, Angelone *et al.* 2013, Tanida *et al.* 2015) is activated by nesfatin-1; 5' AMP-activated protein kinase (AMPK) and phosphatidylinositol-3-kinases (PI3K), however, do not appear to be involved (Tanida *et al.* 2015).

The current findings on intracellular signaling events have to be interpreted with caution as they might not be directly linked to a putative NUCB2/nesfatin-1 receptor. Some of the observed intracellular consequences, e.g. the nesfatin-1-induced activation of a cAMP response element (Cre)-reporter in a transfected neuroblastoma cell line (Ishida *et al.* 2012) and the suppression of cardiac L-type Ca²⁺ channels (Ying *et al.* 2015), are attenuated by the melanocortin receptor (MC) 3/4 antagonist SHU9119 (Ishida *et al.* 2012, Ying *et al.* 2015). Provided that nesfatin-1 is not directly acting at the MC3/4 (Oh-I *et al.* 2006), this could indicate that some observations are not signaling events of a putative NUCB2/nesfatin-1 receptor. They could rather result from intracellular signaling cascades of receptors downstream the alleged NUCB2/nesfatin-1 receptor, e.g. the MC3 or 4.

Genetics

Different polymorphisms of the *Nucb2* gene have been associated with body weight in correlation studies. Based on the frequency of these polymorphisms in lean vs obese subjects some of them were implied to promote obesity (Zegers *et al.* 2012), whereas others rather seem to protect from it (Zegers *et al.* 2011). Specifically, the 1012C>G polymorphism reduces the susceptibility for the development of an obese phenotype. The GG genotype is more frequent in healthy, lean individuals than in obese children (Chen *et al.* 2013) and patients suffering

from the metabolic syndrome (Wang *et al.* 2016), and its presence in the latter patients is associated with lower fasting glucose levels (Wang *et al.* 2016). These findings in metabolic syndrome patients are complemented by the negative association of this polymorphism with blood pressure (Tragante *et al.* 2014).

Energy homeostasis

Homeostatic regulation of food and water intake

Oh-I *et al.* (2006) were the first to demonstrate that both nesfatin-1 and its precursor NUCB2 possess anorexigenic properties. A third ventricle injection of either NUCB2 or nesfatin-1 at similar doses reduces food intake in *ad libitum* fed rats during the dark phase in Oh-I *et al.* (2006). The application of the two other putative cleavage products of NUCB2, nesfatin-2 and/or nesfatin-3 was not effective. Moreover, the anorexigenic effect of nesfatin-1 is independent from leptin because it is still present in leptin-resistant Zucker rats (Brunner *et al.* 1997, Oh-I *et al.* 2006). Conversely, third ventricle acute administration of a nesfatin-1 Ab as well as daily injection of a *Nucb2* antisense oligonucleotide over 10 days increase food intake, thus suggesting that endogenous levels of NUCB2/nesfatin-1 play a role in regulating feeding behavior (Oh-I *et al.* 2006).

Nesfatin-1's anorexigenic effect was confirmed in further studies where nesfatin-1 was injected centrally in *ad libitum* fed rats and mice (Maejima *et al.* 2009, Stengel *et al.* 2009a, Yosten & Samson 2009, 2010, Goebel *et al.* 2011, Könczöl *et al.* 2012, Gotoh *et al.* 2013) and fasted animals (Yosten & Samson 2009, Atsuchi *et al.* 2010, Wernecke *et al.* 2014). Only in one study nesfatin-1 failed to suppress light-phase food intake in fasted animals when given into the cisterna magna (Stengel *et al.* 2009a). When given intranasally, nesfatin-1 reduces cumulative food intake (Shimizu *et al.* 2009b), similar to leptin (Schulz *et al.* 2004, 2012, Fliedner *et al.* 2006), most likely by directly accessing the brain and thus bypassing the BBB. As *Nucb2* mRNA and protein are expressed in the periphery, the effects of its putative cleavage product nesfatin-1 (and segments thereof) in suppressing food intake after peripheral administration were also studied. Intraperitoneal (i.p.) acute administration of nesfatin-1 midsegment decreased food intake in both lean and *db/db* leptin-resistant obese mice (Shimizu *et al.* 2009a), thus suggesting that also peripheral nesfatin-1 induces anorexia in a leptin-independent manner. Peripheral

nesfatin-1 can potentially affect central nervous food intake regulation either via direct access to the brain, vagal afferents or via endocrine messengers, e.g. cholecystokinin (CCK). How and to which extent these different means of signaling are of physiological relevance, remains to be elucidated.

Although endogenous NUCB2/nesfatin-1 itself regulates feeding behavior, its expression can also be regulated by the feeding state. In rodents, *Nucb2* mRNA and NUCB2/nesfatin-1 protein levels were found to be reduced by fasting, and normalized by refeeding, both centrally in the supraoptic nucleus (SON) and PVN (Oh-I *et al.* 2006, Kohno *et al.* 2008, Garcia-Galiano *et al.* 2010) as well as peripherally in subcutaneous adipose tissue (Ramanjaneya *et al.* 2010) and plasma (Stengel *et al.* 2009a). Fittingly, mice fed a high-fat diet for 12 or 20 weeks show an increase in NUCB2 protein expression in subcutaneous adipose tissue (Ramanjaneya *et al.* 2010). In addition, an acute gavage with a high fat bolus also increased serum NUCB2/nesfatin-1 levels in mice (Mohan *et al.* 2014). Finally, peripheral and central acute injection of nesfatin-1 midsegment reduced food intake in mice fed a high-fat diet (Shimizu *et al.* 2009a, Prinz *et al.* 2015), whereas leptin did not (Shimizu *et al.* 2009a), indicating that long term exposure to high-fat diet did not cause 'nesfatin-1 resistance' analogous to the well-described leptin-resistance (Crujeiras *et al.* 2015). Thus, nesfatin-1 could represent a valid alternative in the treatment of metabolic diseases even in the state of leptin resistance.

As mentioned, nesfatin-1 exerts its anorexigenic functions in a leptin-independent manner. Rather, nesfatin-1 signaling seems to be important in mediating leptin-induced anorexia. Recently, it was shown that leptin increases *Nucb2* mRNA expression in the PVN both *in vitro* and *in vivo* (Darambazar *et al.* 2015). In agreement with this, in PVN NUCB2-knockdown mice, both peripheral and central administration of leptin failed to induce anorexia (Darambazar *et al.* 2015). Moreover, central co-administration of leptin and nesfatin-1 did not yield larger effects on energy expenditure than nesfatin-1 or leptin alone, possibly suggesting common downstream signaling mechanisms (Wernecke *et al.* 2014). However, when endogenous nesfatin-1 was blocked by the administration of an Ab, leptin was still capable to induce anorexia (Oh-I *et al.* 2006). Further studies are needed to clarify the functional relationship between these two adipokines.

The nesfatinergic system was shown to interact with a number of systems known to regulate feeding

behavior. For instance, the anorexigenic effect induced by nesfatin-1 is blocked by the central administration of the MC3/4 receptor antagonist SHU9119 (Oh-I *et al.* 2006, Yosten & Samson 2009). Contrary to peripheral leptin actions (Huo *et al.* 2006, Perello *et al.* 2007), central or peripheral administration of nesfatin-1 or its midsegment increased proopiomelanocortin (*POMC*) and cocaine- and amphetamine-related transcript (*CART*) mRNA expression in the nucleus of the solitary tract (NTS), but not at the hypothalamic level in rats (Oh-I *et al.* 2006, Shimizu *et al.* 2009a, Wernecke *et al.* 2014). The interaction between these two systems is further supported by the finding that α -melanocyte-stimulating hormone (α -MSH) administration increased *Nucb2* mRNA expression (Oh-I *et al.* 2006) and activated NUCB2/nesfatin-1 neurons (Sedbazar *et al.* 2014) in the PVN.

The anorexigenic effect of nesfatin-1 seems to be, at least in part, mediated by oxytocinergic neurons. In fact, nesfatin-1 was demonstrated to be co-localized with oxytocin within the SON and PVN (Foo *et al.* 2008, Kohno *et al.* 2008). The excitability of oxytocinergic neurons of the PVN was influenced by nesfatin-1 *in vitro* (Price *et al.* 2008a). Strikingly, endogenous nesfatin-1 was shown to alter oxytocin release in PVN slices (Maejima *et al.* 2009). However, central administration of nesfatin-1 did not affect plasma basal levels of oxytocin in rats (Yosten & Samson 2010), indicating that axonal oxytocin release from magnocellular PVN and/or SON neurons was unaffected. The reduction of cumulative food intake induced by central nesfatin-1 was blocked by the coadministration of a selective antagonist for the oxytocin receptor (H4928) (Maejima *et al.* 2009, Yosten & Samson 2010). When injected site specifically into the PVN, nesfatin-1 suppressed feeding by the stimulation of the oxytocin system, together with an increased in *c-fos* expression in the NTS, suggesting that oxytocinergic neurons projecting from PVN to the NTS are modulated by nesfatin-1 to induce anorexia (Maejima *et al.* 2009).

The nesfatinergic system was also found to interact with the corticotropin-releasing factor (CRF)/CRF₂ receptor system to modulate feeding behavior (see below). Co-localization of nesfatin-1 with CRF within the PVN was observed (Foo *et al.* 2008, Kohno *et al.* 2008). In agreement, nesfatin-1 was shown to influence the excitability of *Crf*-expressing neurons *in vitro* (Price *et al.* 2008a) as well as to increase CRF protein levels (Gotoh *et al.* 2013) in the PVN. The administration of a specific CRF₂ receptor antagonist (astressin₂-B) injected into the forebrain, but not into the hindbrain, fully abolished the anorexigenic effect of nesfatin-1 (Stengel *et al.* 2009a).

NUCB2/nesfatin-1 were shown to be expressed and co-localized with the orexigenic peptide neuropeptide Y (NPY) in the arcuate nucleus (ARC) (Oh-I *et al.* 2006, Brailoiu *et al.* 2007, Inhoff *et al.* 2010). An *in vitro* electrophysiological study demonstrated that the majority of Npy-expressing neurons in the ARC was hyperpolarized by nesfatin-1 (Price *et al.* 2008b), indicating that nesfatin-1 could suppress food intake by downregulating NPY signaling. In agreement, rats administered *i.c.v.* with nesfatin-1 showed decreased *Npy* mRNA expression in the hypothalamus and NTS (Wernecke *et al.* 2014). Also, NUCB2/nesfatin-1 neurons of the PVN were inhibited by NPY *in vitro* (Sedbazar *et al.* 2014), confirming that nesfatin-1 and NPY interact within the PVN-ARC-feeding center. Lastly, it was also found that the orexigenic effect of peripheral ghrelin was blocked by desacyl ghrelin via activation of NUCB2/nesfatin-1 neurons of the ARC (Inhoff *et al.* 2008).

In one study the interaction between nesfatin-1 and the serotonergic system was addressed. The serotonin 5HT_{1B/2C} receptor agonist, m-chlorophenylpiperazine, induced anorexia in a leptin-independent manner by upregulating *Nucb2* expression in the hypothalamus (Nonogaki *et al.* 2008). Such effects were blunted in 5HT_{2C} receptor mutant mice (Nonogaki *et al.* 2008), suggesting that activation of 5HT_{2C} receptors might play a role in regulating *Nucb2* expression and, in turn, feeding behavior. Pharmacological treatment with olanzapine, a neuroleptic, which acts as an inverse agonist on the 5HT_{2C} receptor, was shown to decrease NUCB2/nesfatin-1 expression in hypothalamic feeding-related areas of rats, leading the authors to speculate that olanzapine could enhance food intake and in turn body weight gain partly by downregulating NUCB2/nesfatin-1 expression (Rojczyk *et al.* 2015). Finally, in another study, the involvement of histamine and thyrotropin-releasing hormone (TRH) in mediating nesfatin-1's effects was investigated (Gotoh *et al.* 2013). Histamine is known to potently reduce appetite and (Masaki *et al.* 2004) and NUCB2/nesfatin-1 was found to be expressed in the tuberal hypothalamic area (THA) (Fort *et al.* 2008) where histaminergic neurons are exclusively localized (Giannoni *et al.* 2009). It was found that central administration of histamine increased NUCB2/nesfatin-1 peptide expression in the PVN and in turn, central administration of nesfatin-1 increased the turnover of histamine in the hypothalamus. Moreover, the anorexigenic effect of nesfatin-1 was reduced in animals with disrupted histamine signaling (Gotoh *et al.* 2013). Finally, administration of nesfatin-1 resulted in an increase in *TRH* mRNA expression in the PVN and the

anorexigenic effect of nesfatin-1 was attenuated when an Ab against *TRH* was co-administered (Gotoh *et al.* 2013).

Nesfatin-1 was also shown to alter meal pattern. In mice, central administration of nesfatin-1 reduced meal size and increased inter-meal intervals indicating both satiety (meal termination) and satiation (delayed meal initiation) (Goebel *et al.* 2011), whereas central injection of nesfatin-1 midsegment induced satiety without affecting satiation (Stengel *et al.* 2012). Interestingly, in rats fed normal chow, central nesfatin-1 midsegment induced satiation, whereas satiety was induced in rats with diet-induced obesity (Prinz *et al.* 2015). As suggested by the authors, these mixed results could be explained by differences in species, receptor-binding affinity between full-length nesfatin-1 vs its midsegment and/or the activation of different signaling pathways depending on dietary conditions (Prinz *et al.* 2015). In a recent study, treatment with nesfatin-1 resulted in the upregulation of *Cck* (a satiation peptide) and in the downregulation of peptide *Yy* (PYY, a satiety peptide) mRNA and protein levels both *in vitro* and *in vivo* (Ramesh *et al.* 2016).

For a comprehensive overview of nesfatin-1's effects on homeostatic food intake regulation, please refer to Table 1.

Nesfatin-1 was shown to be involved not only in feeding behavior but also in body fluid regulation (Yosten & Samson 2009, 2010, Könczöl *et al.* 2012, Yosten *et al.* 2012, Yoshimura *et al.* 2014). This was first demonstrated by Yosten and Samson (2009) who showed that nesfatin-1 reduces water intake when injected *i.c.v.*, an effect mediated by MC3/4 and oxytocin receptors (Yosten & Samson 2009, 2010). Interestingly, nesfatin-1's antidipsogenic effect had an earlier onset with respect to the anorexigenic effect (60 min vs 150 min) (Yosten & Samson 2009), raising the possibility that the reduction of food intake might be a consequence of the reduced fluid intake. In addition, reduction of intake elicited by nesfatin-1 was more pronounced in water than in food (70% vs 50%, respectively) (Yosten & Samson 2009).

Intracerebroventricular nesfatin-1 dose-dependently attenuates the water drinking response to angiotensin II, to overnight fluid restriction, and to hypertonic saline (Yosten *et al.* 2012). Consistent with other data (Oh-I *et al.* 2006), a 2-day treatment with antisense morpholino oligonucleotide against *Nucb2* gene resulted in a reduction of NUCB2/nesfatin-1 in the PVN without altering water intake (Yosten *et al.* 2012); however, drinking response upon angiotensin II administration was exaggerated (Yosten *et al.* 2012).

Table 1 Summary of nesfatin-1's actions in rodents: food intake. Unless otherwise specified, synthetic nesfatin-1 (AA 1–82) from Phoenix Pharmaceuticals (Burlingame, CA, USA) was administered.

Species	Nesfatin-1 dose	Procedure	Injection site	Observed outcome	<i>Mucb2</i> mRNA or NUCB2/nesfatin-1 protein	c-fos expression	Reference
Rat	5, 50 pmol		i.v., 4.v., i.c. i.c.	↓ DP food intake ↔ LP food intake (fasted)			Stengel <i>et al.</i> (2009a,b)
Rat	20, 200 pmol		4.v.	↓ DP food intake			Xia <i>et al.</i> (2012)
Rat	500 pmol		i.v.	↓ dopamine in NAcc			Chen <i>et al.</i> (2015)
Rat	5, 25 pmol		3.v.	↓ DP food intake			Oh-I <i>et al.</i> (2006)
Rat	5 pmol (x10 days) (Yanaihara institute; synthetic)		3.v.	↓ DP food intake			
Rat	3 pmol (Sigma; recombinant)		i.v.	↑ CRF in PVN			Gotoh <i>et al.</i> (2013)
Rat	25, 100 pmol		i.v.	↑ TRH in PVN			Könczöl <i>et al.</i> (2012)
Rat	100 pmol		3.v.	↓ DP and LP food intake			Maejima <i>et al.</i> (2009)
Rat	50 pmol		PVN	↓ DP food intake		↑ PVN and NTS	
Rat	5, 50 pmol		i.v., 4.v., i.c.	↓ DP food intake		↑ NTS	Stengel <i>et al.</i> (2009a,b)
Rat	60 pmol		i.v.	↓ DP food intake			Yosten and Samson (2009, 2010)
Rat	180 pmol		i.v.	↓ LP food intake (fasted)			
Rat	25 pmol		i.v.	↓ LP food intake (fasted)	↔ hypothalamus		Wernecke <i>et al.</i> (2014)
Rat	10,000 pmol (not specified)		i.n.	↓ food intake			Shimizu <i>et al.</i> (2009a,b)
Rat	900 pmol		i.v.	↓ DP food intake, ↑ satiation			Prinz <i>et al.</i> (2015)
Rat	8, 24, 73 pmol/g BW (Bachem; synthetic midsegment)		i.p.	↔ DP food intake			
Rat	0.44 pmol/g BW/h (x18 h)		s.c.	↓ DP food intake, ↑ satiety			Gonzalez <i>et al.</i> (2011)
Rat	50 pmol		PVN, LHA VMH	↓ DP food intake ↔ DP food intake ↑ food intake			Chen <i>et al.</i> (2012)
Rat		NUCB2 knockdown (hypothalamus)	i.v.	↓ DP food intake			Wu <i>et al.</i> (2014)
Mouse	300 pmol (Abgent; synthetic)		i.v.	↓ DP food intake			Goebel <i>et al.</i> (2011)
Mouse	300, 900 pmol		i.v.	↑ satiation and satiety ↓ DP food intake ↑ satiety			Stengel <i>et al.</i> (2012)
Mouse	1000 pmol (synthetic midsegment)		i.v.	↓ LP food intake (fasted)			Atsuchi <i>et al.</i> (2010)
Mouse	0.5 pmol (Sigma; recombinant)		i.v.	↓ DP food intake			Gotoh <i>et al.</i> (2013)

Mouse	100 pmol (Yanaihara institute)	3.v.	↓ DP food intake	Maejima <i>et al.</i> (2009)
Mouse	2, 20 pmol	VTA	↓ DP food intake	Chen <i>et al.</i> (2015)
Mouse	250, 1250 pmol/g BW (recombinant)	i.p.	↓ DP food intake	Shimizu <i>et al.</i> (2009a,b)
	250, 500, 1250 pmol/g BW (synthetic midsegment)		↓ DP food intake	
			↑ NTS	

3.v., third ventricle; 4.v., fourth ventricle; AA, amino acid; ACTH, adrenocorticotrophic hormone; ARC, arcuate nucleus; BAT, brown adipose tissue; BMA, basomedial amygdala; BP, blood pressure; CeA, central amygdala; DR, dorsal raphe; CRF, corticotropin releasing factor; CORT, corticosterone; DMNX, dorsal motor nucleus of the vagus; DP, dark phase; EE, energy expenditure; GD, gastricuodenal; GE, gastric emptying; GM, gastric motility; HR, heart rate; i.c., intracisterna magna; i.p., intraperitoneal; ITT, insulin tolerance test; i.v., intravenous; l.v., lateral ventricle; LC, locus coeruleus; LP, light phase; LHA, lateral hypothalamus area; MAP, mean arterial pressure; MR, medial raphe; NAcc, nucleus accumbens; NTS, nucleus of the solitary tract; OGTT, oral glucose tolerance test; PVN, paraventricular nucleus of hypothalamus; RP, raphe pallidus; s.c., subcutaneous; SFO, subfornical organ; SNA, sympathetic nerve activity; SON, supraoptic nucleus; STZ, streptozotocin; TRH, thyrotropin releasing hormone; UCP1, uncoupling protein 1; VLM, ventrolateral medulla; VMH, ventromedial hypothalamus; VTA, ventrolateral medulla; WAT, white adipose tissue.

Nucb2 mRNA is expressed in the subfornical organ (SFO), an area involved in body fluid regulation (Hindmarch *et al.* 2008, Kuksis & Ferguson 2014). An *in vitro* electrophysiological study demonstrated the capability of SFO neurons to respond to nesfatin-1 (Kuksis & Ferguson 2014). Consistently, i.c.v. nesfatin-1 administration increases c-fos immunoreactivity in the SFO as well as in the PVN and central nucleus of the amygdala (CeA). As a functional correlate, nesfatin-1 injected into the SFO increases water intake independent from the angiotensin system; however, this was only observed in the absence of food (Moreau & Ciriello 2013).

Prolonged water deprivation increases *Nucb2* mRNA and NUCB2/nesfatin-1 protein levels in the SFO as well as in the SON and PVN (Yoshimura *et al.* 2014), which are normalized upon rehydration (Yoshimura *et al.* 2014); in contrast, water deprivation reduces *Nucb2* mRNA levels in the total hypothalamus (Yosten *et al.* 2012), thus suggesting that water deprivation might have decreased *Nucb2* mRNA levels in other hypothalamic nuclei than the former.

A summary of nesfatin-1's effects on water intake is given in the [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article.

Hedonic regulation of food intake

A growing body of evidence suggests that nesfatin-1 might be involved not only in regulating homeostatic feeding but also in the reward-related aspects of feeding behavior. In fact, NUCB2/nesfatin-1 protein is expressed in reward-related areas such as amygdaloid nucleus, *nucleus accumbens* (NAcc), lateral hypothalamus and dorsal raphe nuclei (DR) (Goebel-Stengel *et al.* 2011). In an *in vitro* study, Li *et al.* (2014) demonstrated nesfatin-1's capability to hyperpolarize dopaminergic neurons of the *substantia nigra*, a brain area involved not only in sensorimotor functions but also in reward and aversion (Ilango *et al.* 2014). Furthermore, nesfatin-1 decreased the resting membrane potential and firing rate of dopaminergic neurons most likely by a direct mechanism (Li *et al.* 2014). Dopaminergic neurons originating from the ventral tegmental area (VTA) innervate the NAcc and release dopamine in response to rewarding (or aversive) stimuli. Interestingly, i.c.v. nesfatin-1 decreases dopamine release in the NAcc (Chen *et al.* 2015). Furthermore, when nesfatin-1 was injected directly into the VTA, the reduction in chow food intake arises at an earlier onset than when it is injected i.c.v. in mice (1 h vs 3 h; respectively)

(Goebel *et al.* 2011, Stengel *et al.* 2012, Chen *et al.* 2015). However, how and to which extent nesfatin-1 is involved in the modulation of 'wanting', 'liking', and/or other reward-related behaviors is currently unknown.

Whether nesfatin-1 acts directly or also recruits other neurotransmitter systems to modulate reward pathways is yet to be elucidated. One possibility is that NUCB2/nesfatin-1 neurons interact with melanocortin and oxytocin systems, which are mediating nesfatin-1's effects on homeostatic food intake (Oh-I *et al.* 2006, Maejima *et al.* 2009, Yosten & Samson 2009, 2010). In support of this idea, melanocortins and oxytocin were shown to regulate not only homeostatic feeding (Saper *et al.* 2002, Sabatier *et al.* 2013) but also play a role in the modulation of hedonic pathways. Recently, it was shown that motivation to obtain sucrose in rats is downregulated by the activation of MC3 within reward-related areas (Pandit *et al.* 2015, 2016). Similarly, oxytocin reduces methamphetamine-induced seeking behavior in rodents (Cox *et al.* 2013) and reward-driven food intake in humans (Ott *et al.* 2013).

A second possible scenario might be the interaction between the nesfatin-1 and ghrelin systems. Ghrelin and nesfatin-1 are co-expressed in X/A endocrine cells of the gastric mucosa (Stengel & Taché 2009, Stengel *et al.* 2009b). Ghrelin regulates homeostatic feeding by increasing food intake in both rodents and humans (Wren *et al.* 2000, 2001, Gil-Campos *et al.* 2006). An interaction of nesfatin-1 and ghrelin is also supported by data showing their co-localization in goldfish hypothalamus (Kerbel & Unniappan 2012). Moreover, i.c.v. nesfatin-1 suppresses food intake and downregulates preproghrelin and ghrelin receptor mRNA expression in the forebrain of fed fish (Kerbel & Unniappan 2012). In a similar fashion, i.c.v. ghrelin promotes food intake and downregulates *Nucb2* mRNA expression in the forebrain (Kerbel & Unniappan 2012). Accordingly, lipopolysaccharide-induced acute inflammation in rats increased plasma NUCB2/nesfatin-1 levels (Stengel *et al.* 2011), whereas those of acyl and desacyl ghrelin were decreased in conjunction with reduced food intake (Stengel *et al.* 2010), thus suggesting that NUCB2/nesfatin-1 and ghrelin expression might be regulated differentially. As ghrelin participates in regulating hedonic feeding (Egecioglu *et al.* 2010, Skibicka *et al.* 2012), it can be speculated that the two peptides might also interact within reward-related areas.

A summary of nesfatin-1's effects on the hedonic aspects of food intake is given in Table 1.

Gastric distension and gastric acid secretion

NUCB2/nesfatin-1 was not only described as a direct mediator of central nervous regulation of food intake but also as a regulator of gastrointestinal functions, e.g. by slowing down gastric emptying. The latter in turn supports nesfatin-1's central nervous anorexigenic actions by eliciting peripheral satiety signals.

I.c.v. injection of an anorexigenic dose of nesfatin-1 decreased gastric emptying in fasted rats (Stengel *et al.* 2009a) and mice (Goebel-Stengel *et al.* 2011). Additionally, nesfatin-1's involvement in regulating gastroduodenal motility was also shown in mice after a central administration of nesfatin-1's midsegment (Atsuchi *et al.* 2010). Although it was shown previously that the CRF₂ receptor is involved in the regulation of gastric motility (Czimmer *et al.* 2006), nesfatin-1 is recruiting other pathways than this (Stengel *et al.* 2009a).

The brain areas that are thought to be responsible for nesfatin-1's effects on gastrointestinal functions are the PVN, ARC, CeA and basomedial amygdala (BMA). When administered into the PVN, nesfatin-1 dose-dependently decreased gastric motility and emptying, an effect mediated by oxytocinergic neurons (Guo *et al.* 2015). Similarly, when injected into the ARC, CeA or BMA, nesfatin-1 decreased gastric motility and emptying by exploiting the melanocortin system (Li *et al.* 2013b, Wang *et al.* 2014, Xu *et al.* 2015a). Strikingly, the endogenous level of NUCB2/nesfatin-1 seems to be important in the physiological regulation of gastric functions as anti-NUCB2/nesfatin-1 Ab application is also capable to alter the activity of PVN, ARC, CeA and BMA gastric distension-sensitive neurons (Li *et al.* 2013b, Wang *et al.* 2014, Guo *et al.* 2015, Xu *et al.* 2015a).

Gastric distension (e.g. a consequence of reduced gastric emptying, gastric and duodenal motility) might be relayed to the CNS to induce satiety through CCK. Interestingly, the peripheral injection of CCK increased c-fos expression in NUCB2/nesfatin-1 neurons of the SON, PVN, NTS and the area postrema (Noetzel *et al.* 2009, Stengel *et al.* 2009a, Saito *et al.* 2016). Conversely, nesfatin-1 at an anorexigenic dose increased *Cck* mRNA expression in the hypothalamus of fed and unfed goldfish (Kerbel & Unniappan 2012). This positive feedback indicates that the two peptides act in concert to suppress food intake.

In addition to this mechanism, experimental data also support the existence of a neuronal connection between the gastrointestinal system and central nervous NUCB2/nesfatin-1 neurons. NUCB2/nesfatin-1 neurons

of the NTS, which receive gastric and intestinal afferents inputs via the vagus nerve, were activated after gastric distension in rats (Bonnet *et al.* 2013). In addition, oral administration of metformin reduced food intake and gastric emptying in mice, in conjunction with the activation of NUCB2/nesfatin-1 neurons in the NTS and dorsal nucleus of the vagus nerve (DMNX) (Rouquet *et al.* 2014).

Gastric acid secretion was also found to be affected by nesfatin-1. Particularly, a central injection of an anorexigenic dose of nesfatin-1 is also capable to inhibit the 2-deoxy-D-glucose-stimulated gastric acid secretion via vagal efferents in rats, as suggested by a 16-fold increase of c-fos-positive neurons in the DMNX, but not in the NTS (Xia *et al.* 2012). In agreement, 80% of DMNX neurons projecting to the stomach are nesfatin-1 positive (as was shown by retrograde tracing) (Bonnet *et al.* 2013).

Thus, nesfatin-1 appears to contribute to the regulation of food intake by directly and/or indirectly affecting the integration of input and output signals between gut and brain.

An overview of nesfatin-1's effects on gastric distension and gastric acid secretion is provided in the [Supplementary Table 1](#).

Thermogenesis

In contrast to nesfatin-1's role in food intake regulation, its participation in the regulation of energy expenditure, the second aspect contributing to whole body energy homeostasis, is less well investigated and understood. Könczöl and coworkers provided the first evidence for a function of nesfatin-1 in energy expenditure by reporting an increase in core body temperature after its i.c.v. administration (Könczöl *et al.* 2012). This finding was substantiated in a study using direct calorimetry to quantify dry heat loss as a measure for thermogenesis and thus energy expenditure (Wernecke *et al.* 2014). In this study, i.c.v. application of nesfatin-1 increased dry heat loss of rats over eight hours to 8.49 ± 1.09 W/kg^{0.75} compared to 7.09 ± 0.84 W/kg^{0.75} in control animals (Wernecke *et al.* 2014). Thereby other structures than the PVN are involved because NUCB2 knockdown in this nucleus does not affect energy expenditure (Nakata *et al.* 2016).

In contrast to the findings on acute i.c.v. administration of nesfatin-1, subcutaneous (s.c.) osmotic minipump infusion for 7 days elicited a decrease of oxygen consumption and energy expenditure in the

light phase, but no alteration in the dark phase in freely feeding rats (Mortazavi *et al.* 2015). In a short term experiment (1 day), oxygen consumption was reduced in the dark phase; this was, however, not reflected in energy expenditure calculations (Mortazavi *et al.* 2015). In the light phase, no alterations were observed (Mortazavi *et al.* 2015).

The underlying central nervous and peripheral mechanisms of nesfatin-1's impact on thermogenesis remain to be elucidated.

Nesfatin-1's effects on thermogenesis are summarized in [Table 2](#).

Glucose homeostasis

Outside the CNS, *Nucb2* mRNA was detected not only in adipose tissue but also in rodent and human pancreatic islets (Riva *et al.* 2011). The distribution of mRNA expression is matched by the occurrence of NUCB2/nesfatin-1 protein (Gonzalez *et al.* 2009, Stengel *et al.* 2009b, Foo *et al.* 2010, Riva *et al.* 2011), which is colocalized almost exclusively with insulin in the β -cells of pancreatic islets (Gonzalez *et al.* 2009, Foo *et al.* 2010, Riva *et al.* 2011). In fact, NUCB2/nesfatin-1-immunoreactivity is present in all or almost all β -cells (Foo *et al.* 2010, Riva *et al.* 2011), with a subcellular distribution distinct from insulin immunoreactivity (Foo *et al.* 2010). Colocalization of NUCB2/nesfatin-1 with PC1/3 and PC2 suggests that processing into nesfatin-1 may take place physiologically in pancreatic islets (Mohan *et al.* 2016).

In the pancreatic β -cells, intracellular *NUCB2* mRNA and/or NUCB2/nesfatin-1 protein synthesis or release are dynamically regulated by glucose levels: *In vitro*, *Nucb2* mRNA is upregulated in human islets by glucolipotoxic conditions (high glucose and palmitate) and release can be triggered by glucose stimulation in rat islets (Foo *et al.* 2010). This regulation seems to be impaired in glycemic diseases; e.g. in streptozotocin diabetic mice, *Nucb2* mRNA and NUCB2/nesfatin-1 protein expression are significantly reduced in the pancreatic islets (Gonzalez *et al.* 2011b). This was confirmed in the islets of Goto-Kakizaki (GK) rats, a model of type 2 diabetes. However, the opposite was observed in mice with diet-induced obesity and as a consequence type 2 diabetes (Gonzalez *et al.* 2011b). Finally, islets from patients with type 2 diabetes exhibited a reduction in *Nucb2* mRNA compared with islets from healthy donors; this correlated significantly with insulin secretion capacity (Riva *et al.* 2011). However, the difference was not that marked and

Table 2 Summary of nesfatin-1's actions in rodents: thermogenesis and glucose homeostasis.

Species	Nesfatin-1 dose	Procedure	Injection site	Observed outcome	Nucb2 mRNA or NUCB2/nesfatin-1 protein	c-fos expression	Reference
Thermogenesis							
Rat	25, 100 pmol		i.v.	↑ core body temperature			Könczöl et al. (2012)
Rat	25 pmol		i.v.	↑ thermogenesis			Wernecke et al. (2014)
Rat	5 pmol/g BW (x1 day)		s.c.	↔ EE			Mortazavi et al. (2015)
	35 pmol/g BW (x7 days)		s.c.	↓ EE			
	5 pmol/g BW (Abgent; synthetic)		i.p.	↔ EE			
Rat	0.44 pmol/g BW (x18 h)		s.c.	↔ UCP1 (BAT and WAT)			Gonzalez et al. (2011)
Glucose homeostasis							
Rat	25 pmol/h (x6 h)		3.v.	↓ glucose (liver) ↑ insulin sensitivity		↑ PVN, SON, ARC	Yang et al. (2012)
Rat		NUCB2 knockdown (hypothalamus)		↑ glucose uptake (muscles) ↑ glucose (liver)			Wu et al. (2014)
Mouse	2.5 pmol/h (x14 days)	OGTT ITT	s.c.	↓ insulin sensitivity ↓ glucose uptake (muscles, BAT, WAT) ↓ glucose ↓ glucose ↑ insulin ↑ insulin sensitivity			Li et al. (2013a)
Rat	50 pmol	OGTT, ITT	3.v.	↔ glucose			Gonzalez et al. (2011)
Rat	0.44 pmol/g BW/h (x18 h)		s.c.	↑ insulin ↓ glucagon ↔ glucose ↓ glucose			
Mouse		ITT					
<i>wt</i>	10,000 pmol		i.v.	↔ glucose			Su et al. (2010)
<i>db/db</i>	10,000 pmol		i.v.	↓ glucose			
	1,000,000 pmol		i.p.	↔ glucose			
	25 pmol		3.v.	↔ glucose			
STZ	10,000 pmol		i.v.	↔ glucose			
	10,000 pmol (recombinant)	Insulin (s.c.)	i.v.	↓ glucose			

For information on the origin of nesfatin-1 and abbreviations please refer to [Table 1](#).

as the sample size was small, the observations should be treated with some caution (Riva *et al.* 2011). The data on NUCB2/nesfatin-1 in plasma of type 2 diabetic patients are unequivocal as for type 2 patients, both increased (Zhang *et al.* 2012) and decreased (Li *et al.* 2010) levels were reported, whereas plasma NUCB2/nesfatin-1 in type 1 diabetic patients was unchanged compared with healthy individuals (Li *et al.* 2010). It should be noted that plasma NUCB2/nesfatin-1 most likely does not reflect pancreatic production and release but is rather derived from major sources as gastric mucosa (Stengel *et al.* 2009b) and adipose tissue (Ramanjaneya *et al.* 2010). In particular, the variable contribution of the latter might account for the conflicting findings in type 2 diabetic patients in different studies.

A few studies have addressed the effects of glucose challenge on plasma nesfatin-1 levels. Although in rats, in an i.p. glucose tolerance test, NUCB2/nesfatin-1 initially decreased and then returned to basal levels in GK rats and even overshoot in healthy Wistar rats (Foo *et al.* 2010), gavaging with a high-carbohydrate diet was without effect on serum NUCB2/nesfatin-1 in mice (Mohan *et al.* 2014). In the latter, chronic exposure to a high-carbohydrate diet, however, altered the circadian pattern of serum nesfatin-1/NUCB (Mohan *et al.* 2014). In humans, oral glucose administration in healthy humans did not significantly affect plasma NUCB2/nesfatin-1 levels (Li *et al.* 2010).

Although glucose levels have an impact on *Nucb2* mRNA and NUCB2/nesfatin-1 protein release in pancreatic islets (Foo *et al.* 2010), on the other hand, nesfatin-1 enhances glucose-induced insulin secretion by promoting Ca²⁺ influx through L-type channels (Nakata *et al.* 2011). This process, unlike nesfatin-1's actions on hypothalamic neurons (Brailoiu *et al.* 2007), is independent of PKA (Nakata *et al.* 2011). Furthermore, phospholipase A₂ (PLA₂), which releases the putative second messenger arachidonic acid, crucial for glucose-stimulated insulin release (Jones & Persaud 1993), is neither involved in nesfatin-1's insulinotropic effects (Nakata *et al.* 2011). Like in other areas, further advances are hampered by the not yet identified putative nesfatin-1 receptor. Of note, insulin release from pancreatic islets is only stimulated by nesfatin-1 in concentrations that exceed the plasma levels of lean subjects (Tsuchiya *et al.* 2010, Nakata *et al.* 2011). However, the local production of nesfatin-1 in the β -cells and its increased secretion by elevated glucose levels (Foo *et al.* 2010) might either intracellularly or in an autocrine and/or paracrine fashion, participate/

facilitate insulin release upon elevated blood glucose independently from circulating NUCB2/nesfatin-1 levels.

In vivo results from rodents are more complex and thus more difficult to interpret: continuous s.c. infusion of nesfatin-1 in rodents improved glucose utilization by enhancing insulin secretion (Gonzalez *et al.* 2011a, Li *et al.* 2013a). At the same time, due to an activation of intracellular insulin signaling, glucose uptake is ameliorated by increasing insulin sensitivity in liver, muscle and adipose tissue of mice (Li *et al.* 2013a). However, another study in rats could only confirm the effects on glucose uptake for the adipose tissue (Gonzalez *et al.* 2011a) without detecting changes in insulin signal transduction (Gonzalez *et al.* 2011a). Also in the myocardium, nesfatin-1 augments insulin receptor signaling to increase glucose uptake by mobilization of the glucose transporter, supposedly due to local nesfatin-1 production, which is dependent on diet and coronary health (Feijoo-Bandin *et al.* 2013). Interestingly, in normoglycemic fasted *db/db* or freely fed wild-type mice (Su *et al.* 2010), blood glucose was not affected by intravenous nesfatin-1, suggesting that nesfatin-1 is able to correct a pathological hyperglycemic state, but is not of relevance in the normoglycemic range.

In the CNS, nesfatin-1's function with regard to glucose metabolism is less well investigated, but it is implied to be involved both in glucose sensing as well as in the control of glucose metabolism. For instance, the excitability of glucose responsive neurons is modulated by nesfatin-1 in the ventromedial and lateral hypothalamus (Chen *et al.* 2012) and the PVN, where about 27% of NUCB2/nesfatin-1 immunoreactive neurons respond to either glucose and/or insulin (Gantulga *et al.* 2012), the latter implying a physiological role for NUCB2/nesfatin-1 in the modulation of glucose sensing. This seems to be specific to hypothalamic nuclei as in the NTS, another important structure for glucose sensing in the brain (Routh 2002), the response of glucose-sensing neurons is not modulated by NUCB2/nesfatin-1, despite its local expression (Mimee & Ferguson 2015).

In both normal chow and high-fat diet fed rats, reduction of central nervous NUCB2/nesfatin-1 availability by adenoviral-mediated RNA interference induced peripheral insulin resistance, leading to an increase in hepatic glucose flux and a decrease in glucose uptake in peripheral tissues (Wu *et al.* 2014).

Fittingly, nesfatin-1 administered i.c.v. acts in the hypothalamus to increase whole-body insulin sensitivity by stimulating insulin receptor signaling in the liver to

inhibit hepatic gluconeogenesis and in the muscle to improve glucose uptake (Yang *et al.* 2012). However, two other studies failed to observe effects of central nesfatin-1 on blood glucose, glucose tolerance and insulin sensitivity (Su *et al.* 2010, Li *et al.* 2013a).

For a compilation of nesfatin-1's effects on glucose homeostasis please refer to Table 2.

Other systems

Cardiovascular actions

Besides its well-described actions on the regulation of energy homeostasis, the central nervous administration of nesfatin-1 exerts cardiovascular effects. For example, it was shown that that i.c.v. administration of nesfatin-1 increases mean arterial pressure (MAP) (Yosten & Samson 2009, 2010, 2014, Tanida & Mori 2011, Tanida *et al.* 2015); heart rate was also found to be increased (Tanida *et al.* 2015); ERK phosphorylation CRF neurons of the PVN is involved in these effects (Tanida *et al.* 2015). Until now, it is not clear whether central nervous nesfatin-1 activates cardiac sympathetic innervation, but it was shown that it increases renal sympathetic nerve activity (Tanida & Mori 2011, Tanida *et al.* 2015), known to be involved in blood pressure regulation through the renin-angiotensin system (Nakamura & Johns 1995).

Electrophysiological studies have identified the median NTS as a potential site through which nesfatin-1 exerts its cardiovascular actions (Mimee *et al.* 2012). *Nucb2* mRNA and NUCB2/nesfatin-1 protein expression was observed in this nucleus (Brailoiu *et al.* 2007, Foo *et al.* 2008) and both central nervous and peripheral administration of nesfatin-1 induce c-fos expression in its neurons (Maejima *et al.* 2009, Shimizu *et al.* 2009a). Local injection of nesfatin-1 into the median NTS increased both blood pressure and heart rate (Mimee *et al.* 2012). In contrast, site-specific injection into the *nucleus ambiguus*, a key site for parasympathetic cardiac control (Mendelowitz 1999), induced bradycardia, although MAP was not affected (Brailoiu *et al.* 2013). The existence of NUCB2/nesfatin-1 immunoreactivity in this nucleus in rodents (Goebel *et al.* 2009a, Goebel-Stengel *et al.* 2011) implies a physiological function of the peptide.

Nesfatin-1 involvement in cardiovascular regulation is not surprising as many neuropeptides involved in conveying nesfatin-1's anorexigenic signal are also known to contribute to the control of cardiovascular function, e.g. the melanocortin system (Cone 2005).

Hyperstimulation of this system may underlie the development of hypertension in several animal models (da Silva *et al.* 2013, Segal-Lieberman & Rosenthal 2013). Consequently, studies aimed at dissecting the downstream mechanisms of nesfatin-1's cardiovascular action focused on these neuropeptides by blocking their respective receptors. In particular, work from the Samson group has clarified that upon i.c.v. administration, nesfatin-1 acts through sequential activation of POMC neurons (Yosten & Samson 2009, Tanida & Mori 2011), followed by oxytocin (Yosten & Samson 2010) and then CRF (Yosten & Samson 2014) neurons to increase blood pressure (Yosten & Samson 2009, 2014). This circuit matches with nesfatin-1's downstream events involved in the regulation of food intake (Yosten & Samson 2014).

In addition to its central nervous actions, intravenous administration of nesfatin-1 possesses a hypertensive effect (Yamawaki *et al.* 2012), potentially through acting both at the central nervous, but also at the peripheral level by modulating arterial resistance (Yamawaki *et al.* 2012). Both NUCB2 and nesfatin-1 protein have been detected in the heart and nesfatin-1 can directly control heart performance *in vitro* (Angelone *et al.* 2013).

The actions of nesfatin-1 on the cardiovascular system are summarized in Table 3.

Anxiety, behavior and depression

A role of nesfatin-1 in regulating anxiety- and depressive-like behaviors has been suggested. As mentioned previously, substantial NUCB2/nesfatin-1 immunoreactivity was detected in brain areas known to be involved in anxiety-like behavior and stress response, such as amygdaloid nuclei, bed nucleus of the stria terminalis, PVN and hippocampus (Brailoiu *et al.* 2007, Fort *et al.* 2008, Goebel *et al.* 2009a, Goebel-Stengel *et al.* 2011). The i.c.v. administration of nesfatin-1 was shown to increase both adrenocorticotrophic hormone (ACTH) and corticosterone plasma levels in rats (Könczöl *et al.* 2010, Yoshida *et al.* 2010), suggesting that nesfatin-1 might play a role in the modulation of the hypothalamus-pituitary-adrenal (HPA) axis activity. Moreover, bilateral adrenalectomy increased *Nucb2* mRNA expression in the PVN, indicating that its expression is regulated by HPA axis activity (Könczöl *et al.* 2010).

In line with these data, central administration of an anorexigenic dose of nesfatin-1-induced anxiety-like behavior in rats tested in the elevated-plus maze (the gold standard test to measure unlearned anxiety response) and

Table 3 Summary of nesfatin-1's actions in rodents: cardiovascular action, anxiety, behavior and depression.

Species	Nesfatin-1 dose	Procedure	Injection site	Observed outcome	Nucb2 mRNA or NUCB2/nesfatin-1 protein	c-fos expression	Reference
Cardiovascular actions							
Rat	60, 180 pmol		i.v.	↑ MAP			Yosten and Samson (2009, 2010, 2014)
Rat	200 pmol		i.v.	↑ SNA (kidneys, liver, WAT) ↑ MAP and HR			Tanida and Mori (2011), Tanida et al. (2015)
	50 pmol (no specified)		PVN	↑ SNA (kidneys) ↑ MAP and HR			
Rat	25 pmol		ARC	↔ SNA (kidneys)			Könczöl et al. (2012)
Rat	0.05 pmol		i.v.	↑ HR			Mimee et al. (2012)
	0.5, 5 pmol (Sigma)		Medial NTS	↑ BP			Brailoiu et al. (2013)
			Nucleus ambiguus	↑ HR ↓ HR ↔ BP			Yamawaki et al. (2012)
Rat	0.65 pmol/g BW		i.v.	↑ BP			
Anxiety, behavior and depression							
Rat	25 pmol		i.v.	↑ ACTH and CORT			Könczöl et al. (2010)
	500 pmol (not specified)		3.v.				Yoshida et al. (2010)
Rat	5, 25 pmol		3.v.	↑ anxiety			Merali et al. (2008)
	60, 180 pmol		i.v.				Yosten and Samson (2009)
Rat	1, 2, 4 pmol/g BW		i.p.	↑ anxiety			Ge et al. (2015)
Rat	25, 100 pmol		i.v.	↔ locomotion			Könczöl et al. (2012)
Rat	5 pmol/g BW (x1 day)		s.c.	↑ locomotion			Mortazavi et al. (2015)
	35 pmol/g BW (x7 days)		s.c.	↑ locomotion			
	5 pmol/g BW (Abgent; synthetic)		i.p.	↑ locomotion			
Rat	0.44 pmol/g BW (x18 h)		s.c.	↑ locomotion			Gonzalez et al. (2011)
Rat	500 pmol (not specified)		3.v.				Yoshida et al. (2010)
		Restraint stress		↑ CORT	↑ PVN and VLM	↑ PVN, SON, NTS, LC, DR, MR	Goebel et al. (2009a,b)
Rat		Restraint stress			↔ plasma		Yoshida et al. (2010)
Rat		Water avoidance stress			↑ plasma		Xu et al. (2015)
Mouse		Water avoidance stress			↑ hypothalamus	↑ PVN, SON, LC, RP, NTS, VLM	Goebel-Stengel et al. (2011)

For information on the origin of nesfatin-1 and abbreviations please refer to Table 1.

fear conditioning test (learned anxiety response) (Merali *et al.* 2008). In addition, when animals were exposed to a novel environment, nesfatin-1 not only increased the latency to eat palatable food but also decreased the amount consumed (Merali *et al.* 2008). In an open field test, both an acute central nervous (Yosten & Samson 2009) and a peripheral chronic administration of nesfatin-1 (Ge *et al.* 2015) increased anxiety-like behavior. The latter was accompanied by reduced brain-derived neurotrophic factor (BDNF) and ERK 1 and 2 phosphorylation in the prefrontal cortex and hippocampus (Ge *et al.* 2015). Moreover, nesfatin-1-induced anxiety was blocked by the co-administration of SHU9119 (Yosten & Samson 2009), indicating that nesfatin-1 participates in stress response mechanisms by recruiting the central melanocortin system. However, the contribution of the hypothalamic (HPA) and extrahypothalamic circuitries (e.g. amygdaloid nuclei, where nesfatin-1 is abundantly expressed (Goebel *et al.* 2009a, Goebel-Stengel *et al.* 2011)) in inducing anxiety- and fear-related behaviors has yet to be dissected.

Rats exposed to restraint stress did not show altered NUCB2/nesfatin-1 plasma levels (Yoshida *et al.* 2010). On the other hand, in a recent study, NUCB2/nesfatin-1 plasma levels were increased in a water avoidance test (Xu *et al.* 2015b). Such a discrepancy could be explained by the different stress tests (physical vs psychological) or other methodological differences (Yoshida *et al.* 2010, Xu *et al.* 2015b). Interestingly, NUCB2/nesfatin-1 plasma levels were positively correlated with those of corticosterone; in addition, hypothalamic *Nucb2* mRNA expression levels were also found to be increased and positively correlated with those of *Crf* after water avoidance stress acute exposure (Xu *et al.* 2015b).

Exposure to restraint stress increased corticosterone serum levels (Xu *et al.* 2010) and c-fos expression in NUCB2/nesfatin-1 neurons of several brain areas such as PVN, SON, ARC, NTS, locus coeruleus (LC), raphe pallidus (RP) and ventrolateral medulla (VLM) (Goebel *et al.* 2009b, Yoshida *et al.* 2010). Furthermore, it increased *Nucb2* mRNA expression in the PVN and VLM (Könczöl *et al.* 2010). Thus, the NUCB2/nesfatin-1 system is recruited under stress conditions. Together with the finding, that i.c.v. administration of nesfatin-1 increased c-fos expression in PVN, SON, NTS, LC, DR and medial raphe (Yoshida *et al.* 2010), central nervous NUCB2/nesfatin-1 appears to be involved in orchestrating autonomic, neuroendocrine and behavioral responses to stress.

Matching these findings from rodent experimental models, recent data in humans also suggest that a

relationship between nesfatin-1 and stress-related mood disorders might exist. NUCB2/nesfatin-1 plasma levels of patients affected by major depressive disorders were higher than those in healthy subjects (Ari *et al.* 2011). Moreover, a positive correlation was found between circulating NUCB2/nesfatin-1 levels and anxiety in women diagnosed with anorexia nervosa (Hofmann *et al.* 2015a) or obesity (Hofmann *et al.* 2013, 2015b), whereas this relationship was inversed in obese men (Hofmann *et al.* 2015b). Intriguingly, drug-free suicide male victims displayed higher *Nucb2* mRNA expression in the Edinger-Westphal nucleus than in controls, whereas levels were lower in female victims (Bloem *et al.* 2012).

For nesfatin-1's effects in the context of anxiety, behavior and depression, please see Table 3.

Epilepsy

The findings on nesfatin-1 altering neuronal excitability (Price *et al.* 2008a,b, Mimeo *et al.* 2012, Li *et al.* 2014, Chen *et al.* 2015) led researchers to investigate the role of this peptide in neurological disorders such as epilepsy, characterized by imbalanced excitatory and inhibitory neuronal inputs. Subjects diagnosed with primary generalized epilepsy had higher saliva and serum nesfatin-1 levels than control (Aydin *et al.* 2009). This increase could be reduced by antiepileptic drug treatment (Aydin *et al.* 2009). In addition, serum nesfatin-1 levels were found to be higher in epileptic patients up to 48 h after an epileptic attack than in healthy subjects (Aydin *et al.* 2011). Fittingly, kainic acid-induced epileptic seizures increased plasma NUCB2/nesfatin-1 levels in rats (Liu *et al.* 2011). Whether NUCB2/nesfatin-1 plays a facilitative or inhibitory role in triggering epilepsy seizures and in the pathophysiology of epilepsy remains to be determined.

Sleep

As the discovery of co-expression of NUCB2/nesfatin-1 and melanin-concentrating hormone protein (MCH) in the THA (Fort *et al.* 2008), a central nervous structure, which is closely related to the regulation of rapid eye movement (REM) sleep (Luppi *et al.* 2006), a few studies have aimed at investigating potential functional connections between NUCB2/nesfatin-1 protein or *Nucb2* mRNA expression and sleep.

It was shown that abolishment of REM sleep reduces *Nucb2* mRNA and NUCB2/nesfatin-1 protein expression in the dorsal lateral hypothalamus, whose lateral hypothalamus subsection (Papp & Palkovits 2014) is among others involved the regulation of the sleep and wake cycle. REM sleep rebound in turn activates NUCB2/nesfatin-1 neurons (Vas *et al.* 2013). Data on REM sleep alterations upon nesfatin-1 i.c.v. injection are unequivocal as both a reduction (Vas *et al.* 2013) as well as a slight increase (Jego *et al.* 2012) have been published; in line with the latter observation, blockade of endogenous nesfatin-1 expression negatively affects REM sleep (Jego *et al.* 2012).

Besides these few functional studies, in human sleep apnea syndrome an inverse correlation between NUCB2/nesfatin-1 in peripheral circulation and the severity of disease was observed (Aksu *et al.* 2015, Araz *et al.* 2015, Shen *et al.* 2015), whether there is a functional link remains to be elucidated.

Reproduction

Reproductive maturation and function are closely linked to the availability of energy, thereby placing priority to survival of the individual over the thriving of a population. Known endocrine mediators linking metabolic state and reproduction are, e.g., insulin and leptin. Both act at the hypothalamic level to convey information about the metabolic state to gonadotropin-releasing hormone (GnRH) neurons in the PVN and thus in turn control the hypothalamic–pituitary–gonadal (HPG) axis (Hill *et al.* 2008).

Recent data suggest that also NUCB2/nesfatin-1 is involved in the interaction between energy status and reproduction (Garcia-Galiano & Tena-Sempere 2013, Navarro & Kaiser 2013), particularly with respect to the regulation of female pubertal transition, during which *Nucb2* mRNA and protein expression were significantly increased in the hypothalamus (Garcia-Galiano *et al.* 2010). In contrast, during this developmental stage, a negative energy balance decreased both *Nucb2* mRNA and protein expression (Garcia-Galiano *et al.* 2010). Furthermore, central nervous administration of nesfatin-1-induced luteinizing hormone (LH) secretion in freely feeding and to an even greater extent also in short-term fasted pubertal female rats (Garcia-Galiano *et al.* 2010). Fittingly, morpholino oligonucleotide-induced knockdown of hypothalamic *Nucb2* mRNA expression delayed pubertal transition and reduced LH levels and the weights of the ovaries. Food intake was

not affected in these animals, suggesting a direct effect of NUCB2/nesfatin-1 on pubertal maturation (Garcia-Galiano *et al.* 2010).

To date, there is only limited information available on whether nesfatin-1 is also involved in the regulation of the adult HPG axis: in adult female rats, 50 pmol nesfatin-1 i.c.v. did not affect plasma LH (Garcia-Galiano *et al.* 2010). Preliminary data, which were only presented at two conferences, indicate that upon i.c.v. administration of a very high dose of nesfatin-1 (1 nmol), plasma LH and follicle-stimulating hormone (FSH) were increased in male rats (Tadross *et al.* 2010, Patterson *et al.* 2011). Conversely, in the pituitary, *Nucb2* mRNA expression is regulated by 17-estradiol and progesterone sex steroids, suggesting the existence of a feedback-mechanism in the NUCB2/nesfatin-1-HPG-axis interaction (Garces *et al.* 2014).

In a recently published study, however, i.c.v. administration of nesfatin-1 significantly reduced the expression of GnRH mRNA in the hypothalamus and FSH and LH mRNA in the pituitary (Gao *et al.* 2016), suggesting an inhibitory role of nesfatin-1 on the HPG axis. Matching observations were also made in fish after peripheral administration of nesfatin-1 (Gonzalez *et al.* 2012).

The downstream mediators of nesfatin-1 in the context of reproduction have not yet been identified; however, neuropeptides such as oxytocin and α -MSH are known to regulate GnRH action and thus are potential candidates (Parent *et al.* 2008, Roa & Herbison 2012). Furthermore, the recently discovered neuropeptide phoenixin, which is colocalized with NUCB2/nesfatin-1 in a number of hypothalamic nuclei (ARC, PVN, ventromedial and lateral hypothalamus) (Palasz *et al.* 2015) has been suggested as a potential mediator for nesfatin-1's action with regard to reproduction. Phoenixin increases GnRH, GnRH receptor and Kiss1 gene expression (Yosten *et al.* 2013, Treen *et al.* 2016) and potentiates GnRH-stimulated LH release *in vitro* (Yosten *et al.* 2013). These findings were substantiated by an *in vivo* study demonstrating that i.c.v. phoenixin dose dependently increased plasma LH in diestrus female rats (Stein *et al.* 2016).

In the periphery, NUCB2/nesfatin-1 immunoreactivity has been detected in rodent and human testes' Leydig cells; in rat testes, *Nucb2* mRNA and NUCB2/nesfatin-1 are controlled by the pituitary (Garcia-Galiano *et al.* 2012). During pubertal transition, NUCB2/nesfatin-1 protein (but not mRNA) was significantly increased and was suppressed by short-term food deprivation (Garcia-Galiano *et al.* 2012), resembling the findings in the

hypothalamic of female rats under the same conditions (Garcia-Galiano *et al.* 2010). Furthermore, *Nucb2* mRNA and NUCB2/nesfatin-1 protein expression in rat placenta and also plasma NUCB2/nesfatin-1 decrease in the course of gestation (Garces *et al.* 2014).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-16-0361>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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