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DISSERTATION

Nesfatin-1_{30-59} alters food intake microstructure in rats

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Philip Prinz

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1. Table of abbreviations

ANOVA ..... analysis of variance
CART .....cocaine and amphetamine regulated transcript
Cpm ..... counts per minute
CRF^2 ..... corticotropin-releasing factor
DIO ..... diet-induced obesity
EDTA.....ethylenediaminetetraacetic acid
GLP-1 ..... glucagon-like peptide 1
GLUT4 .....glucose transporter type 4
GOAT..... ghrelin-*O*-acyltransferase
HPLC..... high performance liquid chromatography
icv..... intracerebroventricular
ip .....intraperitoneal
NaCl..... sodium chloride
NTS.....nucleus of the solitary tract
NUCB2 ..... nucleobindin 2
POMC ..... proopiomelanocortin
PYY ..... peptide YY
RAPID ..... Reduced temperatures, Acidification, Protease inhibition,
.....Isotonic exogenous controls and Dilution
SEM.....standard error of the mean
TFA.....trifluoroacetate

2. Abstract

2.1 English

Today, assessing the feeding microstructure is essential in order to describe the detailed effects of feeding regulatory peptides (e.g. ghrelin or nesfatin-1) on food intake. Additionally, it is important to detect circulating feeding regulatory peptides in the correct amount and molecular form when investigating their regulation.

Therefore, we validated an automated food intake monitoring system for rats and investigated the effect of the ghrelin-*O*-acyltransferase (GOAT) inhibitor (GO-CoA-Tat; 32, 96 and 288 µg/kg) following peripheral injection in rats. Moreover, the effect of nesfatin-$1_{30\text{-}59}$ injected intracerebroventricularly (icv) in normal weight (0.1, 0.3 and 0.9 nmol/rat) and diet-induced obesity (DIO) rats (0.9 nmol/rat) on food intake microstructure was assessed. Lastly, the RAPID (Reduced temperatures, Acidification, Protease inhibition, Isotopic exogenous controls and Dilution) method, a recently developed blood processing method for the use in rats was established for the use in human blood.

Rats habituated quickly to the automated food monitoring system and food intake was not different compared to manual assessment. Peripheral injection of the GOAT inhibitor had a delayed anorexigenic effect at a dose of 96 µg/kg at 2 h post injection compared to vehicle (-27%). Microstructure analysis showed that food intake was reduced due to reduced meal frequency (-15%), while meal size was not altered. In normal weight rats, nesfatin-$1_{30\text{-}59}$ reduced food intake in the 5th hour (-75%), resulting in a 24-h reduction of cumulative food intake (-12%). The food intake reducing effect was due to a reduction in meal size (-44%), whereas meal frequency was not affected. In DIO rats, nesfatin-$1_{30\text{-}59}$ reduced food intake in the first 4 hour period with a maximum effect in the 3rd and 4th hour post icv injection (-71% and -74%), resulting in a 20-h reduction of cumulative food intake (-13%). The anorexigenic effect was due to a reduced meal frequency (-27%), while meal size was not altered. The RAPID method improved the recovery in 9/9 ^{125}I-radiolabeled peptides (+19% until 39%) and showed the expected peak for acyl ghrelin after performing high performance liquid chromatography compared to standard blood processing (EDTA blood on ice) indicating the correct molecular form.

In summary, peripheral blockade of GOAT reduces food intake by increasing satiety without affecting satiation. Nesfatin-$1_{30\text{-}59}$ is the active core of nesfatin-$1_{1\text{-}82}$ and differently affects food intake under normal weight and DIO conditions. The RAPID method can be used in humans, assesses the correct molecular form and yields higher levels of peptides.

2.1 German

Heutzutage ist es essentiell, die Mikrostruktur der Nahrungsaufnahme zu erfassen, um die Effekte nahrungsregulatorischer Peptide (z.B. Ghrelin oder Nesfatin-1) zu beschreiben. Ebenso wichtig ist die exakte Erfassung der molekularer Form und Menge der im Blutkreislauf zirkulierenden nahrungsregulatorischen Peptide, um deren Regulation zu beschreiben.

Zur Untersuchung des Effekts des Ghrelin-*O*-Acyltransferase (GOAT) Inhibitors (GO-CoA-Tat; 32, 96 und 288 µg/kg) nach peripherer Injektion sowie des Effekts von Nesfatin-$1_{30\text{-}59}$ nach intracerebroventrikulärer (icv) Injektion auf die Nahrungsaufnahme (0.1, 0.3 and 0.9 nmol/Ratte) in normalgewichtigen und *diet-induced obesity* (DIO) Ratten (0.9 nmol/Ratte) wurde ein automatisches Nahrungsaufnahmemesssystem für Ratten etabliert. Die RAPID (*Reduced temperatures, Acidification, Protease inhibition, Isotopic exogenous controls* und *Dilution*) Methode ist eine kürzlich zur Blutaufbereitung in Ratten entwickelte Methode, die zur Anwendung in menschlichem Blut etabliert werden sollte.

Die Ratten haben sich rasch an das automatische Nahrungsaufnahmemesssystem gewöhnt und die Nahrungsaufnahme unterschied sich nicht von der manuellen Erfassung. Die periphere Injektion des GOAT-Inhibitors zeigte einen um zwei Stunden verzögerten anorexigenen Effekt bei einer Konzentration von 96 µg/kg im Vergleich zur Kontrollgruppe (-27%). Die Analyse der Mikrostruktur zeigte eine Reduktion der Nahrungsaufnahme durch eine reduzierte Frequenz der Mahlzeiten (-15%) ohne Veränderung der Größe der Mahlzeiten. Bei einer Dosis von 0.9 nmol/Ratte reduzierte Nesfatin-$1_{30\text{-}59}$ die Nahrungsaufnahme in normalgewichtigen Ratten mit einem maximalen Effekt in der 5. Stunde (-75%). Dies resultierte in einer 24-stündigen Reduzierung der kumulativen Nahrungsaufnahme (-12%). Die reduzierte Nahrungsaufnahme war Folge einer verkleinerten Mahlzeitgröße der Mahlzeiten (-44%), während eine Veränderung in der Frequenz der Mahlzeiten nicht beobachtet wurde. In DIO-Ratten reduzierte Nesfatin-$1_{30\text{-}59}$ die Nahrungsaufnahme innerhalb der ersten vier Stunden mit maximalen Effekten in der 3. und 4. Stunde nach icv Injektion (-71% und -74%). Dies führte zu einer Abnahme der kumulativen Nahrungsaufnahme über 20 Stunden (-13%). Die Analyse der Mikrostruktur zeigte eine Reduktion der Nahrungsaufnahme über eine geringere Mahlzeitenfrequenz (-27%) ohne Veränderung der Mahlzeitengröße. Die RAPID-Methode verbesserte die Ausbeute von 9/9 mit ^{125}Iod markierten Peptiden und zeigte einen stark erhöhten Gehalt von Acyl-Ghrelin (+62%) an der zu erwartenden Position nach Durchführung einer *High Performance Liquid Chromatography* im Vergleich zur Standard-Blutaufbereitung (EDTA-Blut auf Eis). Wir konnten zeigen, dass GOAT-Hemmung die Nahrungsaufnahme durch verstärkte Sattheit reduziert, ohne die Sättigung zu beeinflussen. Nesfatin-$1_{30\text{-}59}$ beeinflusst die Nahrungsaufnahme unterschiedlich in normalgewichtigen und DIO-Ratten. Die RAPID-Methode kann beim Menschen verwendet werden, erfasst die korrekte molekulare Form und erzielt eine höhere Peptidausbeute.

3. Introduction

Nesfatin-$1_{1\text{-}82}$ is an anorexigenic peptide that was discovered in 2006 in the rat hypothalamus and shown to reduce food intake after injection into the third brain ventricle (1). Nesfatin-$1_{1\text{-}82}$ is posttranslationally cleaved from the gene encoding nucleobindin2 (NUCB2) by the pro-hormone-convertase 1/3 (1) (Fig. 1). Interestingly, the other fragments of NUCB2, namely nesfatin-$2_{85\text{-}163}$ and nesfatin-$3_{166\text{-}396}$ did not affect food intake or body weight gain after injection into the third brain ventricle (1). Subsequent research confirmed the anorexigenic effect of nesfatin- $1_{1\text{-}82}$ after injection into the third, fourth and lateral brain ventricle, into the cisterna magna or directly into nuclei of the hypothalamus, namely the paraventricular nucleus or lateral hypothalamic area as well as in the dorsal vagal complex in rats (2–6), mice (7,8) and goldfish (9,10). These results point towards a physiological role of nesfatin-$1_{1\text{-}82}$ in the regulation of feeding. Today it is known, that central nesfatin-$1_{1\text{-}82}$ is also involved in the regulation of gastrointestinal motility (7) and emptying (11), sleep (12), the mediation of anxiety and fear (13,14) as well as glucose metabolism (15,16). It is to note that administration of nesfatin-$1_{1\text{-}82}$ into the third brain ventricle resulted in increased peripheral and hepatic insulin sensitivity and attenuated hepatic gluconeogenesis in rats (16), a finding supported by increased hepatic gluconeogenesis and reduced hepatic and muscular insulin signaling in rats with hypothalamic NUCB2 knockdown (15). Additionally, injection of nesfatin-$1_{1\text{-}82}$ into the third brain ventricle resulted in increased blood pressure in rats indicating a regulatory role in the cardiovascular system (17). Taken together, central nesfatin-1 exerts pleiotropic functions reaching far beyond the initially described anorexigenic effect.

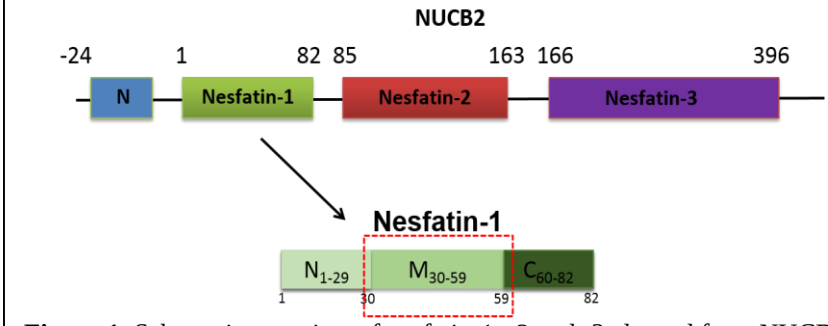

Figure 1. Schematic overview of nesfatin-1, -2 and -3 cleaved from NUCB2 and further processing to nesfatin-$1_{30\text{-}59}$ proposed to be the active core of nesfatin-1.

In the last years, research also focused on the peripheral effects of nesfatin-$1_{1\text{-}82}$. Peripheral nesfatin-$1_{1\text{-}82}$ has an insulinotropic effect and improves glucose stimulated insulin release in rodents (18,19) as well as GLUT-4 translocation in mouse adipose tissue and skeletal muscle (20). Recently, it was shown that peripheral nesfatin-$1_{1\text{-}82}$ is also involved in the reproductive system as part of the hypothalamus-pituitary-gonadal axis in fish (21). However, the food intake modulatory effects of peripheral nesfatin-$1_{1\text{-}82}$ are not completely understood. While acute injection of nesfatin-$1_{1\text{-}82}$ did not alter food intake in Sprague Dawley rats (11) or C57Bl/6 mice (8), chronic peripheral administration reduced food intake in Fischer 344 rats after seven days of administration (22). In the light of the fact

that the rat gastric oxyntic mucosa has higher NUCB2 mRNA expression levels compared to the brain (23) the stomach likely represents the primary site of nesfatin-1_{1-82} production and might be involved in the regulation of food intake. Since it is known that nesfatin-1_{1-82} can cross the blood-brain-barrier (24,25), chronically altered gastric NUCB2 expression and consequently circulating nesfatin-1_{1-82} levels could lead to a central anorexigenic effect. Interestingly, nesfatin-1_{1-82} is co-localized with the orexigenic peptide ghrelin (23) in X/A-like gastric endocrine cells in rats with the occurrence in a different pool of vesicles (23), suggesting a separate release under different metabolic conditions to regulate food intake.

Ghrelin, a peptide of 28 amino acids, is predominantly produced in the stomach and needs to be acylated on its third amino acid in order to bind to its receptor (26). So far it is the only known peripherally produced hormone that centrally stimulates food intake (27,28). This acylation is catalyzed by ghrelin-*O*-acyltransferase (GOAT), an enzyme identified 2008 by two independent groups (29,30). GOAT is expressed in the stomach and also detectable in plasma of rodents (31), suggesting an (additional) activation of ghrelin after release from the stomach. Studies in Siberian hamsters reported a reduction of food intake after intraperitoneal (ip) injection with the GOAT inhibitor (GO-CoA-Tat) (32). However, these studies did not assess the feeding microstructure.
Today, when investigating the effects of feeding regulatory peptides on food intake it is *state of the art* to assess the feeding microstructure (33). The food intake microstructure encompasses parameters such as meals size, meal frequency, meal duration, latency to a meal, eating rate and the inter-meal interval, which are used to comprehensively describe two major characteristics of food intake: 1) satiation [mechanism(s) which cause meal termination] and 2) satiety [mechanism(s) which cause a delay of the next meal after one meal is completed] (34,35). To assess the food intake microstructure, an automated episodic food monitoring system was used that has been established before by our group for murine use (36). In the first study we validated the system for the use in rats and investigated the food intake microstructure following pharmacological peripheral blockade of GOAT signaling in rats.

After the automated food intake monitoring system was successfully established for rats, in the second study it was used to investigate the effects of intracerebroventricularly (icv) administered nesfatin-1_{30-59} on the food intake microstructure in rats. The effect of icv injected nesfatin-1_{30-59} on the feeding microstructure was already characterized in mice (36), however, this information was still lacking in rats. In 2009 it was shown that nesfatin-1_{30-59} is the active core of nesfatin-1_{1-82} (Fig. 1), mimicking the anorexigenic effect of nesfatin-1_{1-82} after ip injection in mice (37). Our group previously extended these findings, by showing that nesfatin-1_{30-59} reduces food intake in mice after injection into the lateral brain ventricle (36). Furthermore, the analysis of the feeding microstructure showed that the anorexigenic effect of nesfatin-1_{30-59} was mediated by increasing satiety, while satiation was not affected (36). In both studies only the mid fragment nesfatin-1_{39-59}, but not the N-

terminal (nesfatin-1_{1-29}) or the C- terminal fragment (nesfatin-1_{60-82}) affected food intake (36,37).
In addition to these pharmacological studies, measurement of endogenous food intake regulatory peptides gains more importance. Thus, studies focusing on the regulation of hunger and satiety frequently assess peptide levels. A main problem is the fast degradation after blood withdrawal due to endogenous enzymes. However, standard blood processing (EDTA blood on ice) bearing the risk of considerable peptide degradation is very common in the clinical and even research setting. This is especially relevant for labile hormones such as acyl ghrelin (38). We previously described the RAPID (Reduced temperatures, Acidification, Protease inhibition, Isotonic exogenous controls and Dilution) method for blood processing in rats which yielded higher peptide levels and allowed for determination of the correct molecular circulating peptide form compared to standard blood processing (EDTA blood on ice) (39). Since feeding regulatory peptides are of great importance in the regulation of hunger and satiety, body weight and subsequently the development of obesity, the RAPID method might be useful in humans as well. Therefore, the RAPID method was established for the use in human blood. Here, recovery of ^{125}I-nesfatin-1 and other radiolabeled peptides in human blood samples, as well as the degradation of ^{125}I-acyl ghrelin compared to standard blood processing were assessed.

4. Material and Methods

4.1 Publication 1: Treatment with the ghrelin-O-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats

Animals. Male Sprague Dawley rats weighing 220-300 g were housed under controlled illumination and temperature. First, animals were housed in groups and afterwards separated into single housing cages. Animals had free access to rodent diet and tap water. After one week of adaptation, animals were handled daily to become accustomed to the investigators and experimental procedures as well as to reduce stress during the experiments. Body weight, appearance and behavior were assessed daily and used as an indicator for well-being of the animals.

Compound. The GOAT inhibitor (GO-CoA-Tat) was stored at -80°C and diluted in 0.9% sterile saline solution directly before the experiments.

Manual food intake monitoring. Food intake was calculated by weighing rat chow before and after a defined time interval.

Automated food intake monitoring. To analyze the microstructural feeding behavior a commercially available food intake monitoring system was used. This system consists of a low spill food hopper placed on an electrical balance, which allows for continuous measurement of food intake at every second with an accuracy of 0.1 mg. Animals are housed in a regular rat single housing cage containing environmental enrichment, bedding material and tap water from regular water bottles. Every time an animal eats, the automated food monitoring system records the start and duration as well as the amount of food. The data is saved on an external hard drive and can be used to calculate the latency to the first meal, meal frequency, meal size, meal duration, time spent in meals, inter-meal-interval and rate of ingestion. The continuous assessment allows the investigator to analyze food intake and the underlying microstructure during any time period chosen.

Habituation to automated food intake monitoring system and comparison with manual assessment. In the first five days, food intake and body weight were assessed manually on a daily basis in group housed rats. Afterwards, rats were separated into single housing cages with eye and odor contact. Food was provided from the top of the cage and food intake and body weight were assessed manually for another three days. In the last step, food was provided from the hopper and food intake was measured by the automatic food intake monitoring system. Body weight was still assessed manually. Data of food intake assessed manually and automatically were compared.

Monitoring of behavior in the automatic food monitoring system. To assess the occurrence of physiological behavior in *ad libitum* fed, single housed rats in cages connected to the automatic food monitoring system, feeding, grooming, locomotion and resting were monitored manually during the first hour of the dark phase in a separate experiment.

Analysis of food intake microstructure in rats after intraperitoneal injection of GOAT inhibitor. *Ad libitum* fed naïve rats were injected ip with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor (32, 96 and 288 µg/kg in 300 µl sterile saline solution) directly before the onset of the dark phase and food intake was monitored using the automated food intake monitoring system. The dose of the GOAT inhibitor that induced the most pronounced reduction of food intake was used for all further analyses.

Assessment of acyl and desacyl ghrelin levels in rats after ip injection of GOAT inhibitor. After ip injection of the GOAT inhibitor (96 µg/kg) or vehicle (saline) at the onset of dark phase, food was removed. Blood was obtained transcardially before injection (0 h) and at 1, 2, and 3 h post injection. Blood was collected in chilled Eppendorf-tubes, containing EDTA and aprotinin and centrifuged directly after blood withdrawal. Plasma was separated and stored at -80°C. Acyl and total ghrelin level were assessed by ELISA. Desacyl ghrelin was calculated as difference of total minus acyl ghrelin for each individual sample.

Monitoring of behavior of rats injected intraperitoneally with GOAT inhibitor. After ip injection of the GOAT inhibitor at the onset of dark phase (96 µg/kg) behavior (food intake, grooming, locomotion and resting) was monitored during the 2nd h post ip injection. This period was chosen since during this time the GOAT inhibitor showed its maximum food intake reducing effect.

Statistical analysis. Distribution of the data was determined by using the Kolmogorov-Smirnov test and expressed as mean ± SEM. Differences between groups were assessed by using the t-test, one-way ANOVA followed by Tukey *post hoc* test and two-way ANOVA followed by Holm-Sidak test. Differences were considered significant when $p < 0.05$.

4.2 Publication 2: Nesfatin-1$_{30-59}$ injected intracerebroventricularly differently affects food intake microstructure in rats under normal weight and diet-induced obesity conditions

Animals. Male Sprague Dawley rats weighing 280-350 g were housed under controlled illumination and temperature. First, animals were housed in groups and afterwards animals were housed in single housing cages with free access to rodent diet and tap water. After an adaptation period, animals were handled daily (including daily control of body weight) to become accustomed to the investigators and experimental procedures (icv and ip injection). Body weight was used as indicator for well-being of the animals and assessed daily.

Compound. Rat nesfatin-1$_{30-59}$ peptide was aliquoted in sterile distilled water and stored at -80°C. Rat nesfatin-1$_{30-59}$ was further diluted in sterile distilled water for icv and in 0.9% pyrogen free saline solution for ip injections to reach experimental concentrations.

Diets. To induce diet-induced obesity, rats were fed a high fat diet containing 45% calories from fat (4.7 kcal/g) for a period of 10 weeks. Control rats were fed standard diet containing 10% calories

from fat (3.9 kcal/g). After the 10-week feeding period, the 50% of rats gaining the most body weight were selected as diet-induced obesity for further experiments.

Intracerebroventricular cannulation. Rats were anesthetized with an ip injection of 100 mg/kg ketamine and 10 mg/kg xylazine. To implant a chronic 22-gauge guide cannula into the right lateral brain ventricle, rats were placed in a stereotactic apparatus (coordinates for the placement from bregma: 0.8 mm posterior, 1.5 mm right lateral and 3.5 mm ventral). Coordinates were based on the atlas of Paxinos and Watson (40). Afterwards the wound was sutured, animals housed in single housing cages and allowed to recover for 5 days. During this time, rats were handled daily to adapt to the icv injection procedure. A 28-gauge cannula was connected to a 25 µl Hamilton syringe to icv inject a volume of 5 µl over a period of 1 min into the conscious rat. After the experiments, the right placement of the cannula was verified by injecting 5 µl toluidine blue and visualizing the spreading of the dye throughout the brain ventricular system.

Automated food intake monitoring. A commercially available food intake monitoring system was used to analyze the microstructural feeding behavior of rats. This system has been established for the use in rats in our previous study (41). Briefly, animals were housed in single cages and food placed on a low spill food hopper placed on an electrical balance, which allowed for continuous measurement of food intake at every second with an accuracy of 0.1 mg. Every time an animal ate, the automated food monitoring system recorded the start and duration as well as the amount of food. The data were saved on an external computer and used to calculate various parameters of the food intake microstructure.

Food intake experiments. To investigate the effect of nesfatin-$1_{30\text{-}59}$ in rats under normal weight conditions, chronically icv cannulated rats fed *ad libitum* with standard rodent diet were icv injected with nesfatin-$1_{30\text{-}59}$ (0.1, 0.3 or 0.9 nmol/rat) or vehicle (5 µl distilled water) directly before the onset of the dark phase. Feeding microstructure was assessed for 24 h post icv injection. Additionally, behavior was determined (locomotor activity and grooming). To assess whether the effect of nesfatin-$1_{30\text{-}59}$ is mediated centrally or peripherally, naïve rats fed *ad libitum* with standard rodent diet were ip injected with nesfatin-$1_{30\text{-}59}$ (8.1, 24.3 or 72.9 nmol/kg) or vehicle (300 µl sterile saline solution) directly before the onset of dark phase. Again, feeding microstructure was assessed for 24 h post ip injection. To investigate the effect of nesfatin-$1_{30\text{-}59}$ under conditions of diet-induced obesity (DIO), chronically cannulated DIO rats fed a high fat diet were icv injected with nesfatin-$1_{30\text{-}59}$ (0.9 nmol/rat, dose based on the first experiment) or vehicle (5 µl distilled water) directly before the onset of the dark phase. Feeding microstructure was assessed for 24 h post icv injection.

Statistical analysis. Distribution of the data was determined by using the Kolmogorov-Smirnov test and expressed as mean ± SEM. Differences between groups were assessed by using one-way ANOVA followed by Tukey *post hoc* test, two-way or three-way ANOVA followed by Holm-Sidak test.

Differences were considered significant when $p < 0.05$.

4.3 Publication 3: A RAPID method for blood processing to increase the yield of plasma peptide levels in human blood

Study participants. Female patients (n=42) hospitalized in the Division of Psychosomatic Medicine at Charité – Universitätsmedizin Berlin were divided into three groups: normal weight (BMI 18.5-25 kg/m²), anorexia nervosa (BMI<17.5 kg/m²) and obesity (BMI>30 kg/m²). Anorexic and obese patients were diagnosed according to the International Classification of Disease-10 (ICD-10).

Blood collection and processing. Blood was collected at day 2 or 3 after patients were hospitalized and before receiving dietary treatment (obese or anorexic patients). Blood collection was performed after an overnight fast. Venous blood was collected in the morning between 07.00 – 08.00 AM. For standard processing, blood was collected in chilled EDTA-containing tubes, centrifuged at 3,000 g for 10 min at 4°C and supernatant was collected and stored at -80°C. For RAPID processing, blood was diluted 1:10 in ice-cold RAPID buffer [0.1 M ammonium acetate, 0.5 M sodium chloride (NaCl) and 1 µg/ml enzyme inhibitors (diprotin A, E-64-d, antipain, leupeptin, chymostatin), pH 3.6], centrifuged at 3,000 g for 10 min at 4°C and the supernatant was collected. Sep-Pak C18 cartridges were charged with 100% acetonitrile, followed by equilibration with 0.1% trifluoroacetate (TFA) and loaded the supernatant at a speed of 1ml/min. Samples were washed with 3 ml 0.1% TFA and eluted with 2 ml 70% acetonitrile containing 0.1% TFA. Vacuum centrifugation was used to dry samples before storage at -80°C for further processing by radioimmunoassay.

Recovery of radiolabeled peptides. ^{125}I-radiolabeled human peptides (acyl ghrelin, glucagon-like-peptide-1 (GLP-1), glucagon, insulin, kisspeptin, leptin, nesfatin-1, PYY_{3-36} and somastatin-28) were diluted in 0.1% acetic acid directly before the experiments. After blood withdrawal, 1 ml blood was transferred to EDTA tubes (containing 50 µl radiolabeled peptides containing 3,000-6,000 cpm) or RAPID tubes (containing 9 ml RAPID buffer and 500 µl radiolabeled peptides containing 30,000-60,000 cpm: due to 1:10 dilution before a 10-times higher concentration was used) and processed as described above. For recovery experiments, samples were not vacuum centrifuged. Recovery of radiolabeled peptides was assessed directly after blood processing using a gamma counter. In standard samples, the whole supernatant was measured, whereas in RAPID samples 1/10 of the total volume was analyzed. Two samples of 50 µl of radiolabeled peptide that were not processed were measured at the same time and set as a 100% control.

High performance liquid chromatography (HPLC) of radiolabeled peptide. For standard processing, 1 ml blood was transferred into an EDTA tube containing 200 µl radiolabeled acyl ghrelin containing 15,000-20,000 cpm and for RAPID processing 1 ml blood was transferred into a tube containing 9 ml RAPID buffer and 200 µl radiolabeled acyl ghrelin containing 15,000-20,000 cpm

and processed as described above. Samples were not dried but directly prepared for reverse phase HPLC. Samples were eluted with a 10 min gradient of 20% acetonitrile, then a 30 min gradient of 35% acetonitrile at a flow rate of 1 ml/min. Fractions of 1 ml were collected every minute and radioactivity was assessed using a gamma counter. As control, HPLC was performed with 200 µl radiolabeled acyl ghrelin containing 15,000-20,000 cpm.

Radioimmunoassay. Frozen (standard) and dried (RAPID) supernatants were thawed at room temperature and re-suspended in distilled water to the original volume of plasma. Kisspeptin as well as acyl- and total ghrelin were assessed by commercial radioimmunoassays. Desacyl ghrelin was calculated as difference of total minus acyl-ghrelin and the acyl/desacyl ghrelin ratio was calculated by dividing acyl by desacyl ghrelin for each individual sample.

Statistical analysis. Distribution of the data was determined by using the Kolmogorov-Smirnov test and expressed as mean ± SEM. Differences between groups were assessed by using the t-test, one-way ANOVA followed by Tukey *post hoc* test or two-way ANOVA followed by Holm-Sidak test. Statistical differences were considered significant when $p < 0.05$.

5. Results

5.1 Publication 1: *Treatment with the ghrelin-O-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats*

Single housing and adaptation to special cages to assess the food intake microstructure does not affect body weight gain in rats. Body weight gain in rats was not affected after separation into single housing cages and after feeding from the hopper, resulting in a continuous increase of body weight over the course of 14 days. Additionally, there were no differences in automated or manual assessment of food intake in dark- or light phases (p = 0.43; data not shown). The food intake monitoring system allowed for assessment of the food intake microstructure in undisturbed rats (Fig. 2). The behavioral satiety sequence, a marker for physiological behavior, was observed in rats housed in the food intake monitoring system (Fig. 3).

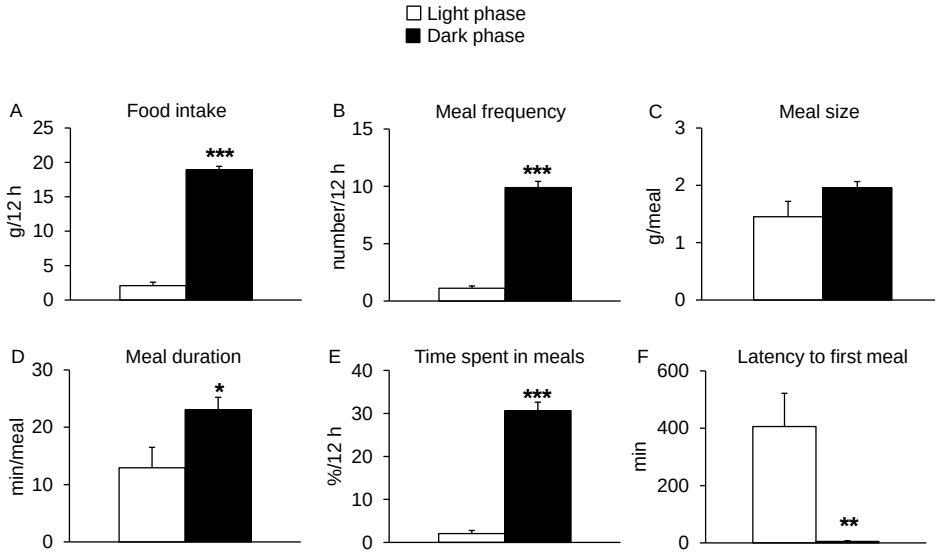

Figure 2. Assessment of the food intake microstructure in undisturbed rats during light phase and dark phase. n = 9

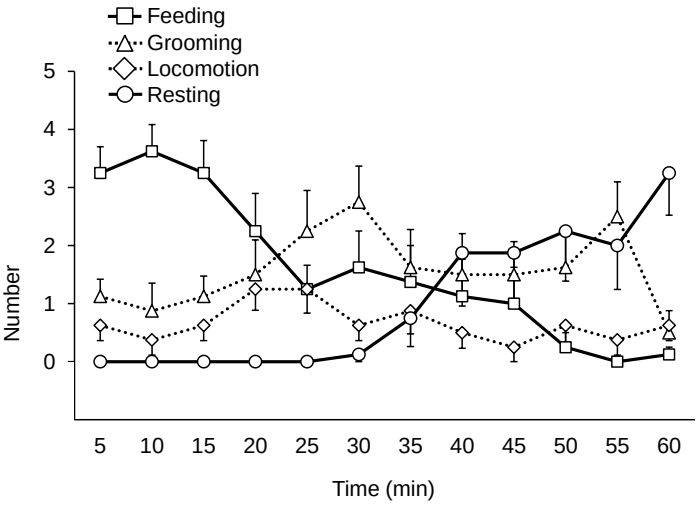

Figure 3. Assessment of the physiological satiety sequence of rats in the automated food monitoring system over 60 minutes. n = 8

GOAT inhibition reduces food intake by a reduction of meal frequency. Intraperitoneal injection of GOAT-Inhibitor caused a significant reduction of food intake in the 2nd hour at the concentration of 96 µg/kg compared to vehicle (-27%, $p < 0.05$), whereas concentrations of 32 and 288 µg/kg GOAT-inhibitor had no effect on food

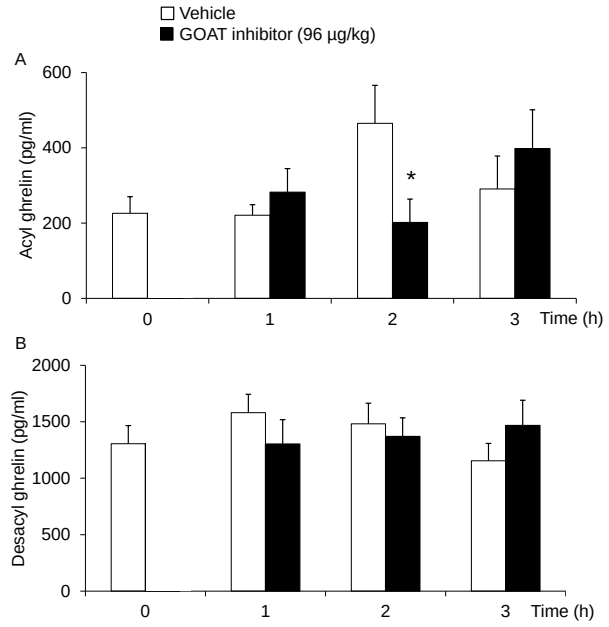

Figure 4. Circulating level of acyl ghrelin and desacyl ghrelin following injection of GOAT inhibitor (96 µg/kg, ip) or vehicle. n = 5-6/time point; *p < 0.05

intake. Therefore, feeding microstructure was assessed for the first 2 h at a dose of 96 µg/kg. The GOAT-inhibitor reduced meal frequency (-15%, $p < 0.05$) and time spent in meals (-39%, $p < 0.05$), while meal size ($p = 0.29$) and duration ($p = 0.33$) were not altered.

GOAT inhibitor reduces circulating acyl ghrelin level. Plasma levels of acyl ghrelin were significantly reduced 2 h post injection (Fig. 4A), while the baseline levels of desacyl ghrelin were not affected at any time point following the injection of the GOAT inhibitor (Fig. 4B).

5.2 Publication 2: Nesfatin-1$_{30-59}$ injected intracerebroventricularly differently affects food intake microstructure in rats under normal weight and diet-induced obesity conditions

Nesfatin-1$_{30-59}$ reduces food intake during the 4-8 h period post icv injection, resulting in a 24-h reduction of cumulative food intake. Nesfatin-1$_{30-59}$ reduced food intake post icv injection in normal weight rats with a delayed onset compared to vehicle. Food intake was reduced during the 4-8 h period at the highest dose of 0.9 nmol/rat (-29%, $p < 0.05$), whereas the other doses did not significantly affect food intake at any time point (Fig. 5A). Analysis of hourly food intake showed that food intake was reduced during the 5th h post icv injection of 0.9 nmol/rat (-75%,

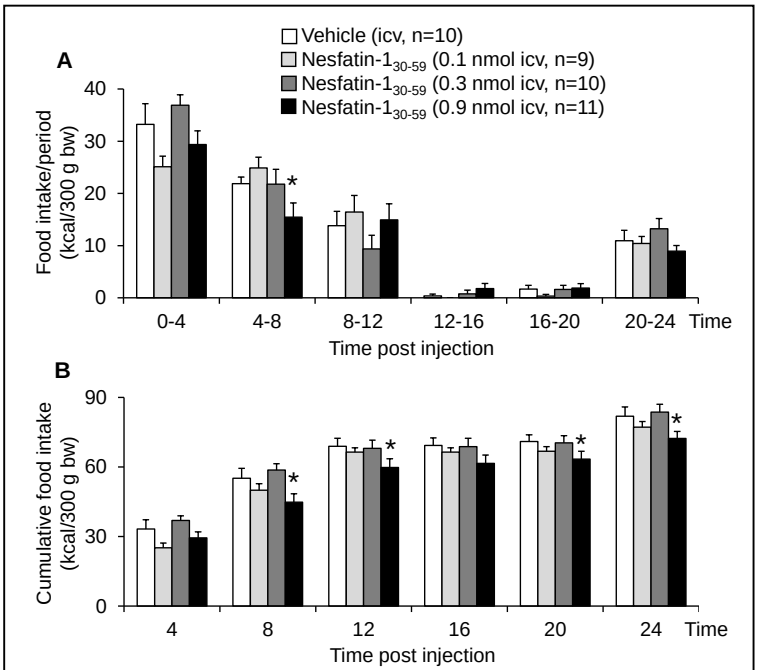

Figure 5. Nesfatin-1$_{30-59}$ was injected icv at a dose of 0.1, 0.3 and 0.9 nmol/rat and food intake monitored using an automated food intake monitoring system. Food intake is expressed as food intake/4 h periods (A) and cumulative food intake (B). n = 9-11/Group; *p < 0.05

compared to vehicle, p < 0.05). Only the dose of 0.9 nmol/rat resulted in a reduction of cumulative food intake over 24 h (-12%, p < 0.05; Fig. 5B). Therefore, this dose was used for all subsequent analyses.

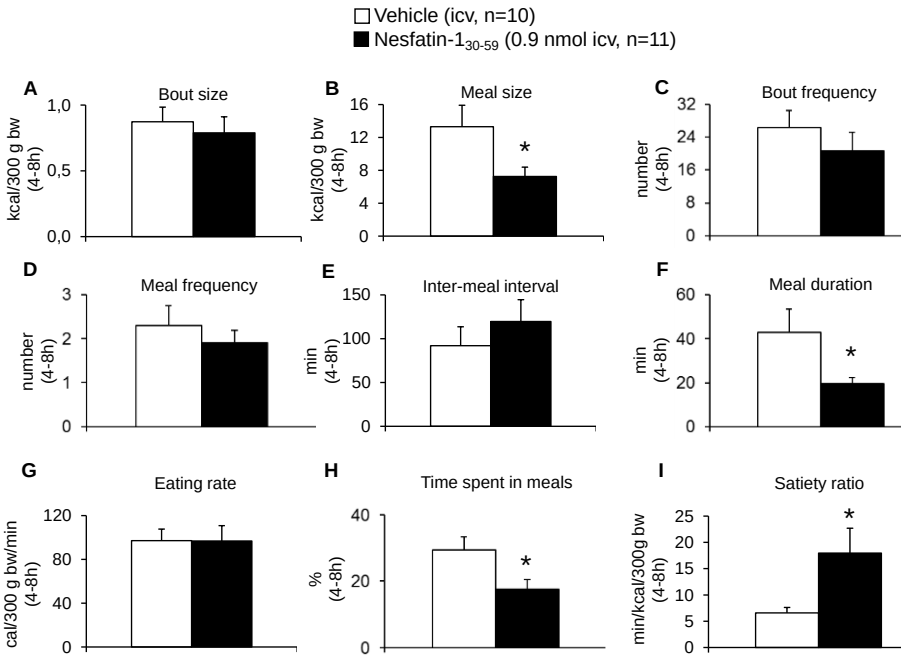

Figure 6. Nesfatin-1$_{30-59}$ (0.9 nmol/rat) or vehicle was injected icv and the food intake microstructure assessed during 4-8 h period. n = 10-11/Group; *p < 0.05

Feeding microstructure analysis of the 4-8 h period showed that icv injected nesfatin-1$_{30-59}$ reduces meal size and duration, while meal frequency remains unaffected. During the 4-8 h post icv injection of nesfatin-1$_{30-59}$ meal size (-45%, p < 0.05; Fig. 6B), meal duration (-54%, p < 0.05; Fig. 6F) and time spent in meals (-41%, p<0.05; Fig. 6H) were reduced, while bout size (Fig. 6A), bout frequency (Fig. 6C), meal frequency (Fig. 6D), inter-meal interval (Fig. 6E) and eating rate (Fig. 6G) were not altered compared to vehicle in normal weight rats. The satiety ratio was increased post nesfatin-1$_{30-59}$ icv injection compared to vehicle (+174%, p < 0.05; Fig. 6I). Although higher doses were used (8.1, 24.3 and 72.9 nmol/rat), nesfatin-1$_{30-59}$ injected ip in *ad libitum* fed rats did not affect food intake during the dark phase compared to vehicle (data not shown).

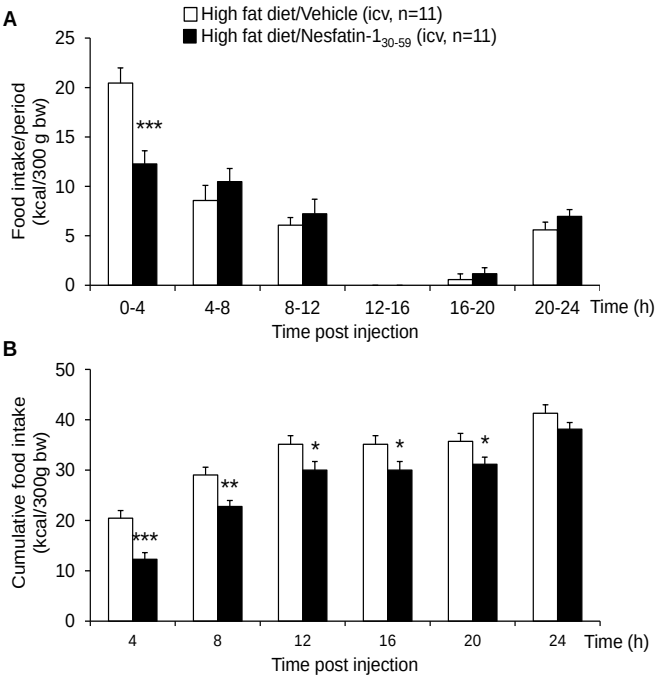

Figure 7. Nesfatin-1$_{30-59}$ (0.9 nmol/rat) or vehicle was injected icv in DIO rats and food intake monitored using the automated food intake monitoring system. Food intake is expressed as food intake/4 h periods (A) and cumulative food intake (B). n = 11/Group; *p < 0.05, **p < 0.01, ***p < 0.001

Nesfatin-1$_{30-59}$ reduces food intake during the first 4 h post icv injection and resultes in a 20-h reduction of cumulative food intake in DIO rats. Nesfatin-1$_{30-59}$ reduced food intake post icv injection in DIO rats with a delayed

onset compared to vehicle. Food intake was reduced during the first 4 h period at the dose of 0.9 nmol/rat (-40%, $p < 0.001$; Fig. 7A). Analysis of hourly food intake showed that food intake was mainly reduced during the 3rd and 4th h post icv injection (71% and 74%, respectively compared to vehicle, $p < 0.05$), resulting in a reduction of cumulative food intake over a period of 20 h (-13%, $p < 0.05$; Fig. 7B).

Feeding microstructure analysis in the period of the first 4 h showed that icv injected nesfatin-1$_{30-59}$ reduces meal frequency, while meal size is not altered. During the first 4 h post icv injection, nesfatin-1$_{30-59}$ reduced bout (-33%, $p < 0.05$; Fig. 8C) and meal frequency (-27%, $p < 0.01$; Fig. 8D) as well as eating rate (-35%, $p < 0.01$; Fig. 8G). Inter-meal intervals (+53%, $p < 0.01$; Fig. 8E) and satiety ratio (-81%, $p < 0.01$; Fig. 8I) were increased. Bout size (Fig. 8A), meal size, meal duration (Fig. 8F) as well as time spent in meal were not affected (Fig. 8H).

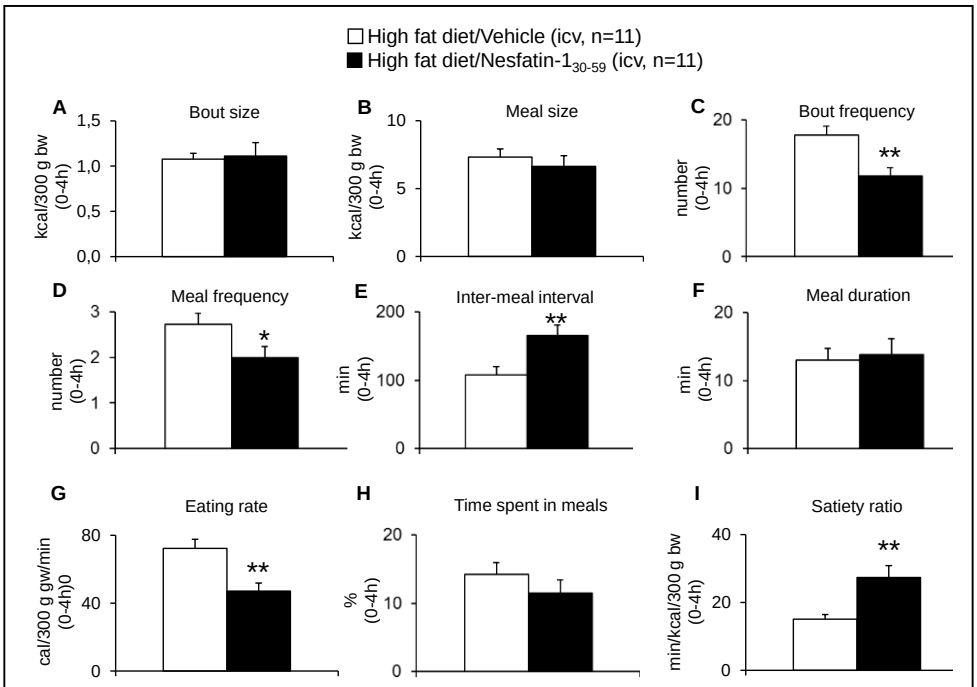

Figure 8. Nesfatin-1$_{30-59}$ (0.9 nmol/rat) or vehicle were injected icv and the food intake microstructure assessed during the 0-4 h period. n = 11/Group; *p < 0.05, ** p < 0.01

5.3 Publication 3: A RAPID method for blood processing to increase the yield of plasma peptide in human blood

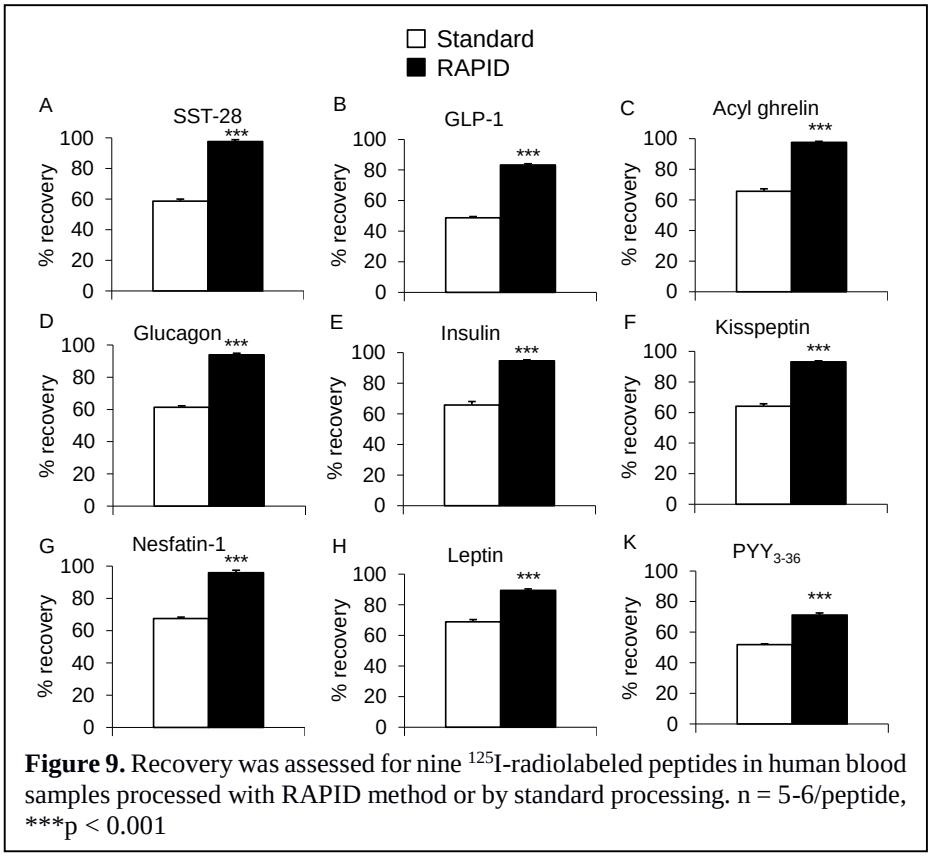

Figure 9. Recovery was assessed for nine ^{125}I-radiolabeled peptides in human blood samples processed with RAPID method or by standard processing. n = 5-6/peptide, ***p < 0.001

The RAPID method increases the yield of ^{125}I-radiolabed peptides in human blood compared to standard procedure. After RAPID blood processing the yield of somatostatin-28 (+39%; Fig. 9A), GLP-1 (+35%; Fig. 9B), acyl ghrelin and glucagon (+32%; Fig. 9C+D), insulin and kisspeptin (+29%; Fig. 9E+F), nesfatin-1 (+28%; Fig. 9G), leptin (+21%; Fig. 9H) and peptide Y_{3-36} (+19%; Fig. 9K) was significantly higher ($p < 0.001$) compared to standard blood processing (EDTA blood on ice).

Ghrelin elutes at the correct position in HPLC following RAPID procedure. To assess the molecular form of the peptide, HPLC was performed after RAPID or standard blood processing. Acyl ghrelin eluted at the expected position in HPLC after RAPID processing, whereas after standard processing an earlier peak was observed most likely representing desacyl ghrelin (Fig. 10). Standard blood processing resulted in a 62% degradation of acyl ghrelin, which was prevented by RAPID processing.

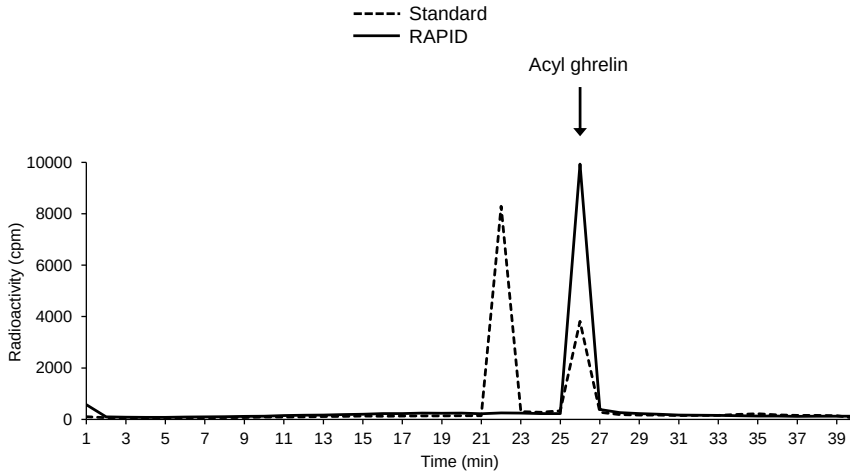

Figure 10. ^{125}I-radiolabeled acyl ghrelin was added to human blood and samples processed using the RAPID method or standard blood processing. HPLC was performed to assess the molecular form of the peptide. The arrow indicates the expected elution position.

6. Discussion

The assessment of food intake is essential for understanding the mechanisms by which food intake regulatory peptides affect hunger and satiety. Since manual measurement disturbs the animals which may affect the results, an automated food intake monitoring device previously established for the use in mice (36,42) has been established in rats. Animals accustomed quickly to the automated food intake monitoring system as indicated by linear body weight gain after separation in single housing cages and feeding from the hopper. Moreover, rats showed a normal food intake and a physiological behavior satiety sequence indicating a transition of feeding towards resting behavior as a well-established surrogate marker for physiological behavior in rodents (43). Lastly, animals did not show any signs of abnormal behavior.

Afterwards the system was used to investigate the effects of a GOAT inhibitor (GO-CoA-Tat) on food intake and feeding microstructure. We showed that an acute intraperitoneal injection of the GOAT inhibitor at the beginning of dark phase reduces food intake in rats. GO-CoA-Tat dose-dependently reduced food intake with a maximum effect at a dose of 96 µg/kg while lower and higher doses had no effects. Whether this U-shaped dose-response curve is due to partial stimulating effects of the GOAT inhibitor at higher doses will have to be further investigated. This reduction was delayed in onset and observed 2 h after ip injection. Rats usually eat during the dark phase (44) and it is well established that circulating levels of ghrelin are up-regulated at the onset of the dark phase (45) likely contributing to the stimulation of food intake (27,28) after acylation by GOAT (26). In the light of the fact that ghrelin has a half-life of approximately 30 minutes (46), the delayed anorexigenic effect of GOAT inhibition may be due to the stimulation of food intake by already acylated ghrelin and then, subsequently the decrease of acyl ghrelin levels due to reduced GOAT signaling. This assumption is supported by the observation of reduced circulating acyl ghrelin levels at 2 h post GOAT inhibitor injection, while desacyl ghrelin levels were not altered.

Analysis of the food intake microstructure of the first 2-h period showed that the inhibition of GOAT reduced dark phase food intake through a reduction of meal frequency, while meal size was not altered. This data shows that peripheral inhibition of GOAT induces satiety without affecting satiation (34,35). One study in mice, investigating the effect of the ghrelin agonist BIM-28131 injected ip, reported an increase of meal frequency and meal size (47). The alteration of satiety described in this study supports the present data. However, the modulation of satiation (in contrast to satiety) observed after ip injection of BIM-28131 is different may be due to species differences (rats *versus* mice), different assessment of food intake (automated food intake monitoring system for solid food *versus* automated dispenser for micropellets) and/or additional pharmacological properties of

BIM-28131 and GO-CoA-Tat. Taken together, the first study showed the validation of an automated food intake monitoring system for the use in rats and described the food intake microstructure following acute pharmacological blockade with an increase in satiety, whereas satiation is not altered.

In the second study we used the previously validated system to assess the effects of icv injected nesfatin-1_{30-59} on feeding microstructure in normal weight and DIO rats. Nesfatin-1_{30-59} reduced dark phase food intake in normal weight rats fed a standard rodent diet with a delayed onset and a maximum effect in the 5th h post icv injection. This food intake reducing effect resulted in a reduction of cumulative food intake over the whole measurement period of 24 h. Previous studies investigating the food intake reducing effect of full length nesfatin-1_{1-82} in rats reported a maximum reduction of food intake in the 3rd post icv injection with a decrease of cumulative food intake over a 6-h observation period (11). These results confirm the assumption of nesfatin-1_{30-59} being the active core of nesfatin-1_{1-82} as previously hypothesized in mice (36,37). Interestingly, nesfatin-1_{30-59} and nesfatin-1_{1-82} exert a different kinetic of anorexigenic action with a more delayed and more prolonged mode of action of nesfatin-1_{30-59}. Whether altered diffusion capacities after icv injection, differential interaction with the nesfatin-1 receptor or additional receptor binding account for these changes will have to be further investigated. The effective dose of nesfatin-1_{30-59} reducing food intake in the present study (0.9 nmol/rat) is comparable to the doses used in mice (0.3 and 0.9 nmol/mouse) (36). However, they were higher than doses of nesfatin-1_{1-82} shown to reduce food intake in rats before (5pmol) (11). One might speculate that nesfatin-1_{1-82} has a higher potency to bind the -yet unknown- nesfatin-1 receptor compared to nesfatin-1_{30-59}. However, this needs to be further investigated.

Analysis of feeding microstructure in the 4-8 h period showed that icv injection of nesfatin-1_{30-59} reduced food intake by reducing meal size, whereas meal frequency and inter-meal intervals were not affected. Therefore, nesfatin-1_{30-59} icv injected increased satiation, while satiety was not altered. Interestingly, these results are different from the feeding microstructure pattern following $_{59}$ icv injection of nesfatin-1_{30-59} observed in mice before where satiety was increased and satiation was not affected (36), pointing towards species differences. Moreover, icv injection of full length nesfatin-1_{1-82} resulted in both increased satiety and satiation in mice (8). These differential effects give rise to different receptor binding of nesfatin-1_{30-59} and nesfatin-1_{1-82}. However, to further answer this question the nesfatin-1 receptor has to be identified.

The signaling of nesfatin-1_{1-82} has already been investigated, indicating three major pathways. First, nesfatin-1_{1-82} reduces food intake by downstream, melanocortin 3/4 signaling, an effect abolished by co-injection of the melanocortin 3/4 receptor agonist SHU9119 (1). Second, nesfatin-1_{1-82} reduces food intake by activation of oxytocin signaling in the paraventricular nucleus, an effect abolished by central injection of H4928 [an oxytocin-receptor agonist (48)]. Third, nesfatin-1_{1-82}

reduces food intake in rats due to activation of the corticotropin releasing factor 2 (CRF_2) receptor (11), an effect abolished by co-injection of astressin$_2$-B (a CRF_2 antagonist). In contrast, signaling pathways for nesfatin-1_{30-59} are not well understood yet. Nesfatin-1_{30-59} induces an up-regulation of proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) in the nucleus of the solitary tract (NTS) in mice (37). The anorexigenic signaling of the major cleavage product of POMC, α-melanocyte stimulating hormone (α-MSH) is mediated by binding of the melanocortin receptor family, including the melanocortin 3/4 receptor (49). Interestingly, injection of the melanocortin 3/4 receptor agonist MTII into the 3rd and 4th brain ventricle reduced meal size, whereas meal frequency was not affected (50,51). This pattern is similar to our observation in this study post icv injection of nesfatin-1_{30-59}, suggesting that nesfatin-1_{30-59} might activate the POMC - α-MSH - melanocortin 3/4 receptor signaling cascade to reduce food intake.

In contrast to the growing evidence on the anorexigenic effects of central nesfatin-1, the role of peripheral nesfatin-1 is not clear and current data are controversial. Although one group showed a reduction of food intake post ip injection of nesfatin-1_{30-59} in mice (37), subsequent studies in rats (11) or mice (8) could not detect any peripheral anorexigenic effects of nesfatin-1. Only one study in male Fischer 344 rats, showed that chronic subcutaneous administration of nesfatin-1_{1-82} (50 µg/kg weight/d) resulted in a reduction of cumulative food intake on the 1st and 7th day of the experiment (22). Interestingly, this study also showed an anorexigenic effect of nesfatin-1_{1-82} following acute ip injection (22). Here, we additionally investigated whether nesfatin-1_{30-59} would affect food intake following ip injection in normal weight rats and could not detect an anorexigenic effect, even at 30-fold higher doses compared to central injection. These findings underline the assumption that the anorexigenic effect of nesfatin-1 is centrally mediated, whereas peripheral nesfatin-1 does not affect food intake after acute injection and seems to have different effects including glucose homeostasis (18) or regulatory processes in the reproductive system (21,52,53).

Since food intake regulatory hormones and signaling pathways are altered under conditions of obesity (54), we investigated the effect of icv injected nesfatin-1_{30-59} on food intake in DIO rats, a well-established animal model mimicking human alimentary obesity (55). Nesfatin-1_{30-59} also reduced food intake at the same dose in DIO rats fed a high fed diet as observed before in normal weight rats fed standard rodent diet, although the time kinetic was different. In DIO rats, the maximum effect occurred during the 3rd and 4th h post injection and resulted in a reduction of cumulative food intake over a period of 20 h. The underlying feeding microstructure was different in DIO rats compared to normal weight rats, showing increased satiety (decreased meal frequency), while satiation (unaltered meal size) was not affected. Apart from the different effects of nesfatin-1_{30-59} and nesfatin-1_{1-82}, these results give rise to the assumption that nesfatin-1_{30-59} acts differently under conditions of DIO compared to normal weight rats, probably due to different downstream signaling

or receptor binding. Rats fed a high fat diet show significantly lower circulating NUCB2/nesfatin-1 levels compared to control (56,57), which might result in a different (more sensitive) response of the unknown nesfatin-1-receptor to exogenously administered nesfatin-1. As described above, our group showed that the CRF_2 receptor plays an important role in the food intake reducing effect of nesfatin-1_{1-82} in rats under normal weight conditions (11). Moreover, brain injection of urocortin 3 (a selective CRF_2 receptor agonist) at a dose of 1µg/rat reduced food intake in rats with a maximum anorexigenic effect during the 3rd and 4th h after injection (34). Furthermore, urocortin 3 decreased meal frequency, whereas meal size was not altered (34). This pattern is very similar to the one observed in our study after nesfatin-1_{30-59} icv injection in DIO rats. Additionally, it was shown, that the cleavage of POMC to α-MSH is reduced under conditions of obesity (58), which might result in a predominance of the CRF_2 receptor signaling pathway to mediate nesfatin-1_{30-59}'s anorexigenic action under DIO conditions compared to normal weight.

In summary, in the second study we demonstrate that nesfatin-1_{30-59} is the active core of nesfatin-1_{1-82} to reduce food intake under normal weight and DIO conditions. However, the feeding microstructure differs with an increase of satiation in normal weight and an increase of satiety under DIO conditions. This is likely due to differential receptor binding and/or signaling pathways activated. Furthermore we shown that the effects of nesfatin-1_{30-59} are centrally mediated, as ip injection of nesfatin-1_{30-59} in much higher concentrations does not affect food intake.

In the third study, we established a blood processing method for the use in human blood. The assessment of endogenous feeding regulatory peptides is important to elucidate their role in the regulation of hunger and satiety. Especially in (e.g. metabolic) diseases with often only subtle differences between groups it is of great importance to yield a high amount of the circulating peptides in order to detect possible differences. In the present study the RAPID blood processing previously established for the use in rats (39) improved the recovery for all nine ^{125}I-radiolabeled peptides tested compared to standard blood processing (EDTA blood on ice). These results are comparable with our previous study showing an improved recovery for 11 of 12 peptides selected to represent a wide range of size and charge, following RAPID blood processing compared to standard blood processing in rats (39). Moreover, the RAPID method allowed the detection of the correct molecular form as shown for acyl ghrelin, while after standard blood processing two thirds of acyl ghrelin were degraded to desacyl ghrelin as shown by HPLC. This is important as desacyl ghrelin counteracts the effects of ghrelin (59,60) and seems to be a physiologically active peptide itself (61). Therefore, it is inalienable to detect the correct molecular form of ghrelin. Established in rats, the RAPID method is likely suited for the use in humans as well. Besides these positive aspects of the RAPID method, some limitations and/or considerations should be mentioned. Compared to standard blood processing the RAPID

method is more expensive, needs more time and requires more training before first use. Therefore, the method may be predominantly used in the research setting.

In conclusion, the RAPID method is transferable to humans and improves recovery of all peptides analyzed. Additionally, the RAPID method allows the detection of the correct molecular form of ghrelin compared to standard processing and should be considered as a tool to analyze circulating peptides when subtle differences are expected. Whether a specific peptide requires processing by this method should be tested beforehand in a pilot study.

Summarized, the three studies presented here show that the automated food intake monitoring previously used in mice before is also suited for the use in rats. We could show that peripheral injection of GOAT inhibitor reduces dark phase food intake in rats due to a reduced meal frequency with a maximum effect in the second hour following ip injection. The same automated food monitoring system was used to investigate the anorexigenic effect of nesfatin-$1_{30\text{-}59}$ in normal weight and DIO rats showing that nesfatin-$1_{30\text{-}59}$ reduces the 24-h cumulative food intake with a maximum effect during the 5^{th} hour post icv injection. This reduction is due to a reduction in meal size, whereas meal frequency was not altered, showing that nesfatin-$1_{30\text{-}59}$ increases satiation in normal weight rats. In DIO rats, nesfatin-$1_{30\text{-}59}$ reduces 20-h cumulative food intake with a maximum effect in the 3^{rd} and 4^{th} hour post icv injection. Here, the reduction is due to reduced meal frequency, while meal size is not altered. These results show, that nesfatin-$1_{30\text{-}59}$ acts differently in DIO rats compared to normal weight rats. In the third study we established the RAPID blood processing method for the use in human that improved the yield of all nine ^{125}I-radiolabeled peptides tested. Furthermore, we showed that the RAPID method allows the detection of the correct molecular form of acyl ghrelin assessed by HPLC, whereas after standard blood processing two thirds of acyl ghrelin were degraded. Therefore, this method is likely to be a tool for peptide research in humans.

7. Literature

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8. Affidavit

I, Philip Prinz certify under penalty of perjury by my own signature that I have submitted the thesis on the topic '**Nesfatin-1$_{30-59}$ alters the food intake microstructure in rats'** I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) corresponds to the URM (s.o) and are answered by me. My contribution in the selected publication for this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

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Date Philip Prinz

<u>Detailed Declaration of Contribution</u>

Philip Prinz contributed to the following publication summarized in this thesis:

Publication 1:

Teuffel P, Wang L, **Prinz P**, Goebel-Stengel M, Scharner S, Kobelt P, Hofmann T, Rose M, Klapp BF, Reeve JR Jr, Stengel A. Treatment with the ghrelin-O-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats. J Physiol Pharmacol. 2015 Aug;66(4):493-503

Contribution in particular: Performance of animal studies (GOAT injection, daily maintenance of animals and automated food monitoring system, measurement of food intake and partial analysis), contributed to paper writing

Publication 2:

Prinz P*, Teuffel P*, Lembke V, Kobelt P, Goebel-Stengel M, Hofmann T, Rose M, Klapp BF, Stengel A. Nesfatin-1_{30-59} injected intracerebroventricularly differentially affects food intake microstructure in rats under normal weight and diet-induced obese conditions. Front Neurosci. 2015 Nov 23;9:422.

* equal contribution

Contribution in particular: Performance of animal studies (daily maintenance of animals and automated food monitoring system, feeding experiments), contribution to data analysis, writing of the first draft of the paper, finalization of the paper

Publication 3:

Teuffel P, Goebel-Stengel M, Hofmann T, **Prinz P**, Scharner S, Körner JL, Grötzinger C, Rose M, Klapp BF, Stengel A. A RAPID method for blood processing to increase the yield of plasma peptide levels in human blood. J Vis Exp. Accepted for publication.

Contribution in particular: Performance of radioimmunoassays, contribution to writing of the paper

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Date Philip Prinz

9. Print copies of the selected publications

Publication 1:

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Publication 2:

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Nesfatin-1_{30-59} Injected Intracerebroventricularly Differentially Affects Food Intake Microstructure in Rats Under Normal Weight and Diet-Induced Obese Conditions

Philip Prinz[1†], *Pauline Teuffel*[1†], *Vanessa Lembke*[1], *Peter Kobelt*[1], *Miriam Goebel-Stengel*[2], *Tobias Hofmann*[1], *Matthias Rose*[1], *Burghard F. Klapp*[1] *and Andreas Stengel*[1*]

[1] *Division of General Internal and Psychosomatic Medicine, Charité Center for Internal Medicine and Dermatology, Charité-Universitätsmedizin, Berlin, Germany,* [2] *Department of Internal Medicine and Institute of Neurogastroenterology, Martin-Luther-Krankenhaus, Berlin, Germany*

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***Correspondence:**
Andreas Stengel
andreas.stengel@charite.de

[†] *These authors have contributed equally to this work.*

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Nesfatin-1 is well-established to induce an anorexigenic effect. Recently, nesfatin-1_{30-59}, was identified as active core of full length nesfatin-1_{1-82} in mice, while its role in rats remains unclear. Therefore, we investigated the effects of nesfatin-1_{30-59} injected intracerebroventricularly (icv) on the food intake microstructure in rats. To assess whether the effect was also mediated peripherally we injected nesfatin-1_{30-59} intraperitoneally (ip). Since obesity affects the signaling of various food intake-regulatory peptides we investigated the effects of nesfatin-1_{30-59} under conditions of diet-induced obesity (DIO). Male Sprague–Dawley rats fed *ad libitum* with standard diet were icv cannulated and injected with vehicle (5 µl ddH_2O) or nesfatin-1_{30-59} at 0.37, 1.1, and 3.3 µg (0.1, 0.3, 0.9 nmol/rat) and the food intake microstructure assessed using a food intake monitoring system. Next, naïve rats were injected ip with vehicle (300 µl saline) or nesfatin-1_{30-59} (8.1, 24.3, 72.9 nmol/kg). Lastly, rats were fed a high fat diet for 10 weeks and those developing DIO were icv cannulated. Nesfatin-1 (0.9 nmol/rat) or vehicle (5 µl ddH_2O) was injected icv and the food intake microstructure assessed. In rats fed standard diet, nesfatin-1_{30-59} caused a dose-dependent reduction of dark phase food intake reaching significance at 0.9 nmol/rat in the period of 4–8 h post injection (−29%) with the strongest reduction during the fifth hour (−75%), an effect detectable for 24 h (−12%, $p < 0.05$ vs. vehicle). The anorexigenic effect of nesfatin-1_{30-59} was due to a reduction in meal size (−44%, $p < 0.05$), while meal frequency was not altered compared to vehicle. In contrast to icv injection, nesfatin-1_{30-59} injected ip in up to 30-fold higher doses did not alter food intake. In DIO rats fed high fat diet, nesfatin-1_{30-59} injected icv reduced food intake in the third hour post injection (−71%), an effect due to a reduced meal frequency (−27%, $p < 0.05$), while meal size was not altered. Taken together, nesfatin-1_{30-59} is the active core of nesfatin-1_{1-82} and acts centrally to reduce food intake in rats. The anorexigenic effect depends on the metabolic condition with increased satiation (reduction in meal size) under normal weight conditions, while in DIO rats satiety (reduction in meal frequency) is induced.

Keywords: anorexigenic, brain-gut-axis, NUCB2, satiation, satiety

INTRODUCTION

Nesfatin-1_{1-82} was discovered in 2006 by Mori et al. as an anorexigenic peptide derived from the rat brain (Oh-I et al., 2006). It is post-translationally processed from the gene encoding nucleobindin2 (NUCB2) by the pro-hormone-convertase 1/3 (Oh-I et al., 2006). NUCB2 consists of a 24 amino acid N-terminal signal peptide and a protein structure containing 396 amino acids, but only the 396 amino acid sequence is cleaved into the N-terminal nesfatin-1_{1-82}, nesfatin-2_{85-163}, and the C-terminal nesfatin-$3_{166-396}$ (Oh-I et al., 2006). It was shown that nesfatin-1_{1-82}, but not nesfatin-2_{85-163} or nesfatin-$3_{166-396}$, reduces food intake and body weight gain in rats after injection into the third brain ventricle (Oh-I et al., 2006). The anorexigenic effect of central nesfatin-1_{1-82} injected into the lateral, third and fourth brain ventricle, into the cisterna magna or directly into the lateral hypothalamic area, the paraventricular nucleus, or the dorsal vagal complex has been confirmed by various independent groups in several studies in rats (Yosten and Samson, 2009, 2010; Chen et al., 2012; Könczöl et al., 2012; Xia et al., 2012; Dong et al., 2014), mice (Atsuchi et al., 2010; Goebel et al., 2011), and goldfish (Gonzalez et al., 2010; Kerbel and Unniappan, 2012). This converging evidence points toward a physiological role of nesfatin-1_{1-82} in the regulation of food intake.

Following these initial studies, the anorexigenic effect of nesfatin-1 was further characterized investigating the underlying food intake microstructure providing deeper insight into the mechanisms underlying the reduction of food intake (Geary, 2005). In mice, nesfatin-1_{1-82} injected intracerebroventricularly (icv) reduced food intake with a delayed onset, an effect due to a reduction in meal size and meal frequency (Goebel et al., 2011) indicating a stimulatory effect on satiation and satiety (Strubbe and Woods, 2004). However, as species differences often play a role, this characterization is pending in rats.

Recently, the active core of nesfatin-1_{1-82} has been identified, namely the mid fragment nesfatin-1_{30-59} (Shimizu et al., 2009), while the N-terminal nesfatin-1_{1-29}, and the C-terminal nesfatin-1_{60-82} had no anorexigenic effect in mice (Shimizu et al., 2009; Stengel et al., 2012a). Although it is not clear yet whether this processing occurs *in vivo*, the characterization is an important step toward a better understanding of the ligand-receptor interaction, keeping in mind that the nesfatin-1 receptor remains to be identified. Again, the characterization of the anorexigenic effect of nesfatin-1_{30-59} is lacking for rats.

After the initial identification of NUCB2/nesfatin-1 in the brain, NUCB2 mRNA, and NUCB2/nesfatin-1 protein expression was also detected in the periphery (Stengel et al., 2009b). Interestingly, the major source of NUCB2/nesfatin-1 is the stomach with much higher NUCB2 mRNA expression levels compared to the brain (Stengel et al., 2009b) likely to act as a gut-brain peptide, a hypothesis supported by the observation that nesfatin-1 is able to cross the blood-brain barrier (Pan et al., 2007). Interestingly, on a cellular level NUCB2/nesfatin-1 is co-expressed with ghrelin, the major orexigenic peptide signaling from the gut to the brain (Al Massadi et al., 2014), within the same cells in rats (Stengel et al., 2009b), and humans (Stengel et al., 2013a). This led to the assumption that these endocrine cells in the stomach can regulate food intake in both directions, either stimulate *via* the release of ghrelin or inhibit *via* the release of NUCB2/nesfatin-1 (Stengel and Taché, 2012b). However, data on the peripheral effects of nesfatin-1 on food intake are inconsistent. While several studies observed no effects of nesfatin-1 on food intake in rats (Stengel et al., 2009a) or mice (Goebel et al., 2011), one study described an anorexigenic effect of nesfatin-1_{30-59} following intraperitoneal (ip) injection of high doses in mice (Shimizu et al., 2009). However, no data in rats exist so far.

The therapeutic potential of NUCB2/nesfatin-1 was discussed in several review articles (Stengel et al., 2013b), especially in the light of its leptin-independent signaling pathway (Oh-I et al., 2006), a hormone known to be less active under conditions of obesity (also known as leptin resistance) (Crujeiras et al., 2015). However, a detailed characterization of the food intake microstructure under conditions of diet-induced obesity (DIO), a well-established animal model for alimentary obesity (Lutz and Woods, 2012) frequently found in humans, has not been performed yet.

Therefore, the aims of the current study were to investigate the effect of icv injected nesfatin-1_{30-59} on the food intake microstructure in normal weight rats. Next, we investigated whether the effect could also be observed following ip injection in rats. Lastly, to characterize the effect of nesfatin-1_{30-59} on the food intake microstructure in obese animals, DIO rats fed a high fat diet were generated and nesfatin-1_{30-59} injected icv.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Harlan-Winkelmann Co., Borchen, Germany) weighing 280–350 g were first group housed (4 rats/group) under controlled illumination (06.00–18.00 h) and temperature (21–23°C). During this time rats were handled daily to become accustomed to the interaction with the investigators (daily control of body weight, light hand restraint for subsequent icv, or ip injections). Rats had *ad libitum* access to standard rodent diet (D12450B, Research Diets, Inc., Jules Lane, New Brunswick, NJ, USA) and tap water. Animal care and experimental procedures followed institutional ethics guidelines, conformed to the requirements and were approved by the state authority for animal research conduct (Landesamt für Gesundheit und Soziales Berlin, LaGeSo Berlin; animal protocol # G 0131/11).

Peptides

Rat nesfatin-1_{30-59} (Bachem AG, Weil am Rhein, Germany) was aliquoted in sterile distilled water and stored at −80°C until further use. Purity was assessed by HPLC and mass spectroscopy (manufacturer's information). Directly before administration, rat nesfatin-1_{30-59} was further diluted in sterile ddH_2O for icv

injection or in sterile 0.9% saline (B. Braun AG, Melsungen, Germany) for ip injection to reach the final concentrations detailed below.

Diets

For the induction of DIO, rats were fed a high fat diet (D12451, 45% calories from fat, 35% from carbohydrates, and 20% from protein, 4.7 kcal/g diet, Research Diets Inc.) for a period of 10 weeks. Control rats were kept on a standard rodent diet (D12450B, 10% calories from fat, 70% from carbohydrates, and 20% from protein, 3.9 kcal/g diet, Research Diets Inc.). Body weight and food intake were assessed daily. At the end of the 10-week feeding period, the 50% of rats gaining the most body weight were selected as DIO.

Intracerebroventricular Cannulation

Rats were chronically icv cannulated as described before (Stengel et al., 2010a,b). Briefly, rats were anesthetized with an ip injection of 100 mg/kg ketamine (Ketanest™, Curamed, Karlsruhe, Germany) and 10 mg/kg xylazine (Rompun™, 2%, Bayer, Leverkusen, Germany). Afterwards, rats were placed in a stereotactic apparatus to implant a chronic 22-gauge guide cannula into the right lateral brain ventricle. The coordinates for the placement (from bregma: 0.8 mm posterior, 1.5 mm right lateral, and 3.5 mm ventral) were based on the atlas of Paxinos and Watson (2006). The chronic 22-gauge guide cannula was fixed by dental cement, anchored by four sterile screws (Plastic One Inc., Roanoke, VA, USA), and the wound was sutured. After the surgery, animals were housed individually and allowed to recover for 5 days. During this time, rats were handled daily to adapt to the icv injection procedure (light hand restraint for 1 min). For the icv injection a 28-gauge cannula was connected to a 25 µl Hamilton syringe by a PE-50 tube (BD intramedic polyethylene tubing, Clay Adams, NJ, USA) and a volume of 5 µl was injected over a period of 1 min into the conscious rat.

The correct placement of the cannula was verified after the experiments by injecting 5 µl of 0.1% toluidine blue and visualizing the spreading of the dye throughout the brain ventricular system. No animals had to be excluded due to erroneous placement of the cannula.

Automated Food Intake Monitoring

The BioDAQ episodic food intake monitoring system (BioDAQ, Research Diets Inc.) was used to investigate the food intake microstructure in rats. This system has been established before for the use in mice (Goebel et al., 2011) and recently also rats (Teuffel et al., 2015).

Rats were habituated for 1 week to the single housing (in regular housing cages with normal bedding and enrichment) and feeding from the hopper and as shown before quickly adapted to these conditions within 2–3 days indicated by normal food intake and regular body weight gain (Teuffel et al., 2015). Water was provided *ad libitum* from regular water bottles. Food was provided in low spill food hoppers placed on a balance. The "bridging phenomenon," that occurs when retained food spillage underneath the gate causes erroneous measurements, was observed very rarely by use of the diet described above and daily maintenance (cleaning) of the hoppers.

The BioDAQ food intake monitoring system weighs the hopper with food (±0.01 g) every second and detects "not eating" as weight stable and "eating" as weight unstable. "Feeding bouts," defined as change in stable weight before and after an event, are recorded as vectors with starting time, duration and amount of food consumed (Teuffel et al., 2015). The feeding bouts are separated by an inter-bout interval (IBI) and meals can consist of one or more bouts. Furthermore, meals are separated by inter-meal intervals (IMI), in rats defined by a duration of 15 min (Teuffel et al., 2015). The minimum meal size in rats was defined as 0.01 g and therefore food intake was considered as one meal when the feeding bouts occurred within 15 min of the previous response and the content of food was equal to or greater than 0.01 g (Teuffel et al., 2015). If the interval between meals was greater than 15 min, the feeding bouts were considered as a new meal. Meal parameters assessed in this study encompassed bout size (g/bout), meal size (g/meal), bout frequency (number/period), meal frequency (number/period), inter-meal interval (min), meal duration (min/meal), eating rate (mg/min), time spent in meals (%) as well as the satiety ratio (min/g food eaten) calculated using the parameters described above. These parameters were extracted from the software and visualized using the Data Viewer (BioDAQ Monitoring Software 2.3.07, Research Diets Inc.). Data analysis was performed in Excel (Microsoft). For better comparability between rats fed control or high fat diet, food intake was expressed in kcal/300 g body weight (bw).

EXPERIMENTAL PROTOCOLS

Food Intake Experiments

In the first experiment icv cannulated rats fed *ad libitum* with standard rodent diet and accustomed to the food intake monitoring system as described above were icv injected with nesfatin-1_{30-59} (0.1, 0.3, or 0.9 nmol/rat) or vehicle (5 µl ddH_2O) directly before the onset of the dark phase. The dose of nesfatin-1_{30-59} was based on our previous study in mice (Stengel et al., 2012a). The food intake microstructure was assessed over a period of 24 h. The experiment was repeated once in a crossover design; rats were allowed to recover for 5 days in between the experiments. During two experiments, behavior was determined by observation of the locomotor activity and grooming (including washing and licking).

In order to assess whether the effect of nesfatin-1_{30-59} is also mediated peripherally, naïve rats accustomed to the food intake monitoring system, handled for ip injections and fed *ad libitum* with standard rodent diet were injected ip with nesfatin-1_{30-59} (8.1, 24.3, or 72.9 nmol/kg) or vehicle (300 µl saline) directly before the dark phase started. These doses were based on the observation that higher doses are required peripherally compared to brain injections (Shimizu et al., 2009). Therefore, up to 30-times higher doses were used peripherally compared

to our icv experiments. The microstructure was assessed over a period of 24 h. This experiment was repeated once in a crossover design. Rats were allowed to recover for 5 days in between the experiments.

Since the hypothalamic regulation of food intake is altered under conditions of DIO (Velloso and Schwartz, 2011), we also investigated the effect of nesfatin-1_{30-59} on the food intake microstructure in chronically icv cannulated DIO rats accustomed to the food intake monitoring system and fed a high fat diet. Nesfatin-1_{30-59} (0.9 nmol/rat, dose based on first experiment of the present study) or vehicle (5 µl ddH_2O) were injected icv directly at the beginning of the dark phase and the food intake microstructure was assessed over a period of 24 h. The experiment was repeated once in a crossover design and rats were allowed to recover for 5 days in between the experiments.

Statistical Analysis

Distribution of the data was determined by using the Kolmogorov-Smirnov test. Data are expressed as mean ± sem and were analyzed by One-way analysis of variance (ANOVA) followed by Tukey *post hoc* test or Two-way or Three-way ANOVA followed by the Holm–Sidak method. Differences between groups were considered significant when $p < 0.05$ (SigmaStat 3.1., Systat Software, San Jose, CA, USA).

RESULTS

Nesfatin-1_{30-59} Injected Intracerebroventricularly at the Onset of the Dark Phase Reduces Food Intake by Decreasing Meal Size in Normal Weight Rats

Nesfatin-1_{30-59} (0.1, 0.3, or 0.9 nmol/rat) injected icv in normal weight rats fed *ad libitum* with standard rodent diet induced a dose-related reduction of dark phase food intake with a delayed onset between the 4–8 h period post injection and observed at a dose of 0.9 nmol/rat compared to vehicle (5 µl ddH_2O, −29%, $p < 0.05$; **Figure 1A**). Therefore, this dose was used for all further analyses. This reduction resulted in a decrease of cumulative food intake which was observed over the 24-h measurement period (−12% vs. vehicle, $p < 0.05$; **Figure 1B**) indicating a long-lasting effect. Two-way ANOVA showed a significant influence of dose [$F_{(3, 239)} = 11.2$, $p < 0.001$] and time [$F_{(5, 239)} = 109.2$, $p < 0.001$].

Analysis of hourly food intake indicated that the main anorexigenic effect occurred during the fifth hour post injection with a −75% reduction of food intake following nesfatin-1_{30-59} compared to vehicle ($p = 0.05$; **Figure 2**). Moreover, parameters of the first meal were mostly unaffected by icv nesfatin-1_{30-59} (**Table 1**) further pointing toward a delayed onset

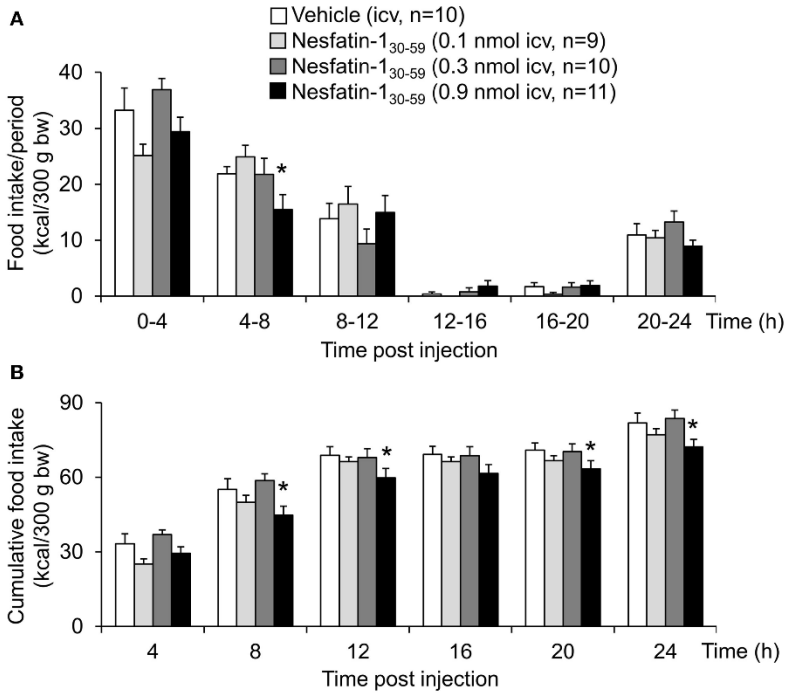

FIGURE 1 | Nesfatin-1_{30-59} reduces food intake for 24 h in *ad libitum* fed normal weight rats. Nesfatin-1_{30-59} injected intracerebroventricularly (0.1, 0.3, and 0.9 nmol/rat) decreases dark phase food intake of standard rodent diet between the fourth and eighth hour and reduces cumulative food intake for 24 h post injection at a dose of 0.9 nmol/rat. Food intake was measured by an automated episodic food intake monitoring system and expressed as food intake (kcal/300 g bw)/4 h periods **(A)** and cumulative food intake over a period of 24 h **(B)**. Each bar represents the mean ± sem. *$p < 0.05$ vs. vehicle.

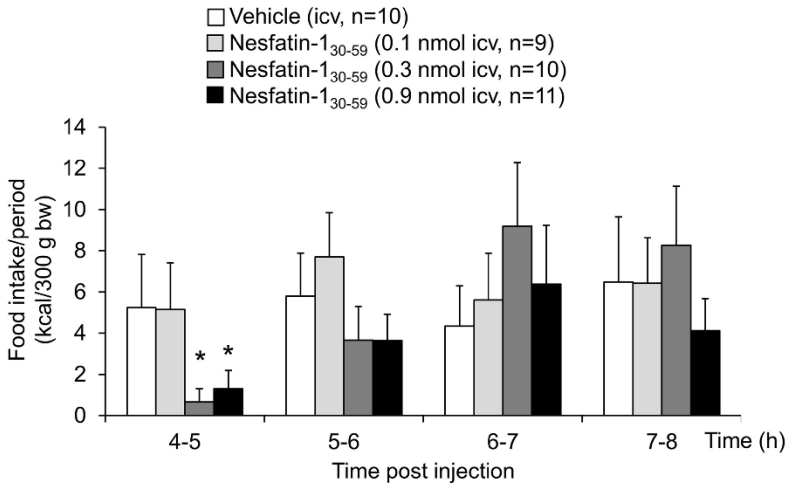

FIGURE 2 | Nesfatin-1_{30-59} reduces food intake during the fifth hour post injection in normal weight rats. In rats fed *ad libitum* with standard rat diet, nesfatin-1_{30-59} injected intracerebroventricularly (0.1, 0.3, and 0.9 nmol/rat) induces a dose-related decrease in dark phase food intake during the fifth hour post injection reaching significance at 0.3 and 0.9 nmol/rat. Food intake is expressed as hourly food intake (kcal/300 g bw) during the 4–8 h period post injection. Each bar represents the mean ± sem. *$p < 0.05$ vs. vehicle.

TABLE 1 | Nesfatin-1_{30-59} injected icv before the dark phase does not alter the parameters of the first meal in *ad libitum* fed normal weight rats.

Parameter	Group	
	Vehicle (5 µl ddH_2O icv)	Nesfatin-1_{30-59} (0.9 nmol icv)
Latency to first meal (min)	2.09 ± 0.55	1.88 ± 0.58
Size of first meal (kcal/300 g bw)	5.85 ± 0.84	7.73 ± 0.90
Duration of first meal (min)	10.09 ± 1.64	13.54 ± 1.68
Eating rate of first meal (cal/300 g bw/min)	35.80 ± 3.94	29.69 ± 2.86
Inter-meal interval (min)	46.71 ± 6.44	69.32 ± 4.59*
Satiety ratio after first meal (min/kcal/300 g bw food eaten)	9.16 ± 1.53	11.80 ± 1.93

*icv, intracerebroventricular; mean ± sem; n = 10–11/group; *$p < 0.05$.*

of the anorexigenic action. However, the interval following the first meal was prolonged by nesfatin-1_{30-59} compared to vehicle (+48%, $p < 0.05$; **Table 1**). Due to the significant reduction of food intake during the 4–8 h period post injection, the underlying food intake microstructure was analyzed during this period. Nesfatin-1_{30-59} (0.9 nmol/rat icv) reduced meal size (−45%, $p < 0.05$; **Figure 3B**), meal duration (−54%, $p < 0.05$; **Figure 3F**), and the time spent in meals (−41%, $p < 0.05$; **Figure 3H**), while bout size (**Figure 3A**), bout frequency (**Figure 3C**), meal frequency (**Figure 3D**), inter-meal intervals (**Figure 3E**), and eating rate (**Figure 3G**) were not significantly affected compared to vehicle ($p > 0.05$). At the same time, the satiety ratio was increased following nesfatin-1_{30-59} compared to vehicle injected icv (+174%, $p < 0.05$; **Figure 3I**). Manual observation indicated no abnormal behavior following icv injection of nesfatin-1_{30-59} (data not shown).

Nesfatin-1_{30-59} Injected Intraperitoneally at the Onset of the Dark Phase Does Not Reduce Food Intake or Alter the Food Intake Microstructure of Normal Weight Rats

Nesfatin-1_{30-59} (8.1, 24.3, or 72.9 nmol/kg, ip) injected at higher doses at the onset of the dark phase did not robustly reduce food intake either expressed as food intake/period or cumulative food intake compared to vehicle ($p > 0.05$; **Table 2**). Only the highest dose (72.9 nmol/kg, ip) led to a slight and short-lasting reduction of food intake during the seventh hour post injection compared to vehicle ($p < 0.05$) that did not translate into a reduction of cumulative food intake ($p > 0.05$; **Table 2**). Also analysis of the food intake microstructure in the 4–8 h period (the period when nesfatin-1_{30-59} injected icv exerted its main effects) indicated no alterations of microstructural parameters compared to vehicle injected ip ($p > 0.05$; **Table 3**). Lastly, parameters of the first meal were not affected by nesfatin-1_{30-59} injected ip compared to control ($p > 0.05$; **Table 4**).

Nesfatin-1_{30-59} Injected Intracerebroventricularly at the Onset of the Dark Phase Reduces Food Intake by Decreasing Meal Frequency of Diet-induced-obese Rats Fed a High Fat Diet

Rats fed a high fat diet (45% calories from fat) for a period of 10 weeks developed DIO and gained significantly more body weight

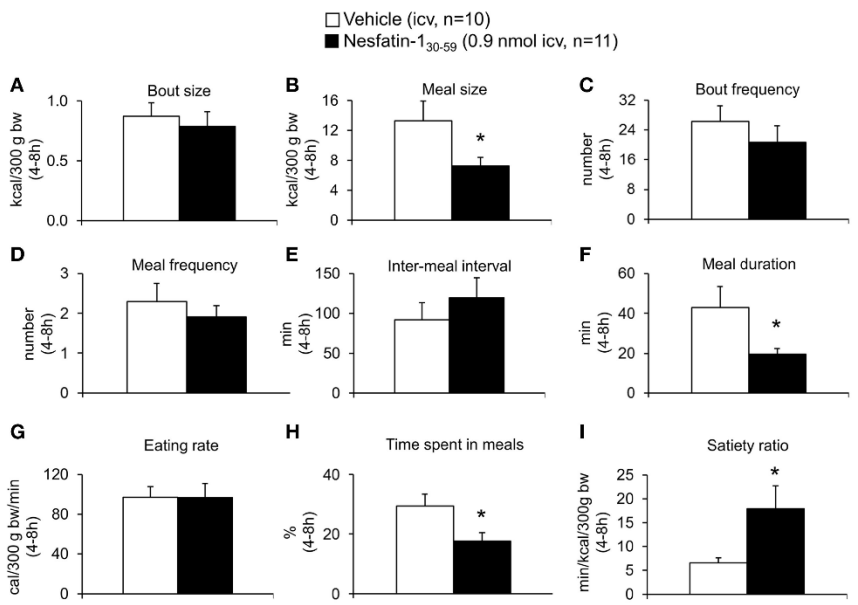

FIGURE 3 | Nesfatin-1_{30-59} reduces meal size, while meal frequency is not altered in normal weight rats. Nesfatin-1_{30-59} (0.1, 0.3, and 0.9 nmol/rat) or vehicle (5 µl ddH_2O) was injected intracerebroventricularly and the food intake microstructure assessed using an automated episodic food intake monitoring system. Due to the strongest reduction in food intake, the dose of 0.9 nmol/rat was used for all further analyses. During the period of 4–8 h post injection, nesfatin-1_{30-59} reduced meal size **(B)**, meal duration **(F)** and the time spent in meals **(H)**, while bout size **(A)**, bout frequency **(C)**, meal frequency **(D)**, inter-meal intervals **(E)** and eating rate **(G)** were not altered compared to vehicle. The satiety ratio was increased following nesfatin-1_{30-59} **(I)**. Each bar represents the mean ± sem. *$p < 0.05$ vs. vehicle.

TABLE 2 | Nesfatin-1_{30-59} injected ip before the dark phase does not alter food intake in *ad libitum* fed normal weight rats.

Period	Group			
	Vehicle (300 µl saline ip)	Nesfatin-1_{30-59} (8.1 nmol/kg ip)	Nesfatin-1_{30-59} (24.3 nmol/kg ip)	Nesfatin-1_{30-59} (72.9 nmol/kg ip)
FOOD INTAKE PER PERIOD (KCAL/300 G BW)				
0–4 h	41.15 ± 2.20	44.00 ± 2.47	44.42 ± 2.21	44.48 ± 2.22
4–8 h	27.70 ± 2.09	22.06 ± 2.54	25.80 ± 2.24	27.39 ± 1.78
8–12 h	13.15 ± 2.03	19.61 ± 2.10	15.56 ± 2.47	17.33 ± 2.24
12–16 h	0.47 ± 0.32	0.63 ± 0.45	1.59 ± 1.06	2.85 ± 1.48
16–20 h	1.21 ± 0.78	0.32 ± 0.28	1.31 ± 0.90	0.78 ± 0.71
20–24 h	12.51 ± 1.28	10.48 ± 0.81	12.54 ± 1.78	10.83 ± 0.91
CUMULATIVE FOOD INTAKE (KCAL/300 G BW)				
0–4 h	41.15 ± 2.20	44.00 ± 2.47	44.42 ± 2.21	44.48 ± 2.22
0–8 h	68.84 ± 2.56	66.05 ± 2.30	70.23 ± 2.33	71.86 ± 3.16
0–12 h	81.99 ± 2.76	85.67 ± 2.61	85.79 ± 2.74	89.19 ± 3.36
0–16 h	82.47 ± 2.69	86.29 ± 2.68	87.38 ± 2.44	92.05 ± 3.32
0–20 h	83.67 ± 2.79	86.62 ± 2.64	88.69 ± 2.35	92.82 ± 3.45
0–24 h	96.18 ± 2.91	97.09 ± 2.57	101.23 ± 2.36	103.65 ± 3.30

ip, intraperitoneal; mean ± sem; n = 14/group; $p > 0.05$.

compared to rats fed standard rodent diet (10% calories from fat; +21% at 10 weeks, $p < 0.01$; **Figure 4**).

When injected icv in DIO rats, nesfatin-1_{30-59} (0.9 nmol/rat) induced a reduction of food intake during the 0–4 h period post injection compared to vehicle (5 µl ddH_2O, −40%, $p < 0.001$; **Figure 5A**). This reduction resulted in a decrease of cumulative food intake over a period of 20 h (−13% vs. vehicle, $p < 0.05$; **Figure 5B**). Two-way ANOVA indicated a significant influence of treatment [$F_{(1, 131)} = 36.3$, $p < 0.001$] and time [$F_{(5, 131)} = 53.4$, $p < 0.001$]. Analysis of hourly food intake during the 0–4 h period showed that the main anorexigenic effect of nesfatin-1_{30-59} occurred during the third and fourth

TABLE 3 | Nesfatin-1_{30-59} injected ip before the dark phase does not alter the food intake microstructure in the 4–8 h period post injection in *ad libitum* fed normal weight rats.

Parameter	Group			
	Vehicle (300 µl saline ip)	Nesfatin-1_{30-59} (8.1 nmol/kg ip)	Nesfatin-1_{30-59} (24.3 nmol/kg ip)	Nesfatin-1_{30-59} (72.9 nmol/kg ip)
Food intake 4–5 h (kcal/300 g bw)	5.96 ± 2.13	6.09 ± 2.15	5.26 ± 2.06	8.35 ± 2.87
Food intake 5–6 h (kcal/300 g bw)	3.22 ± 2.09	4.21 ± 1.98	6.95 ± 2.52	12.20 ± 3.31
Food intake 6–7 h (kcal/300 g bw)	14.18 ± 3.34	5.76 ± 1.97	6.63 ± 2.89	4.29 ± 1.99*
Food intake 7–8 h (kcal/300 g bw)	4.34 ± 2.00	6.00 ± 2.54	6.97 ± 2.25	2.53 ± 1.50
Bout size 4–8 h (kcal/300 g bw)	0.86 ± 0.06	0.82 ± 0.07	0.87 ± 0.04	0.85 ± 0.05
Meal size 4–8 h (kcal/300 g bw)	17.01 ± 2.39	14.32 ± 1.58	15.35 ± 2.25	18.55 ± 2.11
Bout frequency 4–8 h (number)	32.07 ± 3.39	27.29 ± 1.99	31.00 ± 2.86	32.64 ± 2.83
Meal frequency 4–8 h (number)	1.93 ± 0.22	1.71 ± 0.27	2.00 ± 0.23	1.71 ± 0.19
Inter-meal interval 4–8 h (min)	143.61 ± 31.10	101.41 ± 18.08	147.27 ± 27.32	159.46 ± 31.92
Meal duration 4–8 h (min)	53.67 ± 8.20	47.36 ± 7.52	48.12 ± 8.75	57.40 ± 7.42
Eating rate 4–8 h (cal/300 g bw/min)	109.27 ± 10.94	123.77 ± 11.05	107.61 ± 11.40	125.47 ± 18.96
Time spent in meals 4–8 h (%)	36.65 ± 3.17	29.40 ± 3.94	31.89 ± 3.01	35.49 ± 3.43
Satiety ratio 4–8 h (min/kcal/300 g bw food eaten)	8.17 ± 1.08	7.73 ± 1.66	10.33 ± 1.41	8.47 ± 10.4

*ip, intraperitoneal; mean ± sem; n = 14/group; *p < 0.05 vs vehicle.*

TABLE 4 | Nesfatin-1_{30-59} injected ip before the dark phase does not alter parameters of the first meal in *ad libitum* fed normal weight rats.

Parameter	Group			
	Vehicle (300 µl saline ip)	Nesfatin-1_{30-59} (8.1 nmol/kg ip)	Nesfatin-1_{30-59} (24.3 nmol/kg ip)	Nesfatin-1_{30-59} (72.9 nmol/kg ip)
Latency to first meal (min)	4.83 ± 2.24	9.23 ± 3.67	4.74 ± 1.84	7.40 ± 5.39
Size of first meal (kcal/300 g bw)	11.08 ± 1.59	7.70 ± 0.85	8.55 ± 1.26	14.00 ± 2.92
Duration of first meal (min)	21.15 ± 3.84	16.95 ± 3.24	17.70 ± 4.17	29.35 ± 8.89
Eating rate of first meal (cal/300 g bw/min)	156.85 ± 12.37	152.62 ± 17.56	134.24 ± 13.45	176.78 ± 17.44
Inter-meal interval (min)	48.17 ± 6.94	36.54 ± 4.90	43.12 ± 3.20	49.28 ± 7.17
Satiety ratio after first meal (min/kcal/300 g bw food eaten)	5.04 ± 0.57	5.72 ± 1.19	9.08 ± 3.49	4.40 ± 0.57

ip, intraperitoneal; mean ± sem; n = 14/group; p > 0.05.

hour post injection (−71 and −74%, respectively, compared to vehicle, $p < 0.05$; **Figure 6**) pointing toward a delayed effect. In line with this assumption, parameters of the first meal were not significantly altered following nesfatin-1_{30-59} compared to vehicle (**Table 5**).

Based on the finding that nesfatin-1_{30-59} already decreased food intake in DIO rats during the 0–4 h period post injection, the microstructure was analyzed during this period. Nesfatin-1_{30-59} (0.9 nmol/rat, icv) reduced bout frequency (−33%, $p < 0.05$; **Figure 7C**), meal frequency (−27%, $p < 0.05$; **Figure 7D**), and eating rate (−35%, $p < 0.01$; **Figure 7G**), while bout size (**Figure 7A**), meal size (**Figure 7B**), meal duration (**Figure 7F**), and the time spent in meals (**Figure 7H**) were not significantly affected compared to vehicle ($p > 0.05$). Moreover, inter-meal intervals (+53%, **Figure 7E**) and the satiety ratio (+81%, **Figure 7I**) were increased following icv injection of nesfatin-1_{30-59} ($p < 0.01$ vs. vehicle).

The combined analysis of food intake data of rats fed control or high fat diet using Three-way ANOVA indicated a significant influence of diet [$F_{(1, 257)} = 83.5$, $p < 0.001$], time [$F_{(5, 257)} = 108.1$, $p < 0.001$], and interaction of diet and time [$F_{(5, 257)} = 10.6$, $p < 0.001$] as well as an interaction of treatment and time [$F_{(5,257)} = 2.6$, $p < 0.05$].

DISCUSSION

In the present study we investigated the effect of nesfatin-1_{30-59} on feeding behavior in rats under normal weight and diet-induced obese conditions. Nesfatin-1_{30-59} injected icv reduced dark phase food intake in normal weight rats fed a standard rodent diet. This effect was delayed in onset as parameters of the first meal were not altered with a maximum reduction of food intake observed during the fifth hour post injection and long lasting as it resulted in a reduction of cumulative food intake over

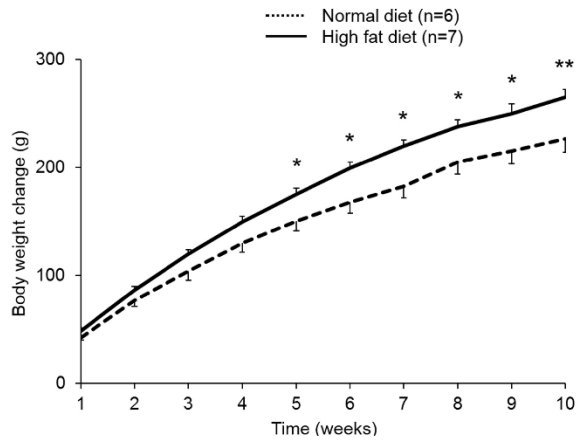

FIGURE 4 | Rats fed a high fat diet develop diet-induced obesity. Rats were *ad libitum* fed either a high fat diet (45% calories from fat, 35% from carbohydrates, and 20% from protein, 4.7 kcal/g) or standard rodent diet (10% calories from fat, 70% from carbohydrates, and 20% from protein, 3.9 kcal/g) over a period of 10 weeks. Rats that gained most body weight (~50%) were selected for the following experiments as diet-induced obese (DIO) rats. DIO rats showed greater body weight gain compared to rats fed standard rodent diet. Data are expressed as mean ± sem. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. vehicle.

the 24 h observation period. These data are in accordance with a previous study in rats where the main reduction following icv full length nesfatin-1_{1-82} was observed during the third hour post injection and also observed over the whole measurement period of 6 h (Stengel et al., 2009a). The difference in the onset of action (third hour after nesfatin-1_{1-82} vs. fifth hour after nesfatin-1_{30-59}) may be due to differential diffusion capacities following icv injection and—more importantly—differential interaction with the yet unknown nesfatin-1 receptor. Lastly, also the interaction with (an) additional receptor(s) has to be considered.

Interestingly, higher doses of nesfatin-1_{30-59} were needed to induce an anorexigenic effect. While nesfatin-1_{1-82} injected icv reduced food intake in rats following injection of 5 pmol, nesfatin-1_{30-59} exerted an anorexigenic effect after icv injection of 0.9 nmol/rat. This difference is likely to result from differential ligand-receptor interaction with full length nesfatin-1_{1-82} being more potent to induce the food intake inhibitory effect at low doses. Whether the difference in potency is due to more selective binding of nesfatin-1_{1-82} to the receptor, slower dissociation or a more potent stimulation of the receptor compared to nesfatin-1_{30-59} will have to be investigated in future studies. The effective dose observed here in rats is comparable to the anorexigenic doses described before in mice (0.3 and 0.9 nmol/mouse nesfatin-1_{30-59} icv; Stengel et al., 2012a).

After establishing nesfatin-1_{30-59} also as an active fragment of nesfatin-1_{1-82} to reduce food intake in rats we investigated the underlying food intake microstructure. Nesfatin-1_{30-59} injected icv reduced food intake by reducing meal size, while meal frequency and inter-meal intervals were not altered. This pattern indicates an increase of satiation (earlier termination of a meal), whereas satiety (later initiation of a new meal after one meal is completed) is not affected. This pattern is different from the one observed before for mice where satiety (reduction in meal frequency) was induced while satiation (meal size was not altered) was not affected by icv nesfatin-1_{30-59} (Stengel et al., 2012a). This difference points toward species differences and highlights the need for cautious translation of results from one species to another. Interestingly, in mice full length nesfatin-1_{1-82} injected icv increased both satiation and satiety (Goebel et al., 2011). This different pharmacodynamics is likely due to a differential receptor binding of full length nesfatin-1_{1-82} and its active core, nesfatin-1_{30-59}.

Early on, the downstream signaling of NUCB2/nesfatin-1 has been investigated. It has been shown that oxytocin is involved in the mediation of nesfatin-1_{1-82}'s anorexigenic effect as nesfatin-1_{1-82} activates oxytocin containing neurons in the paraventricular nucleus of the hypothalamus (PVN) and central administration of the oxytocin-receptor antagonist, H4928 abolished the food intake reduction induced by nesfatin-1_{1-82} in rats (Maejima et al., 2009). Moreover, an involvement of proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) has been indicated as well based on the following observations: First, nesfatin-1_{30-59} was shown to upregulate the mRNA expression of POMC and CART in mice (Shimizu et al., 2009). Second, blockade of α-melanocyte stimulating hormone (α-MSH) signaling, a major anorexigenic cleavage product of POMC (Schwartz et al., 2000), using the melanocortin receptor 3/4 antagonist, SHU9119 also blocked nesfatin-1_{1-82}'s anorexigenic effect (Oh-I et al., 2006). Third, brain ventricular injection of the melanocortin receptor 3/4 agonist, MTII decreased food intake by reducing meal size, while no effect on meal frequency was observed (Azzara et al., 2002; Berthoud et al., 2006), resembling the pattern of action observed after icv injection of nesfatin-1_{30-59} in the present study. Since oxytocin increases the release of POMC in the nucleus of the solitary tract (NTS), nesfatin-1_{30-59} is likely to act *via* an oxytocin→POMC→α-MSH/melanocortin receptor 3/4 pathway to inhibit food intake in rats.

Despite the fact that NUCB2/nesfatin-1 is predominantly expressed in the stomach (Stengel et al., 2009b), the role of peripheral NUCB2/nesfatin-1 is far from being clear. While one study reported an anorexigenic effect following ip injection of nesfatin-1_{30-59} in mice (Shimizu et al., 2009), other studies in rats (Stengel et al., 2009a), or mice (Goebel et al., 2011) did not detect any effect. Similarly, in the present study we did not find an anorexigenic effect of nesfatin-1_{30-59} injected ip in normal weight rats although up to 30-fold higher doses were used. This difference may be due to species differences (mice vs. rats) or different doses used (up to ~40 nmol/mouse vs. ~22 nmol/rat in the present study). However, these findings clearly point toward a central mode of action of nesfatin-1 to reduce food intake, while peripheral nesfatin-1 is likely to have a different main effect such as glucose control (Nakata and Yada, 2013).

Signaling of several food intake regulatory hormones is altered under conditions of obesity (Hellström, 2013). These changes encompass a decrease in circulating ghrelin levels (Ariyasu et al., 2002) which may represent a compensatory action to prevent further overeating. On the other side, the postprandial

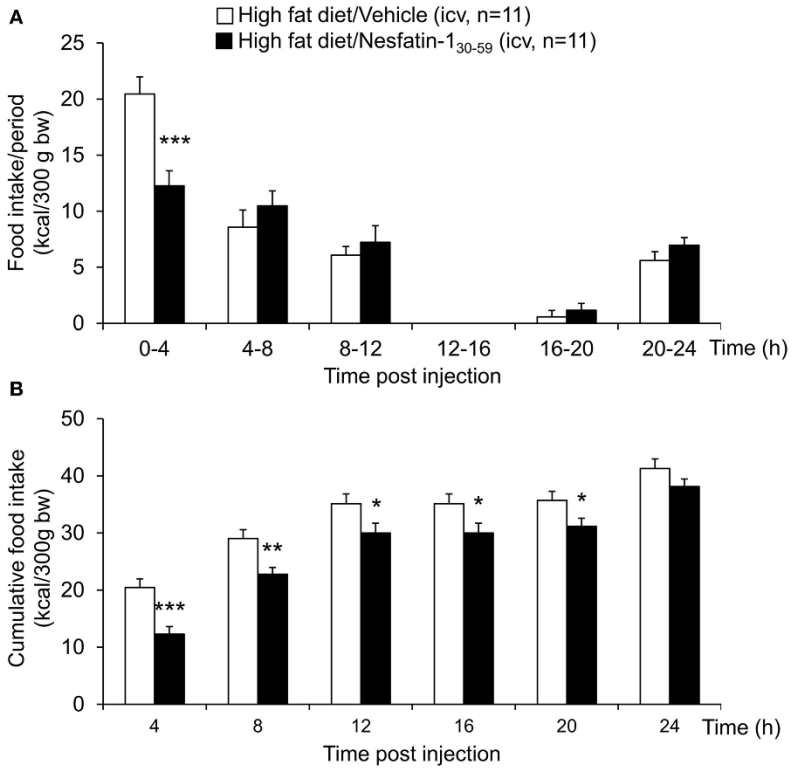

FIGURE 5 | Nesfatin-1_{30-59} reduces food intake for 8 h in *ad libitum* fed diet-induced obese rats. Nesfatin-1_{30-59} (0.9 nmol/rat) injected intracerebroventricularly decreased dark phase food intake in the first 4 h period post injection, an effect that lasted for 8 h compared to vehicle (5 µl dd H_2O). Food intake was assessed using an automated episodic food intake monitoring system and expressed as food intake (kcal/300 g bw)/4 h periods **(A)** and cumulative food intake over a period of 24 h **(B)**. Each bar represents the mean ± sem. $^{*}p < 0.05$ vs. vehicle; $^{**}p < 0.01$ vs. vehicle; $^{***}p < 0.001$ vs. vehicle.

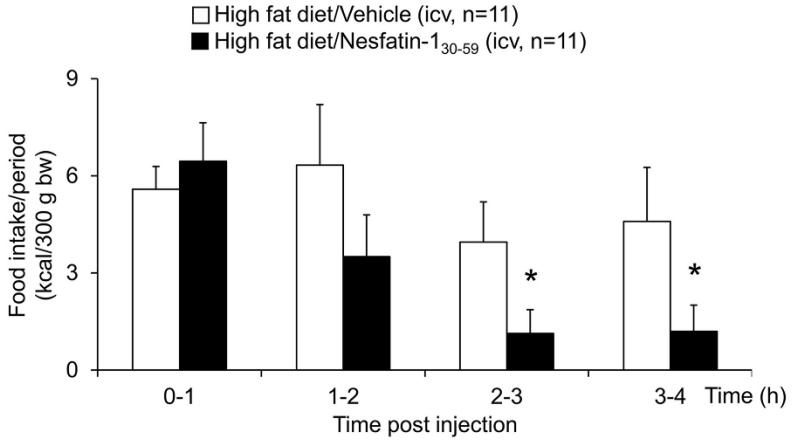

FIGURE 6 | Nesfatin-1_{30-59} reduces food intake in the third and fourth hour post icv injection in diet-induced obese rats. Nesfatin-1_{30-59} injected intracerebroventricularly (0.9 nmol/rat) decreased dark phase food intake in *ad libitum* fed rats during the third and fourth hour post injection compared to vehicle (5 µl dd H_2O). Food intake was assessed using an automated episodic food intake monitoring system and expressed as food intake (kcal/300 g bw)/1 h periods. Each bar represents the mean ± sem. $^{*}p < 0.05$ vs. vehicle.

TABLE 5 | Nesfatin-1_{30-59} injected icv before the dark phase does not alter parameters of the first meal in diet-induced obese rats fed *ad libitum* with high fat diet.

Parameter	Group	
	Vehicle (5 µl ddH_2O icv)	Nesfatin-1_{30-59} (0.9 nmol icv)
Latency to first meal (min)	14.74 ± 6.05	16.62 ± 9.99
Size of first meal (kcal/300 g bw)	5.98 ± 0.46	6.36 ± 0.89
Duration of first meal (min)	12.96 ± 3.11	13.54 ± 2.85
Eating rate of first meal (cal/300 g bw/min)	71.91 ± 8.76	60.26 ± 12.93
Inter-meal interval (min)	80.66 ± 9.92	128.10 ± 24.60
Satiety ratio after first meal (min/kcal/300 g bw food eaten)	13.76 ± 1.77	22.50 ± 5.00

icv, intracerebroventricular; mean ± sem; n = 11/group; $p > 0.05$.

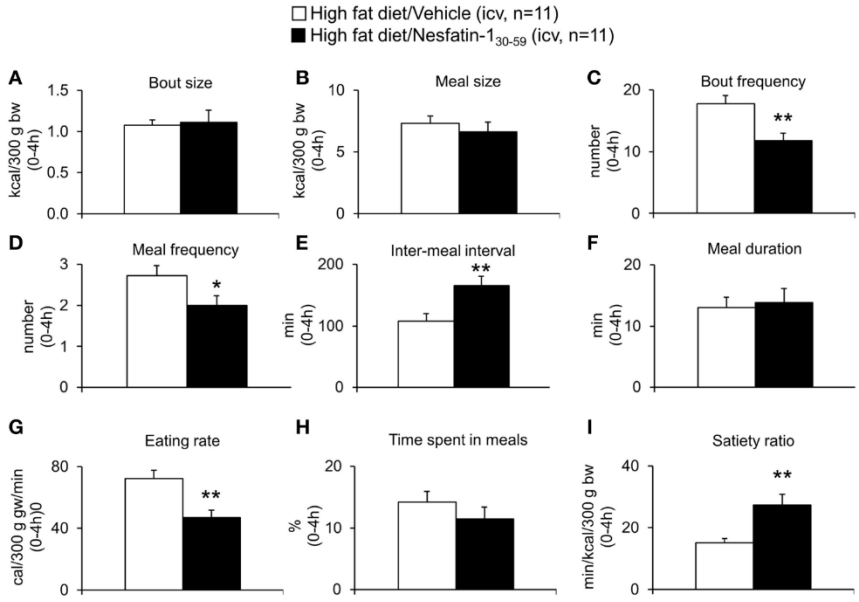

FIGURE 7 | Nesfatin-1_{30-59} decreases meal frequency, while meal size is not altered in diet-induced obese rats. DIO rats fed high fat diet were injected intracerebroventricularly with nesfatin-1_{30-59} (0.9 nmol/rat) or vehicle (5 µl dd H_2O) and the food intake microstructure assessed using an automated episodic food intake monitoring system. During the period of 0–4 h post injection, nesfatin-1_{30-59} reduced bout frequency **(C)**, meal frequency **(D)**, and eating rate **(G)**, while bout size **(A)**, meal size **(B)**, meal duration **(F)**, and the time spent in meals **(H)** were not altered compared to vehicle. Inter-meal intervals **(E)** and the satiety ratio **(I)** were increased following nesfatin-1_{30-59}. Each bar represents the mean ± sem. *$p < 0.05$ and **$p < 0.01$ vs. vehicle.

release of anorexigenic signals such as peptide YY (Xu et al., 2011) is blunted under these condition which is likely to result in reduced anorexigenic signaling. Therefore, we investigated the effect of nesfatin-1_{30-59} also in DIO rats fed a high fat diet. The anorexigenic effect of nesfatin-1_{30-59} was retained in DIO rats with a delayed onset (parameters of the first meal were not changed) and a long duration of action. Interestingly, the main food intake inhibitory effect was already observed during the third hour post injection (−71%), while in normal weight rats the main reduction occurred later in the fifth hour post nesfatin-1_{30-59} icv injection (−75%). Moreover, the effect translated into a reduction of food intake over a 20 h period (−13%), while in normal weight rats the effect lasted for 20 h (−12%). Lastly, the underlying food intake microstructure was different in DIO rats compared to normal weight rats with a decrease in meal frequency and an unaltered meal size under conditions of high fat feeding indicating an increase of satiety while satiation is not altered. Taken together, these findings give rise to differential receptor binding, altered diffusion and—most importantly—point toward a differential downstream signaling under conditions of DIO compared to normal weight rats.

Interestingly, it was recently shown that rats fed a high fat diet display significantly lowered plasma NUCB2/nesfatin-1 levels (Haghshenas et al., 2014; Mohan et al., 2014). Although the cellular action on the so far unknown receptor is yet to be established, the chronically reduced circulating

NUCB2/nesfatin-1 levels may result in a sensitization of the receptor and lead to a stronger/more rapid response to icv nesfatin-1_{30-59}. Early on, it was shown that nesfatin-1_{1-82} acts in a leptin-independent fashion (Oh-I et al., 2006; Maejima et al., 2009). However, the corticotropin releasing factor (CRF) receptor 2, shown to play a role in nesfatin-1_{1-82}'s anorexigenic signaling under normal weight conditions (Stengel et al., 2009a), may be important under DIO conditions as well. An earlier study showed that brain injection of the selective CRF_2 agonist, urocortin 3 (1 μg/rat) reduced food intake with a delayed onset (between 3 and 4 h) by decreasing meal frequency while meal size was not altered in rats (Fekete et al., 2007), a pattern very similar to the one observed following icv nesfatin-1_{30-59} observed under DIO conditions in the present study. The predominance of the CRF_2 downstream signaling pathway in the mediation of nesfatin-1_{30-59}'s anorexigenic action might result from reduced processing of POMC to α-MSH under conditions of obesity due to reduced prohormone convertase two levels (Çakir et al., 2013).

In conclusion, these results provide pharmacological evidence that nesfatin-1_{30-59} is the active core of full length nesfatin-1_{1-82} also in rats that reduces food intake in normal weight rats fed standard rodent diet as well as DIO rats fed a high fat diet. Interestingly, the food intake microstructure differs with an increase of satiation under normal weight and an induction of satiety under DIO conditions. This may result from a downstream nesfatin-1_{30-59} → oxytocin→POMC→α-MSH/melanocortin receptor 3/4 signaling in normal weight rats while in DIO rats the nesfatin-1_{30-59}→CRF/CRF_2 pathway may predominate. The effect of nesfatin-1_{30-59} is centrally mediated as ip injection of higher doses had no effect. Therefore, the main effect of peripheral nesfatin-1 remains to be established.

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Publication 3:

Teuffel P, Goebel-Stengel M, Hofmann T, **Prinz P**, Scharner S, Körner JL, Grötzinger C, Rose M, Klapp BF, Stengel A. A RAPID Method for Blood Processing to Increase the Yield of Plasma Peptide Levels in Human Blood. J Vis Exp. 2016 Apr 28;(110). http://dx.doi.org/10.3791/53959.

10. Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

11. List of publications

Original papers

1) **Prinz P***, Hofmann T*, Ahnis A, Elbelt U, Goebel-Stengel M, Klapp BF, Rose M, Stengel A. Plasma bile acids show a positive correlation with body mass index and are negatively associated with cognitive restraint of eating in obese patients. Front Neurosci. 2015 Jun 3;9:199.

2) Teuffel P, Wang L, **Prinz P**, Goebel-Stengel M, Scharner S, Kobelt P, Hofmann T, Rose M, Klapp BF, Reeve JR Jr, Stengel A. Treatment with the ghrelin-*O*-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats. J Physiol Pharmacol. 2015 Aug;66(4):493–503.

3) **Prinz P***, Teuffel P*, Lembke V, Kobelt P, Goebel-Stengel M, Hofmann T, Rose M, Klapp BF, Stengel A. Nesfatin-1_{30-59} injected intracerebroventricularly differentially affects food intake microstructure in rats under normal weight and diet-induced obese conditions. Front Neurosci. 2015 Nov 23;9:422.

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Abstracts

1.) **Prinz P**, Teuffel P, Lembke V, Kobelt P, Goebel-Stengel M, Rose M, Klapp BF, Stengel A. Nesfatin-1(30-59) injected intracerebroventricularly differentially affects food intake microstructure in rats under normal weight and diet-induced obese conditions. Annual Meeting of the German Society for Neurogastroenterology & Motility, 2015; Freising, Germany.

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3.) Teuffel P, Wang L, **Prinz P**, Goebel-Stengel M, Scharner S, Kobelt P, Hofmann T, Rose M, Klapp BF, Reeve JR Jr, Stengel A. Treatment with the ghrelin-O-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats. Gastroenterology, April 2015

4.) **Prinz P**, Hofmann T, Ahnis A, Elbelt U, Goebel-Stengel M, Klapp BF, Rose M, Stengel A. Plasma bile acids show a positive correlation with body mass index which may affect eating behavior. Neurogastroenterol Motil. 2015.

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7.) Goebel-Stengel M, Teuffel P, Hofmann T, **Prinz P,** Kobelt P, Grötzinger C, Rose M, Klapp BF, Stengel A. Die RAPID-Methode ist auch für die Peptidbestimmung beim Menschen geeignet und verbessert die Ausbeute der gemessenen Peptide. Gastroenterol. 2015.

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“You are only afraid if you are not in harmony with yourself.”

- Hermann Hesse