Aus der Medizinischen Klinik mit Schwerpunkt Psychosomatik
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DISSERTATION

Die Rolle von Peptiden in der Entstehung von Depression und Angst – Implikation von NUCB2/Nesfatin-1

The role of peptides in the pathogenesis of depression and anxiety – implication of NUCB2/nesfatin-1

zur Erlangung des akademischen Grades
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Preface

The following manuscript represents an in-depth summary of the scientific background and current state of research, the methods, the results as well as the discussion of the following three publications:

(1) Nesfatin-1(30-59) Injected Intracerebroventricularly Increases Anxiety, Depression-Like Behavior, and Anhedonia in Normal Weight Rats. Kühne SG*, Schalla MA*, Friedrich T, Kobelt P, Goebel-Stengel M, Long M, Rivalan M, Winter Y, Rose M, Stengel A. Nutrients. 2018;10(12).
*shared first authorship

(2) Central blockage of nesfatin-1 has anxiolytic effects but does not prevent corticotropin-releasing factor-induced anxiety in male rats. Schalla MA, Kühne SG, Friedrich T, Kobelt P, Goebel-Stengel M, Long M, Rivalan M, Winter Y, Mori M, Rose M, Stengel A. Biochemical and biophysical research communications. 2020;529(3):773-7.

(3) Restraint stress affects circulating NUCB2/nesfatin-1 and phoenixin levels in male rats. Schalla MA*, Goebel-Stengel M*, Friedrich T, Kühne SG, Kobelt P, Rose M, Stengel A. Psychoneuroendocrinology. 2020;122:104906.
*shared first authorship

It is to note that the methods described under the sections 2.1.1., 2.1.2., 2.2.1., 2.3.1, 2.3.2, 2.4.1., 2.4.2., 2.4.3., 2.4.4.,2.4.5., 2.4.6., 2.6 as well as results mentioned in the sections 3.1.1., 3.1.2., 3.2., 3.3.1, 3.3.3., 3.3.4., 3.4. are based on the publication from Kühne and Schalla et al. 2018 (1). Moreover, the methods explained in the sections 2.1.1., 2.1.2., 2.1.3., 2.2.2, 2.2.3., 2.2.4., 2.3.1., 2.3.2., 2.4.3, 2.4.4., 2.6 as well as the results depicted in sections 3.1.3., 3.3.2. are taken from the publication from Schalla et al. 2020 (2). Finally, the methods mentioned in the sections 2.1.1., 2.1.2., 2.1.3., 2.3.3., 2.5., 2.6. and the results described in section 3.5. are adapted from Schalla and Goebel-Stengel et al. 2020 (3).

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Abbreviations

ARC	arcuate nucleus
CNS	central nervous system
CRF	corticotropin-releasing factor
DIO	diet-induced obese
DSM	Diagnostic and Statistical Manual of Mental Disorders
EZM	elevated zero maze test
GRP173	G protein-coupled receptor 173
HPA	hypothalamic-pituitary-adrenal
ICD	International Classification of Diseases
icv	intracerebroventricular
iv	intravenous
LH	lateral hypothalamic area
LDB	light/dark box test
MDD	major depressive disorder
NaCl	sodium chloride
NIH test	novelty-induced hypophagia test
PVN	paraventricular nucleus
OFT	open field test
SNRI	serotonin and noradrenalin reuptake inhibitors
SON	supraoptic nucleus
SPT	sucrose preference test
SSRI	selective serotonin reuptake inhibitors
TCA	tricyclic antidepressants
VMH	ventromedial hypothalamic area
YLD	years lost due to disability

Abstract (English):

Background: Depressive and anxiety disorders are highly prevalent diseases causing distinct loss of quality of life. Their pathogenesis is poorly understood and thus currently available treatment options are associated with notable side effects, significant therapy resistance and high relapse rates. Consequently, there is a need for better understanding of their etiology and the present work aims to investigate the role of peptides in their development.

Methods: Therefore, using male Sprague Dawley rats (I) the effect of the active fragment of nesfatin-1 on depressive-like and anxious behavior was examined in several test paradigms, followed (II) by the same investigations in diet-induced obese rats. Additionally, (III) using a nesfatin-1 antibody the endogenous role of nesfatin-1 in depressive-like and anxious behavior was tested under naïve and stress conditions. Finally, since depression and anxiety are closely related to the hypothalamic-pituitary-adrenal axis, (IV) the effect of emotional stress on circulating nesfatin-1 levels was tested.

Results: It could be shown that (I) intracerebroventricularly injected 0.3 nmol nesfatin-$1_{30\text{-}59}$ increased anhedonia reflecting depressive-like behavior, as well as anxiety in normal weight, (II) but not in diet-induced obese rats. Furthermore, (III) an endogenous role of nesfatin-1 in the mediation of anxiety but not in depressiveness could be demonstrated by reduced anxious behavior in nesfatin-1 antibody-treated naïve normal weight rats. In stressed rats acute nesfatin-1 blockade elicited no effect; however, (IV) an increased circulating level of NUCB2/nesfatin-1 was observed after 240 minutes of immobilization.

Conclusion: The present studies showed that nesfatin-1, whose circulating levels are upregulated by stress, is differentially implicated in the development of depression and anxiety. While it induces anhedonia only after exogenous injection, endogenous nesfatin-1 affects anxiety and acts supposedly up-stream from corticotropin-releasing factor signaling. This hypothesis, however, warrants further research.

Abstract (Deutsch):

Hintergrund: Depressive Störungen und Angststörungen sind hochprävalente Krankheitsbilder, die mit einer deutlichen Einschränkung der Lebensqualität einhergehen. Ihre Pathogenese ist nur unzureichend geklärt und so sind die derzeit verfügbaren Behandlungsmöglichkeiten mit erheblichen Nebenwirkungen, häufigen Therapieresistenzen und hohen Rückfallraten verbunden. Daher besteht ein Bedarf für ein besseres Verständnis ihrer Ätiologie und die vorliegende Arbeit zielt darauf ab, die Rolle von Peptiden bei ihrer Entstehung zu untersuchen.

Methoden: Hierzu wurde bei männlichen Sprague Dawley Ratten (I) die Wirkung des aktiven Fragments von Nesfatin-1 auf depressives und ängstliches Verhalten in verschiedenen Testparadigmen untersucht, gefolgt von (II) den gleichen Untersuchungen bei adipösen Ratten. Zusätzlich wurde (III) unter Verwendung eines Nesfatin-1-Antikörpers die endogene Rolle von Nesfatin-1 an depressivem und ängstlichem Verhalten bei naiven Tieren sowie unter Stressbedingungen beleuchtet. Da Depression und Angst eng mit der Stress-Achse assoziiert sind, wurde schließlich (IV) der Effekt von psychischem Stress auf die zirkulierenden NUCB2/Nesfatin-1-Spiegel untersucht.

Ergebnisse: Es konnte gezeigt werden, dass (I) intrazerebroventrikulär injiziertes Nesfatin-1_{30-59} (0,3 nmol) Anhedonie, als eine Form von depressivem Verhalten, sowie Angst bei normalgewichtigen, aber (II) nicht bei adipösen Ratten induzierte. Darüber hinaus konnte (III) eine endogene Rolle von Nesfatin-1 bei der Vermittlung von Angst, aber nicht von depressivem Verhalten nachgewiesen werden, welche durch geringer ausgeprägtes Angstverhalten bei mit Nesfatin-1-Antikörper behandelten naiven normalgewichtigen Ratten demonstriert wurde. Bei gestressten Ratten löste eine akute Nesfatin-1-Blockade keinen Effekt aus, jedoch wurde (IV) ein erhöhter zirkulierender Spiegel von NUCB2/Nesfatin-1 nach 240-minütiger Immobilisation beobachtet.

Schlussfolgerung: Die vorliegende Studie zeigte, dass Nesfatin-1, dessen zirkulierende Spiegel durch Stress erhöht waren, in unterschiedlicher Weise an der Entstehung von Depressivität und Ängstlichkeit beteiligt ist. Während Nesfatin-1 Anhedonie nur nach exogener Injektion induzierte, beeinflusst es Ängstlichkeit über exogene und endogene Mechanismen, die vermutlich dem Corticotropin-Releasing-Faktor-Signalweg vorgeschaltet sind. Diese Hypothese bedarf jedoch weiterer Forschung.

1. Introduction

1.1 Depression

1.1.1. Definition and epidemiology

According to the fifth edition of the “Diagnostic and Statistical Manual of Mental Disorders” (DSM-V) depressive disorders are a group of several different diagnoses all characterized by feeling of sadness and impaired bodily or cognitive functions impacting the daily life of affected individuals (5). The classic condition among depressive disorders is major depressive disorder (MDD) (5), which is a multifarious disease with a diversity of possible symptoms. The DSM-V and 10th edition of the International Classification of Diseases (ICD-10) name depressed mood, anhedonia also known as loss of pleasure, decreased interest in activities, insomnia, early morning awakening or hypersomnia, reduced or increased appetite with weight loss or gain, psychomotor retardation or agitation, loss of energy and fatigue, feeling of guilt and worthlessness, diminished cognitive functions, loss of libido, thoughts of death and suicidal ideation or attempts as common symptoms in depressive episodes (5, 6). When a total of 5 of these symptoms persist for two weeks and cause significant distress and impairment the episode can be classified as major depressive disorder (5).

In 2017, approximately 264 million humans worldwide were suffering from depressive disorders, of which 241 million were diagnosed with MDD (7). Interestingly, MDD is the third leading cause of healthy life lost due to disability (YLD) in females, being responsible for 14.3% of YLDs (7).

1.1.2. Pathogenesis

So far, the etiology of depressive disorders is not fully understood; however, current research suggests the involvement of various physiological systems in the pathogenesis of depression since no single hypothesis or theory can completely explain all the manifold aspects of depressive disorders (8).

The most commonly known theory explaining the development of depression is the monoamine hypothesis (8). Early on in the 1960s it was observed that an elevation of catecholamines at adrenergic receptor sides in the central nervous system (CNS), e.g., by means of monoamine oxidase inhibitors and imipramine-like drugs induced an antidepressant effect (9). Moreover, in 67% of patients with depressive disorders treated with antidepressants acute dietary tryptophan depletion causing a decrease of

87% of tryptophan levels induced a depressive relapse, which subsided again after 24 to 48 hours of regular diet (10).

According to another hypothesis hypothalamic-pituitary-adrenal (HPA) axis hyperactivity could be implicated in depressive symptoms and indeed in atypical, endogenous, melancholic and psychotic forms of depression elevated levels of plasma cortisol were reported (11). Furthermore, modulation of the HPA axis using a corticotropin-releasing factor (CRF) receptor antagonist resulted in antidepressant effects in different animal models of depression (12). However, after 50 years of research, the variability of HPA axis alterations in depression (11) and thus lack of clinical efficacy of human HPA axis modulation (12) strongly emphasize the need for further investigations.

1.1.3. Treatment

The majority of antidepressants available for use in depressive disorders base on the monoamine hypothesis. In a meta-analysis of over 520 trials, testing the efficacy of selective serotonin reuptake inhibitors (SSRI), serotonin and noradrenalin reuptake inhibitors (SNRI) and tricyclic antidepressants (TCA), all were shown to be more effective than placebo (13). However, only two agents, namely agomelatine and fluoxetine, caused fewer dropouts than placebo (13) indicating great potential to improve tolerability of the majority of available antidepressants despite their efficacy. Since, with only agomelatine as exception, all agents were modulators of the monoamine system, depressive symptoms cannot be explained by the monoamine hypothesis alone; therefore, there seems to be a need for alternative agents. This need is further underlined by high prevalence of treatment-resistant depressions (14), which are characterized by a lack of response to at least two trials of antidepressants (15). In a study with patients suffering from MDD 36.8%, 30.6%, 13.7%, and 13.0% of patients achieved remission after one, two, three or four successive treatments, respectively, resulting in a cumulative remission rate of 67% (14). Thus, the prevalence of treatment-resistant depressions was approximately 33%.

1.2 Anxiety

1.2.1. Definition and epidemiology

According to the DSM-V anxiety disorders include several different diagnoses, all characterized by an excessive and reoccurring fear that is impairing the individual's

functioning in daily life (5, 16). Generalized anxiety disorder is the classical disorder in the group of anxiety disorders and was shown to be strongly associated with MDD (17). Thus, here we will focus especially on this condition.

Based on DSM-V generalized anxiety disorder (GAD) is diagnosed when three or more of the symptoms restlessness, reduced cognitive functions, irritability, muscle pain, fatigue, insomnia, impairment in social or occupational activities persist during the majority of days for at least 6 months in several different environmental circumstances (5). In addition, ICD-10 includes more symptoms that can occur with GAD such as constant nervousness, sweating, dizziness, palpitations, lightheadedness and epigastric discomfort (7).

In 2017 over 284 million individuals worldwide were affected by anxiety disorders, with an incidence of 42 million in that year (7) and an estimated global prevalence of 7.3% (18). The analysis of data from 26 countries obtained between 2001 and 2012 showed that the one-year prevalence of GAD fulfilling the diagnostic criteria of DSM-V was 1.8%, while combined lifetime prevalence was even 3.7% (19). With a higher prevalence of anxiety disorders in women then in men (20) anxiety disorders were the eighth leading cause of global YLD in 2017 in women (7).

1.2.2. Pathogenesis

The exact etiopathogenesis of anxiety disorders could not be completely illuminated by research so far. Since the center of processing emotions including fear in the CNS is the amygdala (21), it was suggested that its impairment could cause anxiety disorder-related symptoms. Indeed, a systematic analysis of studies investigating the neural response to emotional stimuli using functional MRI reported a wide variability of limbic and prefrontal region activity in GAD as well as abnormality and inflexibility in mental processing in those patients (22); however, considering the heterogeneity of data further research is needed.

Moreover, due to the presence of symptoms mimicking a stress reaction (sweating, palpitations, lightheadedness) observed in several anxiety disorders the HPA axis, which is the key-regulator of the stress response (23), was proposed to also be implicated in their pathogenesis. However, the relationship between anxiety disorders and HPA axis could not be consistently demonstrated (24), e.g., in one study women with anxiety disorders displayed reduced cortisol responses to stress, while men responded to stress with increased levels (24). Interestingly, since it could be

shown that glucocorticoids have the capacity to modulate limbic metabolic activity (25) abnormalities in emotion-processing brain areas, e.g., due to history of early life stress (26), in anxiety disorders could be related to altered glucocorticoid signaling (25); however, the variability of HPA axis activity in anxiety disorders indicates additional pathophysiological mechanisms responsible for the development of anxiety disorders.

1.2.3. Treatment

The treatment of anxiety disorders mainly consists of psychotherapy and/or medication. In a study including over 37,000 patients pharmacological treatment had a higher effect size than psychotherapy (27). The pharmacological treatment listed in descending order according to their effect size encompassed SNRIs, SSRIs and tricyclic antidepressants (27). In the study it was also observed that therapy results were faster obtained due to pharmacological than psychological therapy (27).

However, although pharmacological therapy was proven to be effective, its effect often is limited to the time span of its intake, as indicated by a relapse rate of 36% within 24 weeks of switching to placebo in patients treated for 20 weeks with sertraline for generalized social phobia (28) and a relapse rate of 37.2% within 5 weeks after drug discontinuation in patients receiving clomipramine for panic disorders (29). In patients with GAD that were treated with venlafaxine for 6 months, that who received placebo in the following 6 months even displayed a relapse rate of 53.7% (30).

1.3 Nesfatin-1

1.3.1. Expression

Nesfatin-1 is an 82-amino acid long polypeptide consisting of an N-terminal, middle and C-terminal domain and is derived from the protein nucleobindin-2 (NUCB2) (31, 32). Since the nesfatin-1 antibody commonly used for immunohistochemical and enzyme-linked immunosorbent assay (ELISA) studies binds an epitope that is present also in nesfatin-1's precursor protein NUCB2 the term NUCB2/nesfatin-1 will be used when describing the expression pattern of nesfatin-1.

NUCB2/nesfatin-1 is widely expressed in the CNS (32-34), predominantly in hypothalamic areas as in the supraoptic (SON), periventricular, paraventricular (PVN), arcuate (ARC) nuclei as well as in ventromedial (VMH), lateral hypothalamic (LH) and medial preoptic area (32, 34). Interestingly, NUCB2/nesfatin-1 is highly co-expressed in the hypothalamus with phoenixin (35), a novel peptide shown to reduce anxiety in

mice (36) and to be negatively correlated with anxiety scores in men (37). However, NUCB2/nesfatin-1- immunoreactivity was also found in the limbic system including the central amygdaloid nucleus, hippocampus, lateral septum, nucleus accumbens, basal ganglia and bed nucleus of the stria terminalis (33, 34). Besides its widespread expression in the CNS, NUCB2/nesfatin-1 expression was also found in various peripheral tissues (38, 39), most importantly in co-expression with ghrelin in endocrine cells of the gastric mucosa (40), but also in adipose tissue (41).

1.3.2. Extracellular and intracellular signaling

So far, the receptor for nesfatin-1 is not known; however, several studies suggest a G-protein coupled receptor to induce alterations of intracellular Ca^{2+} (42-44). Additionally, using ^{125}I-nesfatin-1 autoradiography binding of nesfatin-1 has been demonstrated in the CNS in the cortex, PVN, area postrema, dorsal motor nucleus of the vagus nerve and cerebellum (45). In the periphery radiolabeled nesfatin-1 bound to the gastric mucosa, adipose tissue and various other organs (45). With expression as well as binding sites of nesfatin-1 in the CNS and in peripheral tissues it was suggested that nesfatin-1 secreted in the periphery could also affect central circuitries and vice versa, and indeed it could be demonstrated that nesfatin-1 crosses the blood-brain barrier by a non-saturable mechanism (46).

1.3.3. Role in various physiological systems

The first study investigating nesfatin-1's effects showed that intracerebroventricular (icv) injection of full-length nesfatin-1 diminished feeding in a dose-dependent manner and injection of a nesfatin-1-neutralizing antibody increased food intake (32). Since nesfatin-1's anorexigenic effect was abolished after CRF_2 antagonist application, it was suggested to be CRF-CRF_2 dependent (47). Later it was demonstrated that N-terminal as well as C-terminal fragments of nesfatin-1 did not induce a food intake reduction after icv injection (31, 48); thus, the anorexigenic effect of nesfatin-1 is supposed to be mediated by its midsegment nesfatin-$1_{30–59}$.

Furthermore, nesfatin-1 was shown to regulate body weight (32), have an inhibitory effect on gastrointestinal motility (47, 49-51) as well as a blood glucose-lowering effect (52-55). Nesfatin-1 was also demonstrated to be implicated in lipid and energy metabolism (56-59) as well as in the cardiovascular system (60-63).

Noteworthy, nesfatin-1 was able to increase blood pressure (64), in a CRF_2-dependent manner (65).

1.3.4. Role in depression

In the past it could be shown that in rodents full-length nesfatin-1 induced depressive-like behavior, demonstrated by increased immobility in the forced swimming test (66), reduced motivation for food reward in a sucrose preference test (SPT) (67) and decreased intake of a palatable snack in the novelty-induced hypophagia test (NIH) (68).

Investigations in humans corroborated a role of nesfatin-1 in depression, e.g. in obese women depressiveness correlated positively with NUCB2/nesfatin-1 (69). Moreover, a majority of studies in patients with depression showed increased circulating nesfatin-1 levels in patients with MDD (70-72) as well as a positive correlation between plasma NUCB2/nesfatin-1 levels and Hamilton Depression Rating Scale scores (70, 71, 73). In addition, nesfatin-1 was identified as an independent indicator for severe depression (71) with a sensitivity of 94.3% and specificity of 97.1% to distinguish patients with moderate and severe depressive disorder from healthy volunteers (74). Only in patients suffering from MDD with suicidal ideation serum NUCB2/nesfatin-1 levels were reported to be reduced compared to healthy controls and scores of suicidal ideation to be negatively correlated with nesfatin-1 levels (75). Noteworthy, in patients with MDD NUCB2/nesfatin-1 positively correlated with corticosterone (73), indicating an importance of the HPA axis.

1.3.5. Role in anxiety

Previous investigations suggest an anxiogenic effect of full-length nesfatin-1 in rodents, indicated by increased fear-potentiated startle response and time spent freezing (68), reduced percentage of time spent on the open arms of an elevated plus maze (68), reduced moving distance, time in center, and frequencies of rearing and grooming in the open field test (OFT) (66, 76) as well as reduced moving distance, frequency, and preference index of new arm of the Y maze (76).

In obese women “worries” in the Perceived Stress Questionnaire-20 were positively associated with NUCB2/nesfatin-1 (77). Moreover, in females with anorexia or obesity NUCB2/nesfatin-1 and anxiety correlated positively (77, 78), while in men with obesity this correlation was negative (69). However, therapy-induced

improvement of anxiety scores did neither in women nor in men result in a change of NUCB2/nesfatin-1 plasma levels (79).

1.3.6. Role in stress

Both depression (11, 12) and anxiety (24-26) were shown to be associated with a dysregulation of the HPA axis and also nesfatin-1 was shown to be implicated in stress signaling by the HPA axis. Nesfatin-1 injection was associated with activation of CRF-immunoreactive neurons in the PVN (80), expression of CRF mRNA in the hypothalamus (66) and elevated adrenocorticotropic hormone as well as corticosterone plasma levels (80).

Conversely, NUCB2/nestain-1 in rodents was shown to be affected by various types of stress. Emotional stress stimulated NUCB2/nesfatin-1 positive neurons (34, 80), immunological stress increased NUCB2/nesfatin-1 concentrations in plasma and stomach as well as activated NUCB2/nesfatin-1 immunoreactive neurons in the hypothalamus (81), and physical stress stimulated hypothalamic NUCB2/nesfatin-1 expressing neurons as well (82). Moreover, chronic stress elevated NUCB2/nesfatin-1 expression in the hippocampus (83, 84), in the PVN (85), in the middle brain (83, 85) as well as in the plasma and gastric fundus (84). In human, perceived stress correlated positively with NUCB2/nesfatin-1 in females with obesity (69).

1.4 Aim of study

It can be concluded that depression and anxiety disorders are prevalent diseases which have a significant impact on quality of life as indicated by their ranks in the leading causes of YLD. Unfortunately, their etiopathogeneses are not completely understood, and acute and long-term effects of currently available treatment options are not satisfactory. In the past years emerging evidence points toward a crucial role of peptides in the regulation of behavior, e.g., circulating nesfatin-1 was found to be clearly associated with depressive disorders in human (70-75) and full-length nesfatin-1 induced anxious behavior in rats (68).

Thus, in this study we aimed to (I) investigate the effect of icv injection of the active fragment of nesfatin-1 in normal weight (NW) rats. These experiments were completed by (II) repeating the procedures in diet-induced obese (DIO) rats. Since nesfatin-1's ability to reduce food intake is CRF_2-dependent (47) it was (III) tested whether nesfatin-1 is responsible for CRF-induced behavioral changes using an icv

administered nesfatin-1 antibody. Lastly the (IV) effect of restraint stress on circulating nesfatin-1 was assessed also in relation with the novel anxiolytic peptide phoenixin.

2. Materials and methods

2.1 Animals and diets

2.1.1. Animals

For all experiments male Sprague Dawley rats (Envigo, Germany) weighting between 200 and 250 g were used. After arrival all animals had time to acclimatize for 7 days in groups of 4 to the controlled conditions with a 12-h dark/light cycle with lights on at 6 am, room temperature of 21-23° C and humidity of 45-65% in the animal facility. Rats had ad libitum access to standard rodent chow and water except during experimental procedures as described in detail below. All animals were handled and their body weight, food and water intake were documented daily by one of the two performing investigators MAS (Martha A. Schalla) or SGK (Stephanie G. Kühne). All animal-related procedures were performed according to institutional ethics guidelines for animal care and approved by state authority for animal research.

2.1.2. Experimental group allocation

For the four experiments rats were allocated to four different experimental groups: In group 1, NW animals underwent the SPT, NIH, elevated zero maze (EZM), OFT and light/dark box test (LDB) after icv injection with vehicle or nesfatin-$1_{30\text{-}59}$ (0.3 nmol). Noteworthy, for the SPT approximately half of the used animals received also either 0.1 or 0.9 nmol of nesfatin-$1_{30\text{-}59}$.

For the second experiment, DIO rats underwent also the SPT, NIH, EZM, OFT and LDB after injection with vehicle or 0.3 nmol of nesfatin-$1_{30\text{-}59}$.

In the third group, animals underwent the NIH and EZM after icv injection of vehicle or CRF followed by injection of nesfatin-1 antibody or control IgG antibody.

In the fourth experiment, one half of the group of male rats was allocated to the restraint stress group and the other half was left undisturbed.

2.1.3. Diets

The rats used for experiments 1, 3 and 4 all were fed a standard rodent diet; however, while animals in group 1 and 3 received a diet produced by Research Diets, Inc. (D12450B, 3.9 kcal/g, 10% fat, 70% carbohydrates, 20% proteins, Jules Lane,

New Brunswick, NJ, USA), animals in experimental group 4 had ad libitum access to standard rodent diet produced by sniff Spezialdiäten GmbH (V1534-000, 3.9 kcal/g, 9% fat, 67% carbohydrates, 24% proteins, Soest, Germany). These NW animals displayed an average weight of 259.1 ± 3.1 g at the beginning of experiments.

To induce diet-induced obesity (DIO) animals were fed a high fat diet (D12451, 4.7 kcal/g, 45% fat, 35% carbohydrates, 20% proteins, Research Diets, Inc.) for 10 weeks. As described before only approximately 50% of rats fed this type of diet develop a DIO phenotype after 10 weeks (86); thus, only half of the rats were included into experimental group 2. The average weight in this group before the start of experimental procedures was 426.9 ± 9.5 g.

2.2 Peptides and antibodies

The preparations of peptides and antibodies as described below were performed by MAS with some assistance of SGK and Reinhard Lommel.

2.2.1. Nesfatin-1_{30-59}

For icv injections in experiments 1 and 2 the active fragment of nesfatin-1 was purchased from Bachem AG (Weil am Rhein, Germany). The substance was stored as a lyophilized powder at -80 °C and then aliquoted in sterile water in preparation for the experiments. Based on food intake studies (86) on the day of injection an aliquot with stock solution was thawed and three different solutions with doses of 0.1, 0.3 and 0.9 nmol of nesfatin-1_{30-59} diluted in 5 µL sterile ddH_2O were prepared for the SPT in group 1. As in this experiment the middle dose was shown to induce the most pronounced effect 0.3 nmol of nesfatin-1_{30-59} was used for further experiments.

2.2.2. Corticotropin-releasing factor

For experiment 3, CRF in a lyophilized form, purchased from Phoenixin Pharmaceuticals Inc (catalog no. 019-06, Burlingame, CA, USA) was dissolved in 0.9% sodium chloride (NaCl) before storage at -80 °C. These aliquots that were used for injection had a concentration of 0.6 µg CRF in 5 µL 0.9% NaCl, that was based on previous experiments in rats (87).

2.2.3. Nesfatin-1 antibody

For injections of rats in experimental group 3, nesfatin-1 antibody was provided by Masatomo Mori (Department of Medicine and Molecular Science, Gumna University Graduate School of Medicine, Maebashi, Japan). The antibody against residues 24-38 of amino-acid sequences of rat NUCB2 was generated in rabbits, with a Cys residue added to the C-terminus. The antibody was shipped and stored as a lyophilized powder at -80 °C before being dissolved in ddH_2O on the experimental day. For injections a dose of 8 µg/5 µL per rat was applied based on previous experiments using the same antibody in rats (32).

2.2.4. Control Antibody

For experiment 3, an anti-rabbit IgG antibody (Sigma-Aldrich, Darmstadt, Germany) was aliquoted in sterile water in a concentration of 8 µg/5 µL to be stored at -80 °C and finally be used as a control condition for rats treated with nesfatin-1 antibody.

2.3 Surgeries and substance applications

2.3.1 Intracerebroventricular cannulation

Animals in experimental group 1, 2 and 3 were equipped with an icv cannula (Figure 1). Therefore, firstly rats were anesthetized with a mixture of 10 mg/kg xylazine (Rompun™, 2%, Bayer, Leverkusen, Germany) and 100 mg/kg ketamine (Ketanest™, Curamed, Karlsruhe, Germany). Then, under deep anesthesia the rat was placed in a stereotactic apparatus for fixation. After local skin disinfection a scalp incision was performed exposing the bregma. A guide cannula (22-gauge, Plastic One Inc, Roanoke, VA, USA) was clamped in the cannula holder of the stereotactic apparatus (Figure 1), followed by positioning its tip exactly on top of the bregma. The coordinates of the cannula were assessed. Based on calculations from a rat brain atlas the cannula was then moved 0.8 mm posterior and 1.5 mm right lateral from the bregma using the stereotactic apparatus to determine the location of the hole needed for cannula insertion. After drilling a hole into the skull bone at this exact position the cannula was inserted 3.5 mm into the rat's brain, resulting in its placement into the right lateral ventricle. After the cannula was placed in its final position, it was fixed with three sterile stainless-steel screws (Plastics One Inc., Roanoke, VA, USA) and dental cement (Stoelting Co., Wood Dale, IL, USA), before being closed with a dummy cannula. This

surgical procedure was performed by MAS as operating surgeon with the assistance of SGK.

For pain control rats were subcutaneously injected with 0.03 mg/kg buprenorphine (Essex Pharma GmbH, Munich, Germany) and received 0.1 ml enrofloxacin (2.5% ad. Us. Vet, Bayer Vital GmbH, Leverkusen, Germany) per liter in their drinking water for infection prophylaxis over a period of five days. Altogether rats had five days to recover from surgery. The postoperative observation of all rats and their handling was conducted by MAS and SGK.

After completion of experiments 1, 2 and 3 the correct position of cannula was demonstrated in all rats indicated by visible spreading of dye throughout the ventricular system after injection of 10 µL injection of blue ink in postmortem harvested brain tissue. The finalization and postmortem control of cannula placement was performed by MAS.

Figure 1: Stereotactic apparatus and intracerebroventricularly cannulated rat.

2.3.2 Intracerebroventricular injection

Animals in experimental group 1, 2 and 3 were daily handled with light hand restraint to get accustomed to the investigator and icv injection-related restraint. On the experimental day, 30 minutes before behavioral experiments animals were

removed from their cage and manually fixed in light restraint on an experimental table. Then, the dummy cannula was removed and a 28-gauge cannula (Plastics One, Inc.), that was 1 mm longer than the guide cannula, connected by a PE-50 catheter to a 25-µL Hamilton syringe (Intramedic Polyethylene Tubing, Clay Adams, Parsipanny, NJ, USA) was inserted into the guide cannula. A volume of 5 µL was injected over a time span of 15 seconds, afterwards the 28-gauge cannula was left in the guide cannula for another 60 seconds to allow the whole volume to drain into the ventricle. Then, the 28-gauge cannula was extracted from the guide cannula, which was closed with the dummy cannula. Finally, rats were relieved from the restraint position and placed back in their home cage. The icv injections were performed by MAS and SGK in equal parts.

2.3.3 Intravenous cannulation

For experiment 4 rats were equipped with a chronic intravenous (iv) cannula. Therefore, rats received an injection of a mixture of 100 mg/kg ketamine (Ketanest™, Curamed) and 10 mg/kg xylazine (Rompun™, 2%, Bayer). Then, the skin over the right clavicula was disinfected and incised followed by blunt preparation to expose the right external jugular vein. The vein, after being stripped from attached tissue, was then opened with fine scissors to insert a sterile PE-50 tube (Intramedic Polyethylene Tubing). The tube was advanced several centimeters along the vein, then fixed with suture and blood backflow was tested to ensure that the tube's tip was located just before the right atrium. Using a clamp, the tube was then subcutaneously tunneled along the neck and back of the rat to be then exteriorized through a small skin incision between the left and right scapulae. Finally, the tube was sewed to the skin, filled with 200 units/mL heparin (Heparin-Natrium, Braun, Melsungen, Germany) and closed with wire to prevent clotting. This operation was performed by Miriam Goebel-Stengel (MGS) as operating surgeon and MAS or SGK as her assistance.

Postoperatively all rats received 0.1 ml enrofloxacin (2.5% ad. Us. Vet. Bayer Vital GmbH)/ L of drinking water for 3 consecutive days to prevent postoperative infection. Postoperative observation and handling of animals was conducted by MGS, MAS and SGK.

2.4 Behavior assessment

2.4.1. Automated food intake monitoring

For the SPT and NIH an automated food intake monitoring system (BioDAQ, Research Diets, Inc., Figure 2) was used, allowing a detailed and highly accurate analysis of oral intake of fluid and solid substances (4). Thus, in experiment 1, 2 and 3 all animals that underwent the above-mentioned test were accustomed to the system consisting of standard laboratory rat cages equipped with specialized eating and drinking hoppers provided with microbalances connected to a computer with the according computer software.

The microbalances automatically weigh the fluid and food second by second; when a change in weight occurs, the system registers this event as an eating/drinking bout, also saving information about its start and end; thus, its duration, interval as well as its size in gram. One or more bouts can be considered as a meal if they consist of a consumed amount of at least 0.1 g. If two bouts are separated by an interval of more than 15 minutes, they are classified as belonging to two different meals and this interval is called inter-meal interval (88). Daily observation of all animals exposed to the system and regular maintenance of the system were performed by MAS and SKG.

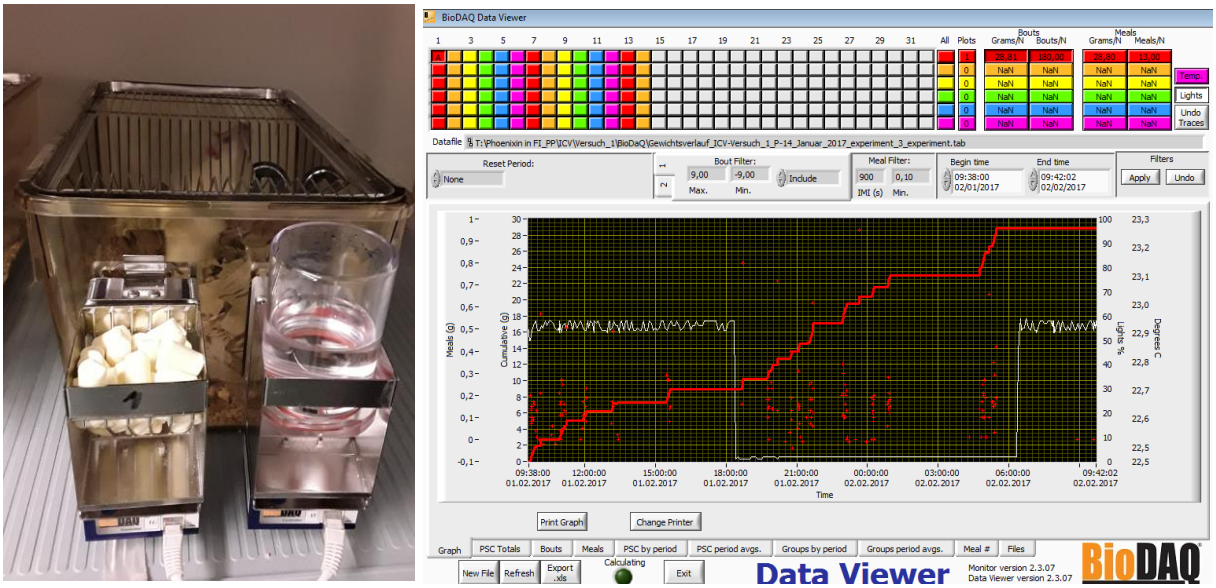


Figure 2: Automated food intake monitoring system.

2.4.2. Sucrose preference test

The SPT (Figure 3) is a well-established test to assesses anhedonia, a common feature of depression-like behavior (89). For these investigations 72 hours before

experimental procedures animals in group 1, 2 and 3 equipped with an icv cannula were exposed to the access and consumption of 1% sucrose solution in addition to regular tap water for 48 hours, with a change of position and content of bottles after 24 hours. After this acclimatization, the sucrose solution was removed from the hopper, so that rats had only access to regular tap water. After 24 hours at the beginning of the dark phase rats were removed from their cages, icv injected and again placed in their home cage. After 30 minutes the rats were given access to 1% sucrose solution and tap water and their intake was monitored for 60 minutes. During all procedures all rats had ad libitum access to chow also during the 60 minutes of SPT. The commonly used SPT protocol was combined with the automated intake monitoring system to ensure a precise recording of fluid consumption. To determine the degree of anhedonia the sucrose to total fluid ratio during the 60 minutes after injection were assessed. This test including the injections was performed by MAS and SGK.

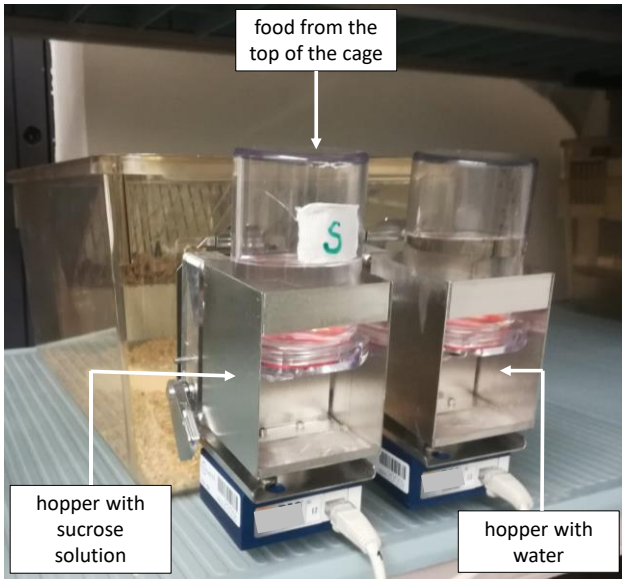


Figure 3 : Sucrose preference test, adapted from (4).

2.4.3. Novelty-induced hypophagia test

To assesses anxious behavior as well as anhedonia (90) the NIH (Figure 4) was performed. Firstly, during five consecutive days at the beginning of the dark phase rats with an icv cannula were exposed to a palatable snack (HoneyMaid™ Graham Cracker Crumbs, Nabisco, East Hanover, NJ, USA) for 30 minutes until their snack intake remained stable. At the beginning of dark phase on the sixth day rats were removed from their cages, received an icv injection and then were again placed in their home cage. After 30 minutes the rats were then removed from their home cage and

transferred into a new cage without bedding. Here, they were given access to the palatable snack for 30 minutes. Noteworthy, when rats were exposed to the palatable snack, they had ad libitum access to regular water but not to chow. During all procedures intake of palatable snack was recorded with the automated intake monitoring system, because the combination of the regular NIH in combination with the automated system allows a more detailed assessment of food consumption (4). MAS and SGK conducted this behavioral test together.

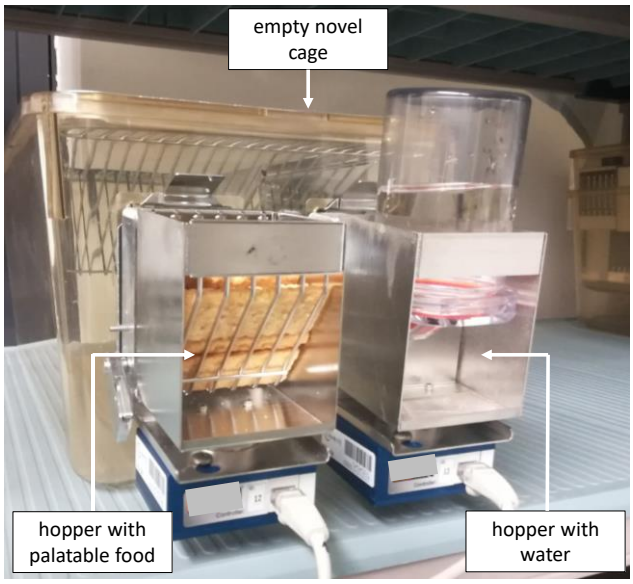
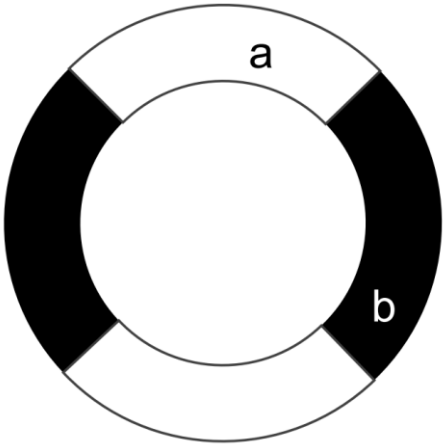


Figure 4: Novelty-induced hypophagia test, adapted from (4).

2.4.4. Elevated zero maze test

The EZM consisting of a zero-shaped elevated platform with two open and two closed wings (Figure 5) was performed to assess anxiety in rodents (91). Naïve animals equipped with an icv cannula were removed from their home cages to receive an injection followed by being again placed in their home cage for 30 minutes. Then, every rat was separately placed in one open arm of the elevated zero maze and left on the apparatus for 5 minutes. During this time using a video camera the behavior of the rat, including total track length, time and number of entries into the different arm and velocity, was recorded before being analyzed by the means of a connected computer software (Biobserve GmbH, Bonn, Germany). Anxious behavior was indicated by reduced explorative behavior meaning low locomotory activity, decreased track length and time in open arms (92). These experiments and icv injections were performed by MAS and SGK with assistance from Melissa Long (ML).

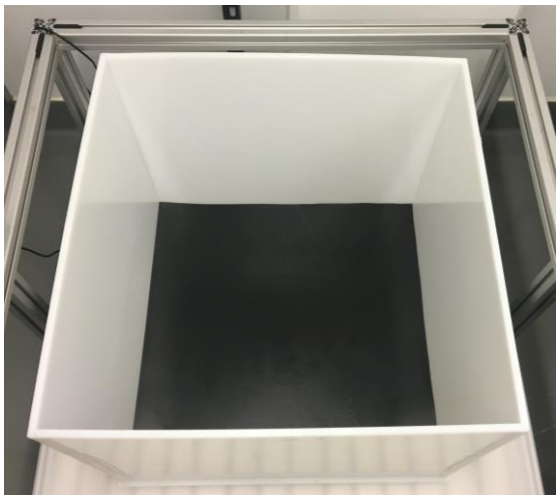


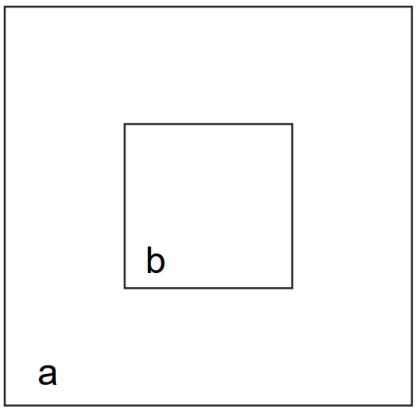
a open arm
b closed arm

Figure 5: Elevated zero maze test, adapted from (1).

2.4.5. Open field test

The OFT is used to assess anxiety by examining exploratory behavior in a 50x50 cm white polyvinylchloride box with a black floor (Figure 6). Naïve rats equipped with an icv cannula were taken from their home cage to be icv injected and then again placed in their home cage. After 30 minutes each rat was placed in the central zone of the box and its movement, including locomotion, total distance as well as duration and entries into the central and outer zone of the box, was recorded for 5 minutes with a camera. The video was analyzed using a computer software (Biobserve GmbH) and multiple entries and an increased duration in the outer zone of the box were interpreted as reduced exploratory and thus anxious behavior (93). MAS and SGK performed these procedures together with assistance from ML.



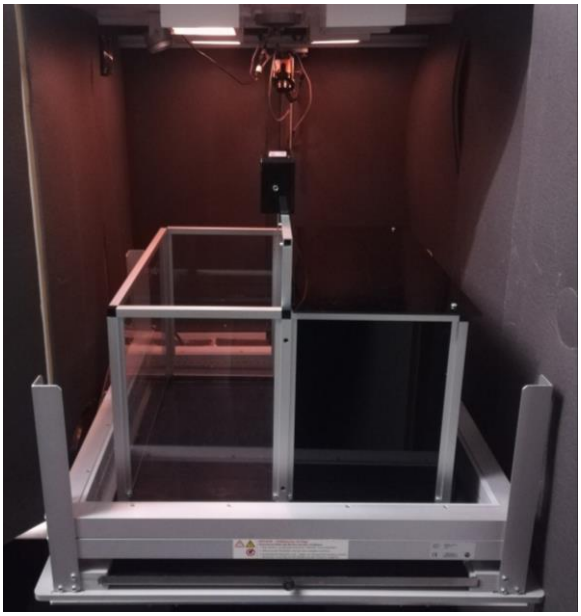


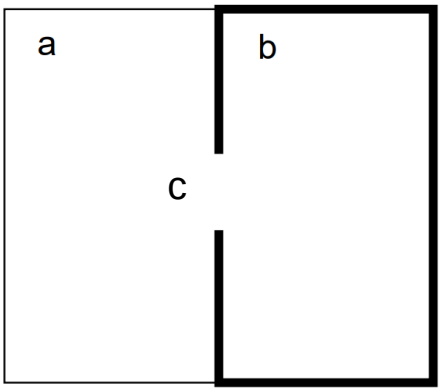
a outer zone
b center zone

Figure 6: Open field test, adapted from (1).

2.4.6. Light/Dark box test

To test for the extent of anxiety in rodents the LDB can be used (94), which consists of one illuminated and one not illuminated compartment connected by a hole (Figure 7). For this test, naïve icv cannulated rats after being removed from their home cage to be icv injected were transferred back to their home cage where they were left undisturbed for 30 minutes. At the beginning of the test each rat was then placed in the illuminated compartment of the box and left there for 10 minutes. Using the recordings of a sensor in the hole between the illuminated and not illuminated compartment the movements of the rat were analyzed by a computer software (Biobserve GmbH) that quantified the latency to first leave the light and enter the dark compartment, total number of crossing and total time spent in each compartment. Short latency to first enter and increased time in the dark compartment were interpreted as reduced exploratory and thus anxious behavior (95). This behavioral test including the injections were performed by MAS and SGK with assistance from ML.





a light compartment
b dark compartment
c opening

Figure 7: Light/Dark box test, adapted from (1).

2.4.7. Restraint stress

For experiment 4 iv cannulated, separately housed male rats were transferred in their home cages from their housing room into a procedure room. One half of the animals were then restrained in a Decapi-Cone (DecapiCones, Braintree Scientific, Inc. Braintree, MA) that was prepared to allow ventilation and heat exchange (96, 97) and put back into their home cage in an immobile position, while the other half was left undisturbed in their home cages. The rats were then left in an immobile or undisturbed condition for 240 minutes only interrupted by blood withdrawal. The experiment was performed between 8 am and 2.30 pm by MGS and MAS in equal part.

2.5. Blood withdrawal and analysis

2.5.1. Blood withdrawal via intravenous cannula

As mentioned above for experiment 4, restrained and undisturbed rats equipped with an iv cannula underwent blood withdrawal 0, 15, 30, 60, 120 and 240 minutes after start of restraint stress or controlled conditions. The withdrawn blood was collected in pre-cooled tubes with aprotinin (1.2 trypsin inhibitory unit per 1 mL blood; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and EDTA (7.5%, 10 µL/0.5 mL blood; Sigma-Aldrich Chemie GmbH, Munich, Germany) and placed immediately on ice. These withdrawals of blood were performed by MGS and MAS. Afterwards, probes were centrifuged at 4 °C for 10 minutes at 3,000 x g and separated plasma was stored at -80 °C by SGK before further analysis.

2.5.2. Blood analysis

Blood withdrawn from rats in experiment 4 was analyzed using commercially purchased ELISA according the manufacturer's instructions by Petra Busse. ELISA assays of nesfatin-1 (#EK-003-22, Phoenix Pharmaceuticals, Inc.) displayed a linear detection range of 1.26–17.7 ng/mL and an intra-assay variability of 6.0 %. The linear detection range of ELISA assays of phoenixin (#EK079-01, Phoenix Pharmaceuticals, Inc.) was 0.07–2.1 ng/mL and intra-assay variability was 9.7 %. In assays of cortisol (#KGE008B, R&D Systems® Bio-Techne GmbH Wiesbaden-Nordenstadt, Germany) the linear detection range was 0.2–10 ng/mL and intra-assay variability was 12.2 %.

2.6. Statistical analysis

For statistical analysis SPSS 25 (IBM Corp. 2017, IBM SPSS Statistics for Windows, Version 25.0, Armonk, NY, USA) as well as SigmaStat 3.1. (Systat Software, San Jose, CA, USA) were used.

All data was tested for normality with the Kolmogorov-Smirnov test before further analysis. For experiments 1 (except data from SPT), 2 and 4 comparison of two groups was performed with a t-test for normally distributed data or using Mann-Whitney-U test when data was not normally distributed. For experiments 1 (data from SPT) and 3, when data from four groups were compared with each other, normally distributed data was analyzed using ANOVA followed by Tukey *post hoc* test and not normally distributed data was tested for significance with Kruskal-Wallis. For experiment 4 effect of time and treatment was analyzed with two-way ANOVA and correlation with Pearson's analysis. Data was expressed as mean ± SEM with significance defined as $p<0.05$.

3. Results

3.1. Nesfatin-1_{30-59} and depression-like behavior in normal weight rats

3.1.1. Nesfatin-1's effect on sucrose preference

The icv injection of nesfatin-1_{30-59} in male NW rats accustomed to the consumption of 1% sucrose solution decreased the sucrose/water intake ratio significantly at a dose of 0.3 nmol (0.66 ± 0.13, n=11) compared to vehicle (ddH_2O, 1.00 ± 0.00, n=11, $p<0.05$) and nesfatin-1_{30-59} at a dose of 0.1 nmol (1.00 ± 0.00, n=10, $p<0.05$). However, the sucrose/water ratio after icv injection of 0.9 nmol nesfatin-1_{30-59}

(0.88 ± 0.09, n=10, *p*=0.30) was not significantly decreased compared to that after the injection of 0.3 nmol nesfatin-$1_{30\text{-}59}$ (Figure 8).

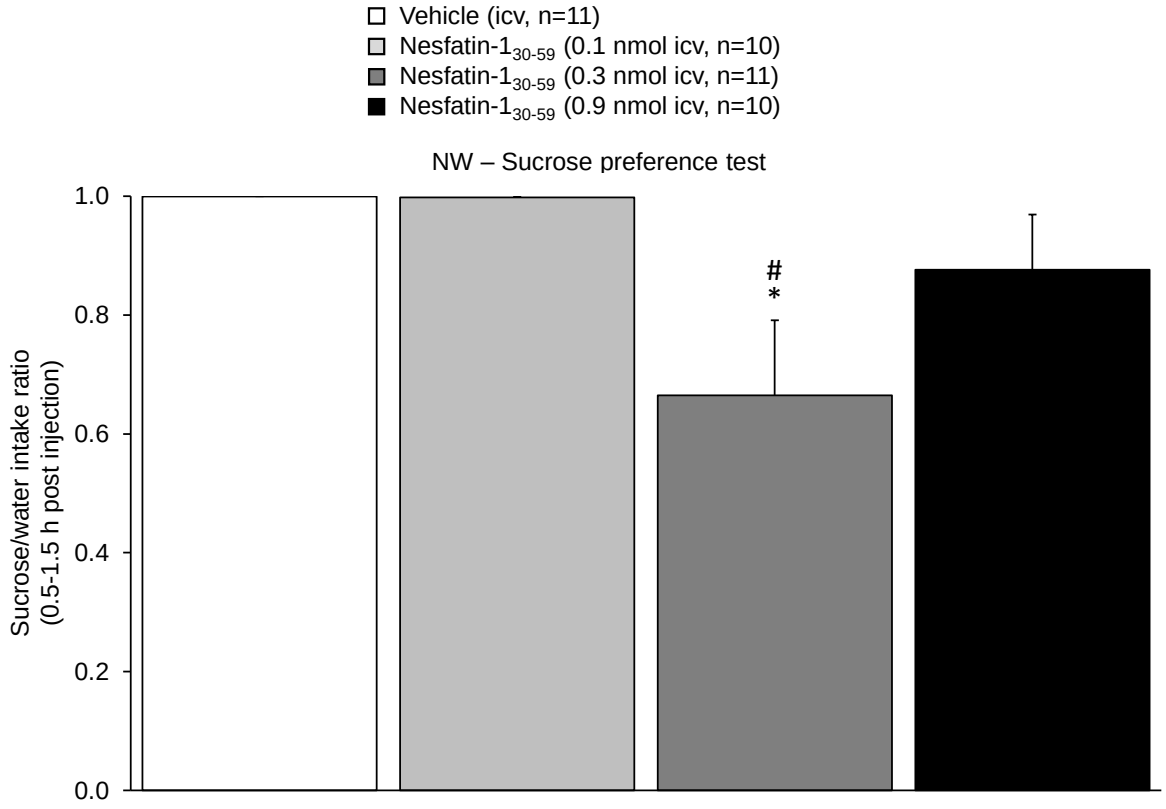


Figure 8: Nesfatin-$1_{30\text{-}59}$ reduced sucrose preference in normal weight male rats, adapted from (1). * *p*<0.05 vs. vehicle, # *p*<0.05 vs. nesfatin-$1_{30\text{-}59}$ 0.1 nmol.

3.1.2. Nesfatin-1's effect on novelty-induced hypophagia

In male NW rats that previously underwent a training period during which they developed a stable baseline of palatable snack intake, an icv injection of 0.3 nmol nesfatin-$1_{30\text{-}59}$ significantly reduced the amount of palatable snack intake in a novel environment (1.09 ± 0.32 kcal, *p*=0.04, n=8) compared to vehicle treated animals (ddH_2O, 4.36 ± 1.31 kcal, n=8). In contrast, the latency to approach the palatable snack in a novel environment was not different between rats treated with nesfatin-$1_{30\text{-}59}$ at a dose of 0.3 nmol (207.0 ± 58.4 s, *p*=0.68) and those injected with vehicle (ddH_2O, 241.3 ± 48.8 s; Figure 9).

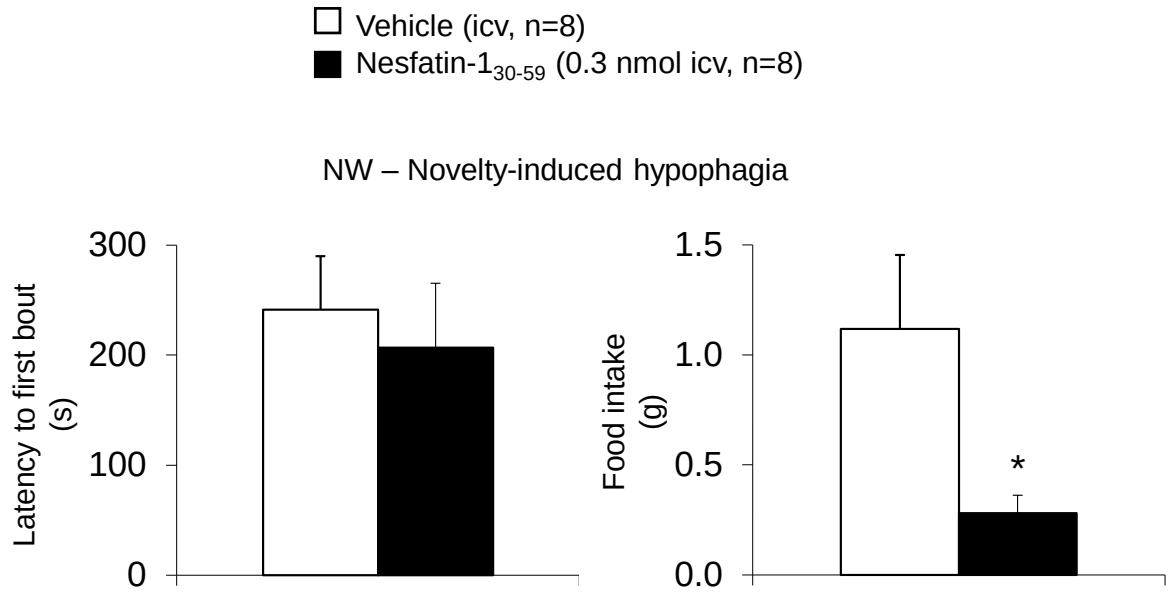


Figure 9: Nesfatin-1$_{30-59}$ evoked novelty-induced hypophagia in normal weight male rats, adapted from (1). * p<0.05 vs. vehicle.

3.1.3. Nesfatin-1 antibody's effect on novelty-induced hypophagia

When male rats that were accustomed to the access to a palatable snack were icv injected with a nesfatin-1 antibody and vehicle (0.9% NaCl, n=11) they displayed no difference to animals that received a control antibody and vehicle (n=12) regarding latency to the first feeding bout (1.08 ± 0.18 vs. 3.98 ± 1.96 min, p>0.05), number of bouts (7.09 ± 1.45 vs. 4.67 ± 0.86, p>0.05) or palatable snack intake (1.02 ± 0.36 vs. 1.02 ± 0.36 g, p>0.05) in a novel environment.

Furthermore, when male rats displaying a stable baseline of palatable snack intake in a familiar environment were icv injected with a nesfatin-1 antibody and CRF (n=11) no differences in their behavior compared to rats treated with a control antibody and CRF (n=11) were observed regarding latency to the first feeding bout (1.07 ± 0.21 vs. 3.09 ± 0.96 min, p>0.05), number of bouts (6.18 ± 1.28 vs. 4.64 ± 0.8, p>0.05), or palatable snack intake (0.51 ± 0.17 vs. 0.35 ± 0.07 g, p>0.05).

Additionally, comparing rats that underwent the NIH protocol and received control antibody and CRF with rats that were icv injected with control antibody and vehicle no differences in behavior regarding latency to the first feeding bout (0.8-fold, p>0.05), number of bouts (1.0-fold, p>0.05) or palatable snack intake (0.3-fold, p>0.05) in a novel environment were observed.

3.2. Nesfatin-1_{30-59} and depression-like behavior in diet-induced obese rats

3.2.1. Nesfatin-1's effect on sucrose preference in obesity

In male DIO rats that were accustomed to the consumption of a 1% sucrose solution an icv injection of 0.3 nmol nesfatin-1_{30-59} (0.89 ± 0.06, n=8, *p*=0.69) did not alter sucrose/water intake ratio 30 to 90 minutes after injection compared to vehicle (ddH_2O, 0.85 ± 0.07, n=8).

3.2.2. Nesfatin-1's effect on novelty-induced hypophagia in obesity

When nesfatin-1_{30-59} in a dose of 0.3 nmol (n=5) was icv injected in male DIO rats displaying a stable palatable snack intake in a familiar environment, no difference compared to vehicle (ddH_2O, n=5) was observed regarding latency to approach the food (105.2 ± 16.16 vs. 76.2 ± 16.17 s, *p*=0.64) or palatable snack intake (2.97 ± 0.69 vs. 3.57 ± 0.73 kcal, *p*=0.61) in a novel environment.

3.3. Nesfatin-1_{30-59} and anxious behavior in normal weight rats

3.3.1. Nesfatin-1's effect on the elevated zero maze test

The icv injection of nesfatin-1_{30-59} at a dose of 0.3 nmol (n=9) in male NW naïve rats significantly reduced the number of visits in the open arms of the maze (19.8 ± 1.8, *p*=0.002) when compared with vehicle treated rats (ddH_2O, 32.5 ± 2.8, n=8-9/group; Figure 10). Furthermore, the treatment with 0.3 nmol nesfatin-1_{30-59} tended to reduce the time in the open arms of the EZM (92.7 ± 11.9 s, *p*=0.05) compared to vehicle (128.9 ± 15.6 s), while no difference was observed between the two experimental groups regarding overall track length (25.6 ± 1.4 vs 27.9 ± 1.1 m, *p*=0.14).

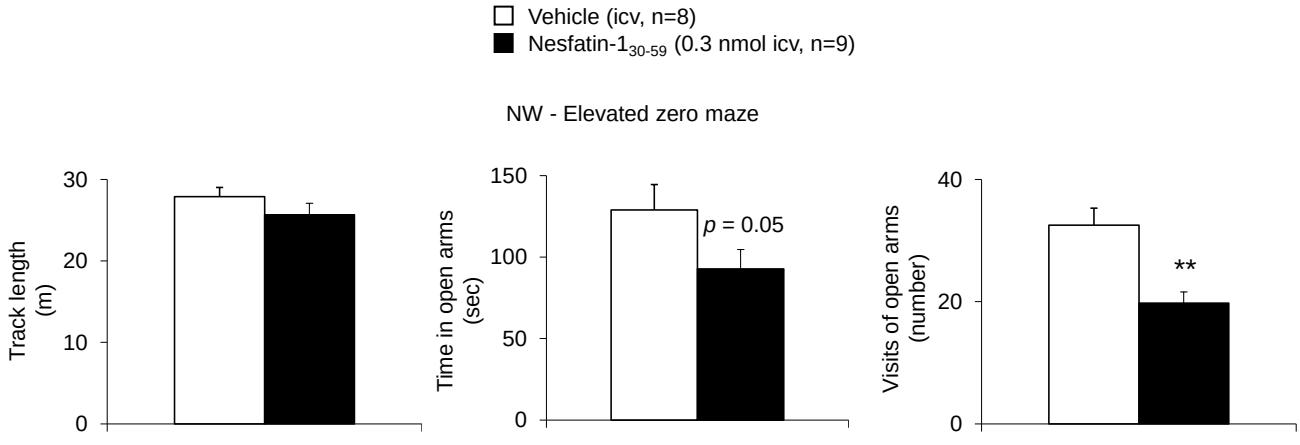


Figure 10 Nesfatin-1_{30-59} increased anxious behavior in the elevated zero maze test in normal weight male rats, adapted from (1). ** *p*<0.01 vs. vehicle.

3.3.2. Nesfatin-1 antibody's effect on the elevated zero maze test

When NW naïve rats icv injected with nesfatin-1 antibody and vehicle (n=11) were compared to rats that received a control antibody and vehicle (0.9% NaCl, n=11) an increased number of entries into the open arms of the EZM (33.18 ± 2.04 vs. 20.36 ± 2.68, $p<0.05$) and an increased overall track length (35.96 ± 1.92 vs. 25.84 ± 1.7 m, $p<0.05$; Figure 11) was observed.

Moreover, when male naïve NW rats that were treated with nesfatin-1 antibody and vehicle were compared to rats that received nesfatin-1 antibody and CRF (n=9) an increased number of entries into the open arms (33.18 ± 2.04 vs. 14.89 ± 3.69, $p<0.001$), time in open arms (134.5 ± 12.68 vs. 71.16 ± 16.15 s, $p<0.05$), track length in the open arms (18.43 ± 2.08 vs. 7.72 ± 2.75 m, $p<0.01$) and overall track length (35.96 ± 1.92 vs. 18.17 ± 3.58 m, $p<0.001$) was detected.

When comparing nesfatin-1 antibody- and vehicle-treated male naïve NW rats with rats that were icv injected with control antibody and CRF the number of entries into the open arms (33.18 ± 2.04 vs. 13.58 ± 2.34, $p<0.001$), time in open arms (134.55 ± 12.68 vs. 71.07 ± 10.81 s, $p<0.05$), track length in open arms (18.43 ± 2.08 vs. 6.13 ± 1.18 m, $p<0.001$) and overall track length (35.96 ± 1.92 vs. 18.83 ± 1.28 m, $p<0.001$) were significantly increased.

No significant differences were observed between rats treated with control antibody and CRF (n=12) compared to those that received nesfatin-1 antibody and CRF regarding number of entries into the open arms (13.58 ± 2.34 vs. 14.89 ± 3.69, $p=0.99$), time in the open arms (71.07 ± 10.81 s vs. 71.16 ± 16.15 s, $p=1.00$), track length in open arms (6.13 ± 1.18 m vs. 7.72 ± 2.75 m, $p=0.95$) or overall track length (18.83 ± 1.28 m vs. 18.17 ± 3.58 m, $p=1.00$).

Control antibody- and CRF-injected male naïve NW rats tended to display decreased time in the open arms (71.07 ± 10.81 s, $p=0.17$) and track length in the open arms (6.13 ± 1.18 m, $p=0.23$) when compared with rats that were treated with control antibody and vehicle (113.53 ± 16.49 s and 11.56 ± 1.89 m).

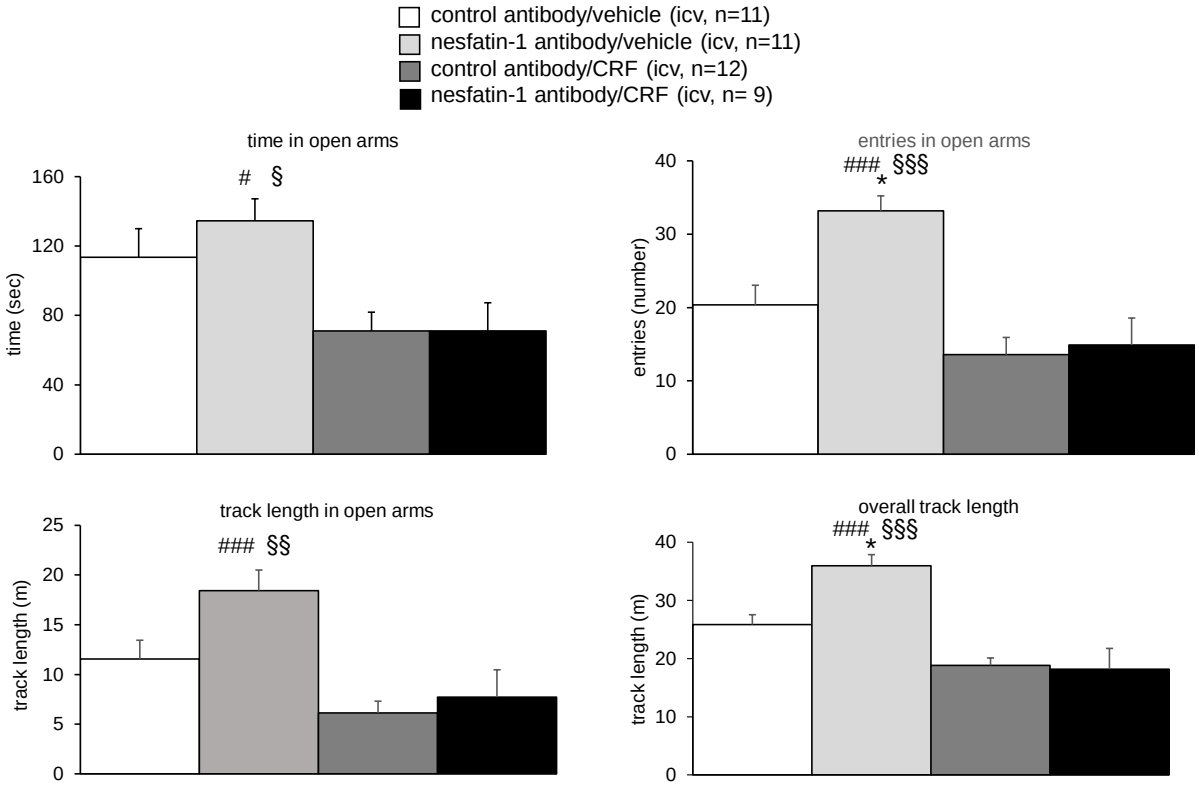


Figure 11 Nesfatin-1 antibody decreased anxious behavior in the elevated zero maze test in normal-weight naïve male rats, adapted from (2). * p<0.05 vs. control antibody/vehicle; # p<0.05 and ### p<0.001 vs. control antibody/CRF; § p<0.05, §§ p<0.01 and §§§ p<0.001 vs. nesfatin-1 antibody/CRF.

3.3.3. Nesfatin-1's effect on the open field test

Naïve male NW rats receiving an icv injection of 0.3 nmol nesfatin-$1_{30\text{-}59}$ (n=12) decreased the number of entries into the center zone (20.6 ± 2.0, p=0.008) and duration spent in this zone during the OFT (11.5 ± 0.7 s, p=0.04) when compared to vehicle-treated rats (dd$H_2$0, 37.8 ± 6.1 and 16.3 ± 2.6 s, respectively, n=11; Figure 12). However, average velocity in the OFT (7.4 ± 0.4 vs. 7.4 ± 0.5 cm/s, p=0.47) and overall distance (22.3 ± 1.2 m vs. 22.6 ± 0.9 m, p=0.82) did not differ between nesfatin-$1_{30\text{-}59}$-treated and vehicle-treated animals.

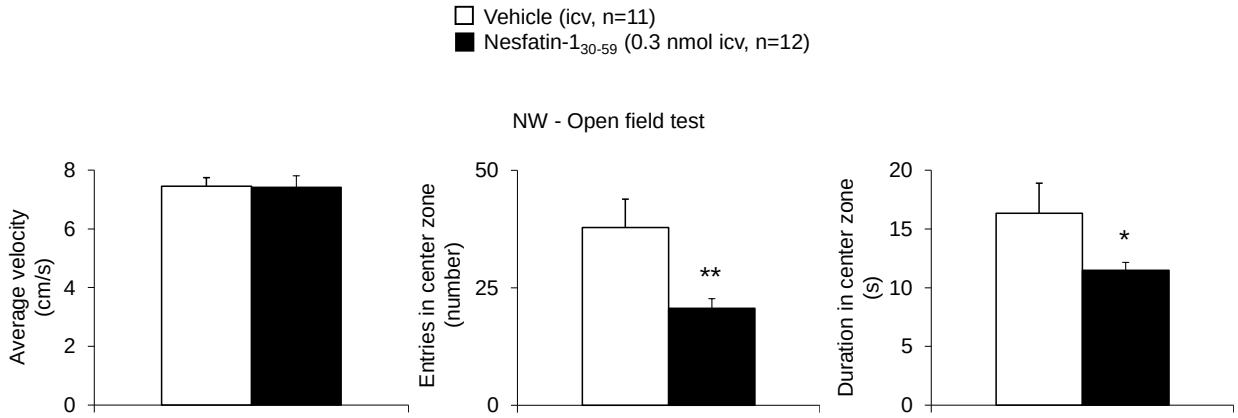


Figure 12 Nesfatin-1_{30-59} increased anxious behavior in the open field test in normal weight male rats, adapted from (1). * p<0.05 and ** p<0.01 vs. vehicle.

3.3.4. Nesfatin-1's effect on the light/dark box test

The behavior of naïve NW male rats in the LDB did not differ between those that were icv injected with nesfatin-1_{30-59} at a dose of 0.3 nmol (n=7) and those that received vehicle (ddH_2O, n=8) regarding time in the dark compartment (381.9 ± 12.2 vs. 413.0 ± 15.9 s, p=0.10), number of visits of the bright compartment (12.0 ± 0.9 vs. 11.5 ± 0.6, p=0.33) and time in the bright compartment (218.1 ± 12.2 vs. 187.0 ± 15.9 s, p=0.10), although a trend of a decreased latency to cross to the dark compartment was observed after nesfatin-1_{30-59} injection (21.5 ± 3.2 vs. 32.6 ± 5.6 s, p=0.08).

3.4. Nesfatin-1_{30-59} and anxious behavior in diet-induced obese rats

3.4.1. Nesfatin-1's effect on the elevated zero maze test in obesity

In DIO male rats icv injection of 0.3 nmol nesfatin-1_{30-59} (n=5) had no effect on behavior in the EZM, as reflected by similar visits of the open arms (15.6 ± 2.77, p=0.94), time in open arms (80.58 ± 15.62 s, p=0.93) and overall track length (27.13 ± 1.74 m p=0.93) when compared to vehicle (ddH_2O, n=5, 15.0 ± 5.95, 77.92 ± 22.35 s and 26.7 ± 3.71 m).

3.4.2. Nesfatin-1's effect on the open field test in obesity

When comparing the behavior in the OFT of DIO rats that received nesfatin-1_{30-59} at a dose of 0.3 nmol (n=5) with those that were injected with vehicle (ddH_2O, n=5) no difference of number of entries into the center zone (42.75 ± 10.73 vs. 32.8 ± 3.94, p=0.47), duration spent in this zone (12.98 ± 3.82 vs. 15.8 ± 2.94 s, p=0.63), average

velocity in the open field test (5.53 ± 0.53 vs. 5.97 ± 0.15 cm/s, *p*=0.50) or overall distance (16.9 ± 1.6 m vs. 18.5 ± 0.7 m, *p*=0.41) was observed between both groups.

3.4.3. Nesfatin-1's effect on the light/dark box test in obesity

All parameters assessed in the LDB were similar between DIO rats treated with 0.3 nmol nesfatin-1_{30-59} (n=5) and those injected with vehicle (n=5), including time in the dark compartment (528.6 ± 15.34 vs. 527.44 ± 17.07 s, *p*=0.97), latency to cross to the dark compartment (8.79 ± 2.53 vs. 13.03 ± 5.17 s, *p*=0.57), number of visits of the bright compartment (10.75 ± 3.07 vs. 8.6 ± 1.89, *p*=0.63) and time in the bright side (71.4 ± 15.34 vs. 72.56 ± 17.07 s, *p*=0.97).

3.5. NUCB2/nesfatin-1 under restraint conditions

Restraint stress in NW male rats equipped with an iv cannula did not affect circulating NUCB2/nesfatin-1 levels compared to undisturbed animals at 0, 15, 30, 60 and 120 minutes; however, after 240 minutes there was a significant increase in plasma levels of NUCB2/nesfatin-1 in restraint compared to undisturbed rats (5.30 ± 0.33 vs. 3.95 ± 0.32 ng/mL, *p*<0.05, Figure 13). In addition, a trend indicating an effect of time on NUCB2/nesfatin-1 levels ($F_{(5,78)}$=2.356, *p*=0.05) was observed in the two-way ANOVA.

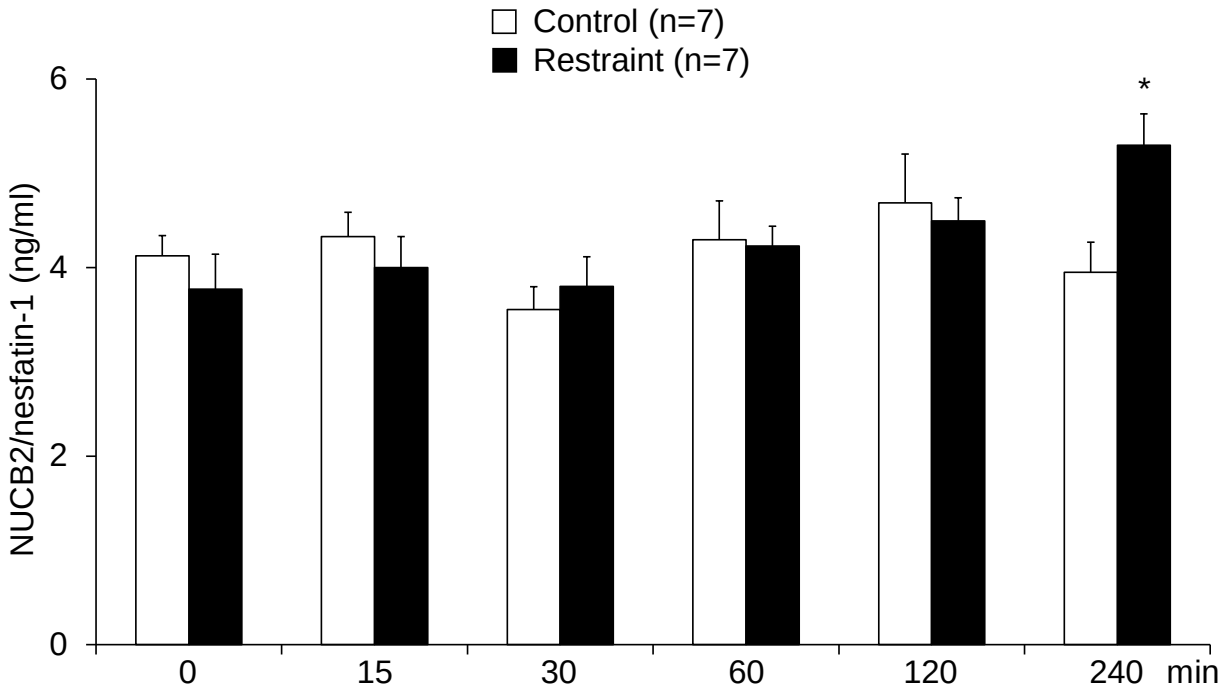


Figure 13 Circulating NUCB2/nesfatin-1 levels were elevated at 240 minutes of restraint stress in normal weight male rats, adapted from (3). * p<0.05 vs. control.

The Pearson's analysis indicated no significant correlation between circulating NUCB2/nesfatin-1 and plasma cortisol levels (r=-0.143, p>0.05); however, a significant positive correlation between plasma NUCB2/nesfatin-1 and phoenixin concentrations (r=0.378, p<0.001) was observed (Figure 14).

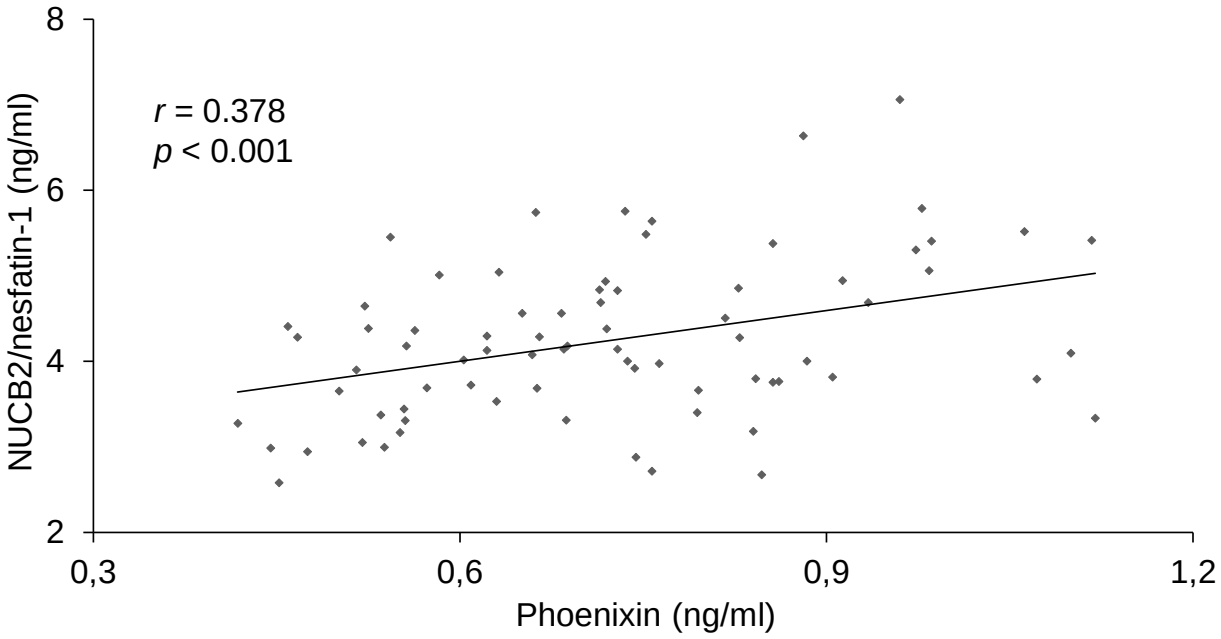


Figure 14 Circulating NUCB2/nesfatin-1 was positively correlated with circulating phoenixin in stressed and naïve male rats, adapted from (3).

4. Discussion

Since depression and anxiety disorders are highly prevalent enormously impacting on the quality of life of affected patients (7), their pathogeneses are merely understood and treatments lack efficacy especially in terms of long-term outcome (8, 16), the performed studies aimed to shed some light on the pathological mechanism responsible for depressive-like and anxious behavior. Since emerging data indicate pleotropic effects of several novel peptides including nesfatin-1 (98) in these studies the focus was to illuminate its role in the development of depression and anxiety.

4.1. Nesfatin-1's depression-like inducing effect

A previous study indicated that full-length nesfatin-1 increases anhedonia in rats (68). In detail, icv injected full-length nesfatin-1 reduced food intake of a palatable snack under novelty conditions (68); however, another study failed to show that intraperitoneally injected full-length nesfatin-1 elicited an anhedonic effect, since it did not affect 2% sucrose solution intake over 6 hours in a SPT (66). Although in the same study it was shown that acute and chronic peripheral administration of full-length nesfatin-1 increased immobility in the forced swim test (66) and authors concluded that peripheral full-length nesfatin-1 was able to evoke depressive-like behavior, it is to note that there is evidence that the forced swim test does not accurately measure depression-like behavior but rather a stress-coping strategy (99). Thus, this data indicates that full-length nesfatin's anhedonic effect is predominately centrally mediated.

To further illuminate this property of nesfatin-1, in this study it was tested whether the fragment of nesfatin-1, that mediates centrally-mediated anorexia, is also responsible for its depression-like effect. Indeed, it was observed for the first time that 0.3 nmol of nesfatin-$1_{30\text{-}59}$ robustly reduced the sucrose/water intake ratio and palatable snack intake in a novel environment 30 minutes after icv injection in NW male rats (1), indicating an anhedonic effect. Interestingly, icv injection of 0.1 nmol and 0.9 nmol of nesfatin-$1_{30\text{-}59}$ did not significantly reduce sucrose/water intake ratio, resulting in a U-shaped relation dose-effect curve. One possible reason for the ineffectiveness of nesfatin-$1_{30\text{-}59}$ in a dose of 0.9 nmol on anhedonia could be supraphysiological stimulation of its receptor leading to receptor endocytosis and desensitization. Additionally, it could be suggested that nesfatin-$1_{30\text{-}59}$ functions in a self-regulatory system, where its increased action is balanced due to endogenous antagonistic substances. Both theories warrant further investigations. Noteworthy, it is assumed that the effect on palatable snack intake is not due to nesfatin-$1_{30\text{-}59}$'s anorexigenic property, since it was previously shown that full-length nesfatin-1 icv injection did not change palatable snack intake in the home cage (68) and nesfatin-$1_{30\text{-}59}$'s anorexigenic effect was reported to have a delayed onset of 4 hours after icv injection (86).

Since these studies showed effectiveness of nesfatin-1/ nesfatin-$1_{30\text{-}59}$ only after exogenous injection (1, 66, 68) it was unclear whether the depression-like inducing effect was physiological or rather pharmacological. In the present study it was consequently investigated whether the acute blockage of central nesfatin-1 resulted in an anti-depressant effect. Icv injection of male rats with a nesfatin-1 antibody in

combination with a vehicle did not alter palatable snack intake in a novel environment compared to animals that received control antibody and vehicle (2). Taken together, the present study observations suggest that endogenous nesfatin-1 is not involved in the mediation of anhedonia in naïve rats under basal conditions.

In order to test if this was also true under conditions of stress, male rats were treated with icv CRF and then the effect of acute pre-treatment with nesfatin-1 antibody was evaluated. However, since CRF was not able to induce a significant anhedonic effect (2), the anti-anhedonic effect of nesfatin-1 antibody under stress conditions could not be sufficiently examined.

Although these results argue against an endogenous role of nesfatin-1 in the mediation of anhedonia in NW rats, the present investigations nevertheless may suggest an endogenous effect of nesfatin-1 on anhedonia in obesity. Despite the lack of an anhedonic effect of 0.3 nmol nesfatin-$1_{30\text{-}59}$ in DIO rats, still in both DIO groups (nesfatin-$1_{30\text{-}59}$ and vehicle) sucrose water intake ratio was 0.89 ± 0.06 and 0.85 ± 0.07, what seemed to be decreased compared to a ratio of 1.00 ± 00 in vehicle-treated NW rats (1). Similarly, both latency to approach the food (105.2 ± 16.16 and 76.2 ± 16.17 s) and palatable snack intake (2.97 ± 0.69 and 3.57 ± 0.73 kcal) in DIO rats with or without nesfatin-$1_{30\text{-}59}$ treatment appeared to be reduced when compared to vehicle treated NW animals (241.3 ± 48.8 s and 4.36 ± 1.31 kcal) (1). However, due to the fact that those animals were not tested at the same time their behavior was not directly compared statistically; thus, it cannot be assured if differences between NW and DIO rats were significant. Noteworthy, there is evidence pointing towards increased NUCB2/nesfatin-1 levels in DIO rats, indicated by nesfatin-1 expression in adipose tissue, increased levels in obese mice (41) and a positive correlation between gastric expression and BMI in humans (100). It could thus be concluded that increased nesfatin-1 in states of obesity could be responsible for an endogenous anhedonic phenotype. Moreover, the lack of pronounced depression-like behavior in DIO rats due to application of 0.3 nmol nesfatin-$1_{30\text{-}59}$ (1) could be explained by central desensitization of nesfatin-1 signaling under conditions of supposed hyper-nesfatinemia in obesity, as seen with a dose of 0.9 nmol nesfatin-$1_{30\text{-}59}$ in NW rats (1). Whether this hypothesis can be corroborated needs to be tested in following investigations.

4.2. Nesfatin-1’s anxiety-inducing effect

Previous studies showed that full-length nesfatin-1 increases anxiety in rats (68, 76). Firstly, icv injection of full-length nesfatin-1 reduced the number of entries into and the proportion of time spent in open arms of an elevated plus maze (68); additionally daily intraperitoneal injection with high doses of nesfatin-1 of 2-8 µg/day for three weeks reduced moving distance and duration in the center of the OFT (76). The latter study could indicate that also peripherally applied full-length nesfatin-1 is able to induce anxious behavior (76), pointing towards a not exclusively centrally mediated anxiety-inducing effect of nesfatin-1. However, since nesfatin-1 was shown to be able to cross the blood-brain barrier (46, 101) and additionally only three-week-long but not acute treatment resulted in increased anxiety (76), it can be hypothesized that it is crucial that circulating nesfatin-1 reaches the CNS to modulate anxiety.

To further shed light on the mechanisms responsible for full-length nesfatin-1-induced anxiety, in the present experiments the effect of the fragment responsible for nesfatin-1's anorexigenic effect on anxious behavior was tested. Indeed, icv injection of 0.3 nmol nesfatin-$1_{30\text{-}59}$ reduced explorative behavior of NW rats in the EZM and OFT indicating increased anxious behavior (1). Interestingly, this effect could not be reproduced in the LDB. This could be explained by nesfatin-1's sensitivity to light. Nesfatin-1's anorexigenic effect was reproduced several times under low illumination of 30-40 lx (32) or during dark phase (47, 86, 102); for the LDB however, rats were initially placed in a strongly illuminated compartment of the box (135 lx), which could have prevented an effect, while the OFT and EZM were located in a procedure room with dimmed light. Moreover, since in mice it was shown that starting in the light compared to the dark compartment in the LDB induced an approach behavior (103), it could be hypothesized that this phenomenon also affected the present experiments. In conclusion, although it could not be reproduced in the LDB the present study found significant evidence that exogenous injection of the middle fragment of full-length nesfatin-1 increases anxiety in NW rats (1).

Noteworthy, the injection of the same dose of nesfatin-$1_{30\text{-}59}$ did not increase anxious behavior in DIO rats (1). Presumably, the obese phenotype impacted the activity of those rats, as corroborated by differences (that were not statistically tested) in average velocity in the OFT compared between DIO and NW vehicle-treated rats (5.53 ± 0.53 and 5.97 ± 0.15 vs. 7.4 ± 0.5 cm/s) (1). An altered activity level could be a reason for reduced validity of tests assessing anxiety by measuring exploratory behavior, as in the present study.

Another reason for the lack of effect of nesfatin-1_{30-59} in DIO rats could be increased baseline anxiety compared to NW rats. This is indicated by a supposed difference between DIO (with or without nesfatin-1_{30-59} injection) and NW vehicle-treated NW rats in the EZM regarding visits (15.6 ± 2.77 and 15.0 ± 5.95 vs. 32.5 ± 2.8) and time in open arms (80.58 ± 15.62 and 77.92 ± 22.35 vs. 128.9 ± 15.6 s) or LDB including time in bright (71.4 ± 15.34 and 72.56 ± 17.07 vs. 187.0 ± 15.9 s) (1), which were not tested for significance because experiments were performed at different times. These possible differences could indicate that DIO rats display an endogenous anxious phenotype, possibly due to increased nesfatin-1 levels as mentioned above (41, 100), in which the addition of nesfatin-1_{30-59} at a dose of 0.3 nmol cannot further increase anxiety.

To test the hypothesis that nesfatin-1 is implicated in the endogenous mediation of anxiety the effects of acute blockade of central nesfatin-1 on exploratory behavior were observed. Indeed, based on more frequent entries into the open arms and an increased overall track length in the EZM due to icv nesfatin-1 antibody injection (2) the present study points towards a physiological anxiogenic effect of endogenous nesfatin-1 in the CNS. However, this was not observed in rats with CRF-induced stress (2), indicating that nesfatin-1 does not act downstream of CRF in the mediation of anxiety, but nevertheless displays an endogenous and not pharmacological anxiety-inducing effect. Based on the present study it cannot be excluded that nesfatin-1 modulates CRF-induced stress by affecting up-stream signaling. This hypothesis could be corroborated by increased hypothalamic CRF mRNA expression due to acute peripheral injection of full-length nesfatin-1, in relation to increased immobility in the forced swim test (66). However, it warrants further research to understand by which mechanism nesfatin-1_{30-59} induces its anxiogenic effect.

4.3. Nesfatin-1's modulation due to stress

Previous literature was able to show that stress in various forms influences nesfatin-1. Physical stress in form of abdominal surgery led to the activation of NUCB2/nesfatin-1 positive brain areas (82). Immunological stress induced by lipopolysaccharide injection activated NUCB2/nesfatin-1 immunoreactive neurons (81) and increased circulating NUCB2/nesfatin-1 levels (104). Emotional stress during the acute water avoidance test elevated hypothalamic mRNA expression and circulating concentration of NUCB2/nesfatin-1 (105). Between 15 and 60 minutes of restraint

stress activated NUCB2/nesfatin-1 immunoreactive brain nuclei (97), in detail c-Fos expressions was detected in NUCB2/nesfatin-1-expressing neurons in the PVN, SON, nucleus of the solitary tract, locus coeruleus and dorsal raphe nucleus. Noteworthy, this short duration of restraint stress did not lead to an alteration of plasma NUCB2/nesfatin-1 levels (80), while chronic immobilization stress for 21 days increased concentrations of NUCB2/nesfatin-1 not only in the PVN but also in the serum (85).

In this study it was thus tested if acute emotional stress for a longer duration could alter circulating NUCB2/nesfatin-1 levels. Indeed, restraint stress in NW male rats led to a distinct stress reaction as suggested by a significant increase in plasma cortisol compared to undisturbed rats (3). Noteworthy, there was no correlation between the increase in cortisol and NUCB2/nesfatin-1 in plasma, since NUCB2/nesfatin-1 concentrations were similar between stressed and undisturbed rats throughout the first part of the experiment (3). Only after 240 minutes of immobilization stress circulating NUCB2/nesfatin-1 increased in comparison to undisturbed rats (3).

Unfortunately, since the last blood withdrawal before 240 minutes after the start of the immobilization was at 120 minutes, it cannot be clearly determined when exactly between 2 and 4 hours of restraint stress the rise of NUCB2/nesfatin-1 levels began. Nevertheless, it can be said that the emotional stress-induced increase of circulating NUCB2/nesfatin-1 is delayed, which could be caused by long duration of absorption of NUCB2/nesfatin-1 from expression sites, such as the hypothalamus (106) which is stimulated during stress (85), into the circulation.

It is to doubt that the observed increase in circulating NUCB2/nesfatin-1 has a consequence on physiological functions since it was altered only about +30 % compared to controls. Interestingly, the present experiments could show that there was a significant positive correlation between NUCB2/nesfatin-1 and phoenixin (3). Since phoenixin is a hypothalamic peptide that is closely co-expressed with nesfatin-1 in the hypothalamus (35) it could be assumed that the stress-induced alteration of nesfatin-1 is more a hypothalamic than a peripheral phenomenon. Consequently, it can be hypothesized that it is rather the increased hypothalamic than the circulating NUCB2/nesfatin-1 level that is clinically significant.

This assumption is supported by studies indicating an effect of nesfatin-1 on the HPA axis. In this context it was shown that centrally applied nesfatin-1 activated CRF positive neurons in the PVN (80), increased hypothalamic mRNA expression of CRF

(66) and upregulated expression of CRF peptide (107). Moreover, nesfatin-1 injected into the third ventricle increased circulating levels of adrenocorticotropin hormone and corticosterone (80). These studies indicate that centrally applied nesfatin-1 could induce hyperactivity of the HPA axis and the present study was able to corroborate those studies by specifying that this effect is endogenous but probably not induced by modulation of signaling downstream from CRF (2). Since in depression CRF neurons were found to be strongly activated and thus the HPA axis is frequently found to be hyperactive (108) it could be suggested that nesfatin-1 is implicated in this activation of CRF neurons and thus the development of depression.

4.4. Conclusion

Several conclusions can be drawn from the present work. Firstly, it was observed that nesfatin-$1_{30\text{-}59}$ is able to induce depressive and anxious behavior after icv injection in NW but not DIO rats, suggesting a desensitization in obesity to nesfatin-1. Moreover, nesfatin-1 was shown to be differentially implicated in the development of depression and anxiety. While it induces anhedonia only after exogenous injection, it increases anxiety due to exogenous and endogenous mechanisms. The distinct signaling pathways warrant further research. Additionally, in our investigations nesfatin-1 blockade did not abolish a CRF-induced stress reactions causing anxiety and anhedonia; thus, nesfatin-1 is probably not implicated in CRF downstream signaling. However, since circulating NUCB2/nesfatin-1 was increased due to prolonged acute immobilization stress associated with increased corticosteroid plasma level, a significant role of nesfatin-1 in the HPA axis is likely. Thus, more investigations should follow studying whether CRF inhibition could prevent nesfatin-1-induced depressiveness or anxiety as well as experiments further corroborating the anxiolytic effect of nesfatin-1 blockade in other species.

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Statutory Declaration

"I, Martha Anna Schalla, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Die Rolle von Peptiden in der Entstehung von Depression und Angst – Implikation von NUCB2/Nesfatin-1; The role of peptides in the pathogenesis of depression and anxiety – implication of NUCB2/nesfatin-1", independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

Declaration of your own contribution to the publications

Martha Anna Schalla contributed the following to the below listed publications:

Publication 1: Kühne SG*, Schalla MA*, Friedrich T, Kobelt P, Goebel-Stengel M, Long M, Rivalan M, Winter Y, Rose M, Stengel, A Nesfatin-1(30-59) Injected Intracerebroventricularly Increases Anxiety, Depression-Like Behavior, and Anhedonia in Normal Weight Rats, Nutrients, 2018:
Preparation of experiments and materials (equal contribution as SGK), performing intracerebroventricular cannulations of DIO rats as surgeon, performing intracerebroventricular cannulations of NW rats as assistance, monitoring of the experimental animals (equal contribution as SGK), performing all intracerebroventricular injections as well as all behavioral experiments (equal contribution as SGK and with assistance of ML), results shown in section 3.1. and 3.2. concerning the DIO rats as well as the figures 1B, 2C, 2D, 3D, 3E, 3F, 4D, 4E, 4F, 5E, 5F, 5G and 5H in the publication were created on the basis of my statistical evaluation, extensive corrections to the first draft of the manuscript made by SGK, revision of the manuscript after peer review (equal contribution as SGK and with assistance of all other co-authors).

Publication 2: Schalla MA, Kühne SG, Friedrich T, Kobelt P, Goebel-Stengel M, Long M, Rivalan M, Winter Y, Mori M, Rose M, Stengel A, Central blockage of nesfatin-1 has anxiolytic effects but does not prevent corticotropin-releasing factor-induced anxiety in male rats, Biochemical and biophysical research communications, 2020:
Preparation of experiments and materials (equal contribution as SGK), performing all intracerebroventricular cannulations as surgeon, monitoring of the experimental animals (equal contribution as SGK), performing all intracerebroventricular injections as well as all behavioral experiments (equal contribution as SGK and with assistance of ML), results shown in section 3. and figures 1 and 2 in the publication were created on the basis of my statistical analysis, designing the drafts of figure 1 and figure 2, writing the entire first draft of the manuscript, manuscript submission, revision of the manuscript after peer review (with assistance of all other co-authors).

Publication 3: Schalla MA*, Goebel-Stengel M*, Friedrich T, Kühne SG, Kobelt P, Rose

M, Stengel A, Restraint stress affects circulating NUCB2/nesfatin-1 and phoenixin levels in male rats, Psychoneuroendocrinology, 2020:
Designing and preparation of experiments and materials (equal contribution as MGS and SGK), assisting with half of the intravenous cannulations (MSG was surgeon, SGK assisted with other half of iv cannulations), monitoring the experimental animals (equal contribution as MGS and SGK), performing the stress experiments including blood sampling (equal contribution as MGS and SGK), writing the entire first draft of the manuscript, conversion of the published figures 1, 2, 3 and 4 from excel to power point format and subsequent minor editing, designing of the first draft of the graphical abstract of the publication, manuscript submission, revision of the manuscript after peer review (with assistance of all other co-authors).

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Signature of doctoral candidate

Publications

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI Selected Categories: **"NUTRITION and DIETETICS"** Selected Category Scheme: WoS

Gesamtanzahl: 81 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	Annual Review of Nutrition	5,528	8.886	0.005230
2	PROGRESS IN LIPID RESEARCH	5,302	8.435	0.006750
3	Advances in Nutrition	3,937	6.853	0.012870
4	AMERICAN JOURNAL OF CLINICAL NUTRITION	58,213	6.549	0.055760
5	CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION	10,197	6.015	0.011670
6	NUTRITION REVIEWS	7,526	5.788	0.010600
7	International Journal of Behavioral Nutrition and Physical Activity	8,371	5.548	0.019780
8	CLINICAL NUTRITION	10,558	5.496	0.016870
9	PROCEEDINGS OF THE NUTRITION SOCIETY	5,238	5.347	0.006230
10	INTERNATIONAL JOURNAL OF OBESITY	22,185	5.151	0.032040
11	FOOD CHEMISTRY	90,665	4.946	0.101120
12	NUTRITION RESEARCH REVIEWS	2,164	4.586	0.001840
13	CURRENT OPINION IN CLINICAL NUTRITION AND METABOLIC CARE	4,842	4.534	0.007130
14	EUROPEAN JOURNAL OF NUTRITION	5,669	4.423	0.011650
15	JOURNAL OF NUTRITIONAL BIOCHEMISTRY	9,815	4.414	0.014150
16	JOURNAL OF NUTRITION	38,804	4.398	0.029930
17	JOURNAL OF PARENTERAL AND ENTERAL NUTRITION	5,287	4.249	0.007990
18	Nutrients	12,031	4.196	0.032520
19	Obesity	17,578	4.042	0.037840

Selected JCR Year: 2017; Selected Categories: "NUTRITION and DIETETICS"

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Article

Nesfatin-1_{30-59} Injected Intracerebroventricularly Increases Anxiety, Depression-Like Behavior, and Anhedonia in Normal Weight Rats

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Abstract: Nesfatin-1 is a well-established anorexigenic peptide. Recent studies indicated an association between nesfatin-1 and anxiety/depression-like behavior. However, it is unclear whether this effect is retained in obesity. The aim was to investigate the effect of nesfatin-1_{30-59}—the active core of nesfatin-1—on anxiety and depression-like behavior in normal weight (NW) and diet-induced (DIO) obese rats. Male rats were intracerebroventricularly (ICV) cannulated and received nesfatin-1_{30-59} (0.1, 0.3, or 0.9 nmol/rat) or vehicle 30 min before testing. Nesfatin-1_{30-59} at a dose of 0.3 nmol reduced sucrose consumption in the sucrose preference test in NW rats compared to vehicle (–33%, $p < 0.05$), indicating depression-like/anhedonic behavior. This dose was used for all following experiments. Nesfatin-1_{30-59} also reduced cookie intake during the novelty-induced hypophagia test (−62%, $p < 0.05$). Moreover, nesfatin-1_{30-59} reduced the number of entries into the center zone in the open field test (−45%, $p < 0.01$) and the visits of open arms in the elevated zero maze test (−39%, $p < 0.01$) in NW rats indicating anxiety. Interestingly, DIO rats showed no behavioral alterations after the injection of nesfatin-1_{30-59} ($p > 0.05$). These results indicate an implication of nesfatin-1_{30-59} in the mediation of anxiety and depression-like behavior/anhedonia under normal weight conditions, while in DIO rats, a desensitization might occur.

Keywords: behavior; depression; gut-brain axis; NUCB2; obesity; psychosomatic

1. Introduction

Nesfatin-1 was identified in the rat hypothalamus and early on established as an anorexigenic peptide [1]. The 82-amino acid polypeptide is derived from its precursor protein nucleobindin-2 (NUCB2) by post-translational cleavage and consists of an N-terminal, middle, and C-terminal domain [2]. Expression of NUCB2/nesfatin-1 in the arcuate nucleus (Arc), the paraventricular nucleus

(PVN), and the nucleus of the solitary tract (NTS) further suggested a role in the regulation of food intake [3]. Intracerebroventricular (ICV) injection of the peptide resulted in a robust reduction of dark phase food intake [4], a finding subsequently replicated for the mid fragment of nesfatin-1, nesfatin-1_{30-59} [2,5] representing the active core of nesfatin-1.

Later studies also identified the expression of NUCB2/nesfatin-1 in the periphery such as in adipose tissue [6], endocrine pancreatic beta cells [7], testis [8] and a major source in the stomach, namely in endocrine X/A-like cells of the stomach where it is co-localized with ghrelin [9]. These findings were subsequently confirmed in humans [10]. Despite the fact that this finding suggested an implication of peripheral nesfatin-1 in the regulation of food intake, the anorexigenic effect was more readily observed after central injection while peripheral application had no effect on food intake [4] or required very high doses [2], although nesfatin-1 was shown to cross the blood-brain barrier in both directions [11,12]. Later on, studies identified other effects of peripheral nesfatin-1 such as a role in glucose homeostasis [13], an anti-inflammatory action [14], and an increase of blood pressure [15] pointing towards more pleiotropic effects of the peptide, an assumption recently supported by the widespread distribution of nesfatin-1 autoradiographic signals [16], a surrogate parameter for the expression of the nesfatin-1 receptor which is still to be identified [17]. These signals were detected in the gastric mucosa, duodenum, jejunum, ileum, pancreas, adrenal gland, testis, visceral adipose tissue, heart, skeletal muscle, lung, liver and kidney as well as in the pituitary, cortex, paraventricular nucleus of the hypothalamus, area postrema, dorsal motor nucleus of the vagus nerve and cerebellum [16].

In the brain, the release of food intake-regulatory peptides is often affected by aversive situations [18], indicating an involvement of these peptides in the stress response. This was shown e.g., for bombesin [19] or ghrelin [20] or nesfatin-1, for which several links with the stress-signaling system have been observed. First, nesfatin-1's food intake-inhibitory action is mediated by downstream corticotropin-releasing factor (CRF) receptor 2 signaling [4]. Second, ICV injection of nesfatin-1 activates CRF-positive neurons subsequently increasing circulating adrenocorticotropic hormone (ACTH) and corticosterone levels [21]. Lastly, several stressors activate brain NUCB2/nesfatin-1 signaling and also increase circulating NUCB2/nesfatin-1 levels [22,23]. These include psychological (restraint or water avoidance) [22,24], physical (abdominal surgery) [25], and immunological (injection of lipopolysaccharide) stressors [26].

A recent study described that circulating NUCB2/nesfatin-1 levels were significantly higher in patients with major depressive disorder compared to healthy controls [27]. Additionally, elevated plasma levels of NUCB2/nesfatin-1 have been reported in female obese inpatients with high anxiety scores resulting in a correlation between NUCB2/nesfatin-1 and self-reported anxiety [28]. However, these associations do not allow us to draw a causal conclusion. In rats, nesfatin-1 was shown to increase anxiety and fear-related behavior after an ICV injection [29], and also repeated intraperitoneal injection of nesfatin-1 increased anxiety and decreased exploratory behavior in rats [30]. However, it is unclear whether the short fragment, nesfatin-1_{30-59}, also affects anxiety and depression-like behavior in rats and whether these effects are retained under conditions of obesity often leading to a reduction/loss of function of several food intake-regulatory peptides [31].

Therefore, in the present study we investigated the effects of ICV injected nesfatin-1_{30-59}, proposed to be the active core of nesfatin-1, on anxiety behavior using the open field test, elevated zero maze, and light/dark box as well as on anhedonic/depression-like behavior using the sucrose preference and the novelty-induced hypophagia test in normal weight rats. To investigate whether these effects are retained under conditions of obesity, we also performed these tests in diet-induced obese (DIO) rats.

2. Materials and Methods

2.1. Animals

For the experiments, male Sprague Dawley rats (Envigo, Germany) weighing 200–250 g were used. Rats were group housed during the acclimatization period under controlled conditions with a 12-h dark/light cycle with lights on at 6 AM, humidity of 45–65% and at a temperature of 21–23 °C with *ad libitum* access to water and standard rodent diet (D12450B, Research Diets, Inc., Jules Lane, New Brunswick, NJ, USA). Body weight and food intake were measured daily, during this time rats were handled daily to become accustomed to the investigators. Animal care and experimental procedures followed institutional ethics guidelines and were approved by the state authority for animal research (Landesamt für Gesundheit und Soziales Berlin, LaGeSo Berlin).

2.2. Diets

The rats were divided into two groups: one group received a standard rodent diet (D12450B, Research Diets, Inc., Jules Lane, New Brunswick, NJ, USA, 3.9 kcal/g, 10% fat, 70% carbohydrates, 20% proteins) and the other group received a high-fat diet (D12451, Research Diets, Inc., Jules Lane, New Brunswick, NJ, USA, 4.7 kcal/g, 45% fat, 35% carbohydrates, 20% proteins). After 10 weeks rats developed DIO, a well-established animal model for obesity. Since only 50% of rats develop DIO, these rats were selected after 10 weeks as reported before [5]. The average body weight of the DIO rats at the beginning of the experiments was 426.9 ± 9.5 g, while the average body weight of the normal weight rats was 259.1 ± 3.1 g at the start of the experiments (p <0.001).

2.3. Intracerebroventricular Cannulation

Before surgery, the rat was anesthetized with ketamine (100 mg/kg; Ketanest™, Curamed, Karlsruhe, Germany) and xylazine (10 mg/kg; Rompun™, 2%, Bayer, Leverkusen, Germany) as described before [32]. Then, the animal was placed in a stereotaxic apparatus and a small incision in the scalp was made to expose the bregma. After localization of the bregma, the right location to implant a guide cannula (0.8 mm posterior, 1.5 mm right lateral, and 3.5 mm ventral from bregma) was determined using the rat brain atlas [33], and a hole was drilled into the scull. The guide cannula (22-gauge, Plastics One Inc., Roanoke, VA, USA) was inserted into the right lateral ventricle and fixed on the scull with four sterile stainless-steel screws (Plastics One Inc., Roanoke, VA, USA) and dental cement (Stoelting Co., Wood Dale, IL, USA). The guide cannula was closed with a dummy cannula. To reduce postoperative pain and to avoid infection, rats received buprenorphine (0.03 mg/kg subcutaneously for three days) and enrofloxacin (2.5% ad us. vet. 0.1 ml/L in drinking water, Bayer Vital GmbH, Leverkusen, Germany).
After surgery, rats were singly housed. They had five days to recover and were handled daily with light restraint to get used to the injection procedure. The correct position of the ICV cannula was verified after the last experiment when rats were sacrificed by pentobarbital overdose. A volume of 10 µL of 0.1% toluidine blue was injected into the lateral brain ventricle as described before [32]. Correct placement of the cannula was evaluated under the microscope and indicated by spreading of the dye throughout the brain ventricular system. No rats had to be retrospectively excluded from analyses.

2.4. Peptide and Intracerebroventricular Injection

Rat nesfatin-$1_{30\text{-}59}$ (Bachem AG, Weil am Rhein, Germany) was stored as a powder at –80 °C and aliquoted in sterile distilled water before the experiments. For ICV injections, nesfatin-$1_{30\text{-}59}$ was applied at three different doses, namely 0.1, 0.3 and 0.9 nmol diluted in 5 µL sterile double distilled H_2O to ensure the sterility of the injection. Doses were based on previous experiments investigating the effects of nesfatin-$1_{30\text{-}59}$ on food intake [5].

On the day of the experiment, lightly hand restrained rats were slowly (over 15 s followed by 60 s time to drain from the ventricle) injected ICV with vehicle (5 µL sterile H_2O) or nesfatin-1_{30-59} (0.1, 0.3 or 0.9 nmol in 5 µL sterile H_2O) using a 28-gauge cannula (Plastics One Inc., Roanoke, VA, USA) 1 mm longer than the guide cannula and connected to a 25-µL Hamilton syringe by a PE-50 catheter (Intramedic Polyethylene Tubing, Clay Adams, Parsippany, NJ, USA). Since the dose of 0.3 nmol/rat exerted the most pronounced effect in the sucrose preference test, this dose was used for all subsequent experiments.

2.5. Experimental Design and Procedures

To assess anxiety, exploratory behavior, depression-like behavior, and anhedonia, several well-established tests were performed in normal weight as well as DIO rats. Animals were ICV injected with vehicle or nesfatin-1_{30-59} at the beginning of the dark phase as the main effect of nesfatin-1 on food intake was observed during this photoperiod [4], and were tested 30 min later. All animals performed at least two tests and rats had at least three days to recover in between the tests. To reduce the number of animals, an in-between test crossover design was used. All experiments were conducted at the beginning of the dark phase.

2.5.1. Sucrose Preference Test

The sucrose preference test examines anhedonic behavior, a component of depression-like behavior [34] and was performed as follows: Three days before the experiment animals received a 1% sucrose solution for 48 h additionally to their regular drinking water. At 24 h before the experiment, the sucrose solution was removed. On the day of the experiment, normal weight rats were ICV injected with nesfatin-1_{30-59} (0.1, 0.3 or 0.9 nmol/rat) or vehicle and 30 min later rats had access to the 1% sucrose solution in addition to regular water and solid diet. Water and sucrose intakes were monitored by an automated food intake monitoring system (BioDAQ, Research Diets Inc., Jules Lane, New Brunswick, NJ, USA) as established before [35].
Briefly, food and water were placed on microbalances that weigh the food and water hoppers every second (± 0.01 g) and detect "not eating/drinking" as weight stable and "eating/drinking" as weight unstable. Feeding/drinking bouts (changes in stable weight before and after a bout) are recorded with a start time, duration and amount consumed. Bouts are separated by an inter-bout interval (IBI). Meals consist of one or more bouts separated by an inter-meal interval (IMI) defined as 15 min with a minimum meal size of 0.1 g as in our previous study [36]. Rats were habituated to the system for five days before the test started.
Water and sucrose intakes were monitored in an automated fashion for 1 h and the "sucrose to total fluid intake" ratio determined. A decreased sucrose intake is a surrogate of anhedonic behavior [37].

2.5.2. Novelty-Induced Hypophagia

The novelty-induced hypophagia test assesses anxious and depression-like behavior [38]. We used an adapted form as described before [29]. During a training period, rats received a palatable snack (HoneyMaid™ Graham Cracker Crumbs, Nabisco, East Hanover, NJ, USA) and water *ad libitum* at the beginning of the dark phase for 30 min for five consecutive days. Food intake was assessed in an automated fashion (BioDAQ, Research Diets Inc., Jules Lane, New Brunswick, NJ, USA) as described above to assess a stable baseline food intake.
On the day of the experiment, rats were ICV injected with nesfatin-1_{30-59} (0.3 nmol/rat) or vehicle, placed back in their home cages and, 30 min later, moved in a novel cage without bedding or enrichment inducing novelty stress. Here, animals had access to the familiar palatable snack and water *ad libitum* for 30 min and food intake microstructure was assessed for another 30 min.

2.5.3. Open Field Test

The open field test exposes the rat to a new and unfamiliar environment [39] and assesses explorative behavior as a surrogate for anxiety. The test was performed in a 50 × 50 cm white polyvinylchloride box with a black floor. This box can be divided into a center zone and an outer zone (Figure S1). A camera records duration and entries into the center zone as well as the locomotion of the animal including the total distance crossed. A long total distance and high locomotion along with a long duration in center zone and multiple entries into the center zone are interpreted as explorative behavior, whereas a reduction of these parameters reflects anxious behavior.
On the day of the experiment, rats were ICV injected with nesfatin-$1_{30\text{-}59}$ (0.3 nmol/rat) or vehicle and 30 min later placed in the center of the open field box. Behavior was recorded for 5 min and analyzed using a connected software (Biobserve GmbH, Bonn, Germany). After every test, the apparatus was cleaned with 5% ethanol.

2.5.4. Elevated Zero Maze

The elevated zero maze is a well-established tool to assess explorative behavior and anxiety [40] and is a further development of the elevated plus maze with the advantage of a higher sensitivity [41]. It consists of a zero-shaped elevated platform with the North and South wings being open arms, while the East and West wings are closed (Figure S1). A video camera records the total track length, the time and number of entries into open and closed arms and the average velocity. Animals are considered as being in the open/closed area when all four paws are in the respective arm. A high average track length and velocity represent high locomotor activity indicative of explorative behavior. Likewise, the time in open arms indicates explorative behavior, whereas the time in closed arms reflects anxious behavior. Besides track length, direct locomotor activity was not measured outside of this test, since previous studies indicated that nesfatin-1 ICV has no effect on locomotion [4,42].
On the day of the experiment, rats were ICV injected with nesfatin-$1_{30\text{-}59}$ (0.3 nmol/rat) or vehicle, 30 min later placed in the open arm of the elevated zero maze and their behavior was recorded for 5 min and analyzed using a connected software (Biobserve GmbH, Bonn, Germany). After every test, the apparatus was cleaned with 5% ethanol.

2.5.5. Light/Dark Box

The light/dark box is another test to assess the rats' explorative behavior and anxiety [43]. The apparatus consists of two compartments, one dark and one light, connected by a small hole (Figure S1). Anxious animals tend to spend more time in the dark compartment [44]. As in the experiments described above, a camera records the movements of the animal.
On the day of the experiment, rats were ICV injected with nesfatin-$1_{30\text{-}59}$ (0.3 nmol/rat) or vehicle, placed in the bright compartment 30 min later and their behavior, including latency to cross to the dark compartment, entries into, and duration in both compartments was recorded for 10 min and analyzed using a connected software (Biobserve GmbH, Bonn, Germany). After every test, the apparatus was cleaned with 5% ethanol.

2.6. Statistical Analysis

The Kolmogorov-Smirnov test was used to test the distribution of the data. Normally distributed data were assessed using the *t*-test, whereas for non-normally distributed data the Mann–Whitney-U test was applied. For the comparison of multiple groups, a one-way ANOVA followed by Tukey *post hoc* test was applied. For better comparability, all data were displayed as mean ± sem. Differences between groups were considered significant when $p < 0.05$ (SigmaStat 3.1., Systat Software, San Jose, CA, USA).

3. Results

3.1. Nefatin-1$_{30-59}$ Injected Intracerebroventricularly Induced Anhedonic and Depression-Like Behavior in Normal Weight but Not DIO Rats

Nesfatin-1$_{30-59}$ injected in normal weight rats ICV at a dose of 0.3 nmol significantly reduced the sucrose/water intake ratio to 0.66 ± 0.13 (n = 11) compared to vehicle (1.00 ± 0.00, n = 11) and nesfatin-1$_{30-59}$ at a dose of 0.1 nmol (1.00 ± 0.00, p = 0.03; n = 10), while compared to nesfatin-1$_{30-59}$ at a dose of 0.9 nmol (0.88 ± 0.09, n = 10) did not reach significance (p = 0.30; Figure1) reflective of an anhedonic effect. Based on this experiment the dose of 0.3 nmol nesfatin-1$_{30-59}$ was used for all subsequent experiments. In contrast, when nesfatin-1$_{30-59}$ was injected at 0.3 nmol in DIO rats, no difference in sucrose intake was observed as reflected by a similar sucrose/water intake ratio in nesfatin-1$_{30-59}$ and vehicle treated rats (p = 0.69; n = 8/group, Figure1).

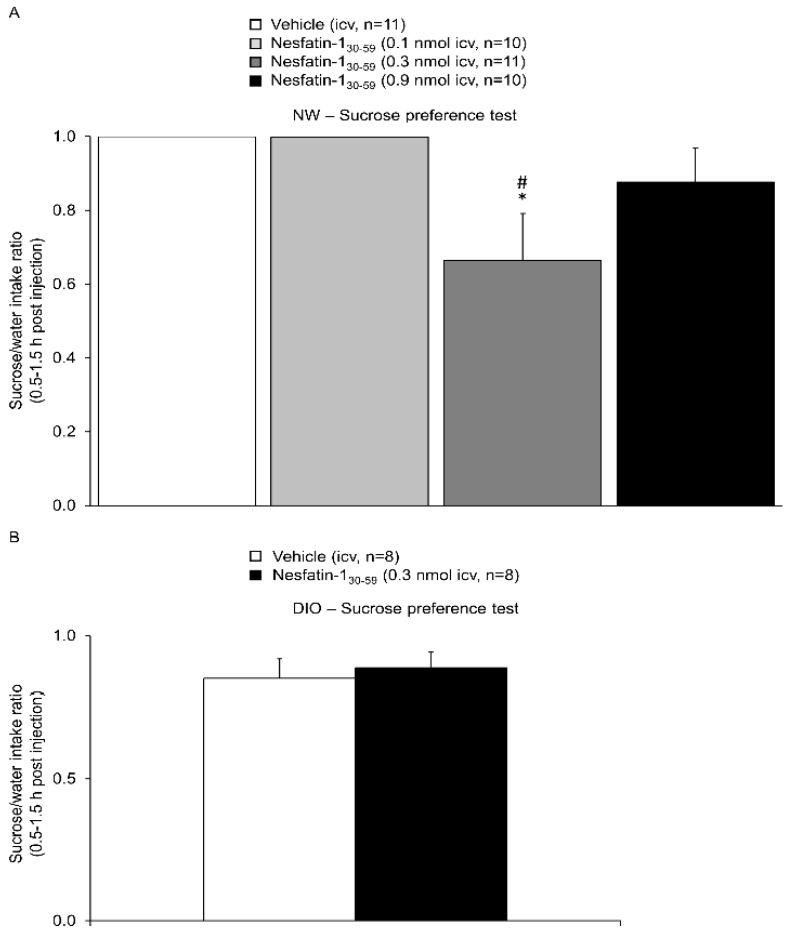

Figure 1. Nesfatin-1$_{30-59}$ decreased sucrose preference in normal weight but not in diet-induced obese rats. (**A**) Intracerebroventricularly cannulated normal weight rats were injected with vehicle (5 µL H_2O, n = 11) or nesfatin-1$_{30-59}$ (0.1 nmol/rat, n = 10, 0.3 nmol/rat, n = 11, or 0.9 nmol/rat, n = 10, in 5 µL H_2O) at the beginning of the dark phase and water as well as sucrose intake assessed between 0.5 and 1.5 h post injection. Nesfatin-1$_{30-59}$ reduced the sucrose/water intake ratio at a dose of 0.3 nmol/rat; this dose was used for all further analyses. (**B**) Diet-induced obese rats injected with nesfatin-1$_{30-59}$ (0.3 nmol/rat in 5 µL H_2O, n = 8) did not show significant differences in sucrose preference compared to diet-induced obese rats injected with vehicle (5 µL H_2O, n = 8). Data were not normally distributed, for better comparability all data are expressed as mean ± SEM. Data were analyzed using one way ANOVA (**A**) or Mann–Whitney-U test (**B**). Abbreviations: DIO, diet-induced obesity; NW, normal weight. * p < 0.05 vs. vehicle; # p < 0.05 vs. Nesfatin-1$_{30-59}$ 0.1 nmol.

In the novelty-induced hypophagia test normal weight rats treated with nesfatin-1_{30-59} (0.3 nmol/rat) showed a significantly reduced intake of the palatable food compared to vehicle (1.09 ± 0.32 vs. 4.36 ± 1.31 kcal, $p = 0.04$), while the latency to approach the food was not significantly altered (207.0 ± 58.4 vs. 241.3 ± 48.8 s, $p = 0.68$; $n = 8$/group, Figure2). Overall food intake (24-h) of the standard rodent diet was not significantly different between the nesfatin-1_{30-59} and vehicle group before (58.3 ± 5.7 vs. 65.6 ± 3.7 kcal, $p = 0.73$) and after (60.5 ± 1.8 vs. 55.5 ± 3.1 kcal, $p = 0.07$) the novelty-induced hypophagia test. In DIO rats, none of these parameters was significantly altered during the novelty-induced hypophagia test by 0.3 nmol nesfatin-1_{30-59} compared to the vehicle ($p = 0.61$ and $p = 0.64$; $n = 5$/group, Figure2). Similarly, the 24-h food intake of the high-fat diet was not different between the nesfatin-1_{30-59} and vehicle group before (50.8 ± 2.3 vs. 59.6 ± 4.0 kcal, $p = 0.13$) and after (67.7 ± 8.5 vs. 45.8 ± 9.5 kcal, $p = 0.29$) the novelty-induced hypophagia test.

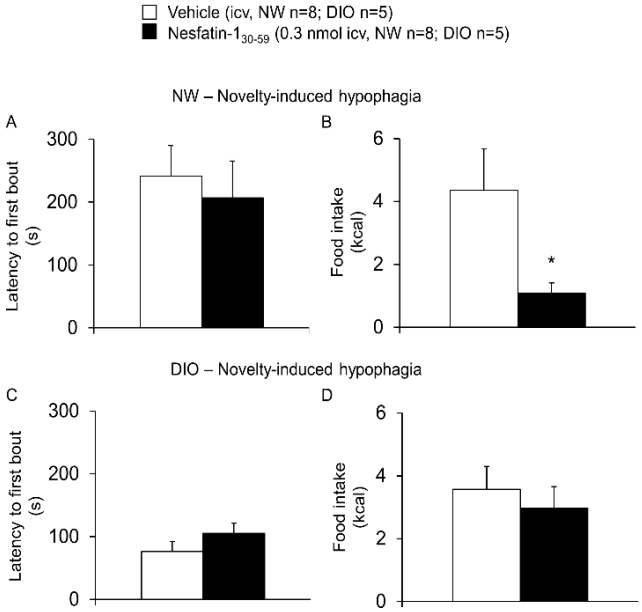

Figure 2. Nesfatin-1_{30-59} increased novelty-induced hypophagia in normal weight but not in diet-induced obese rats. Intracerebroventricularly cannulated normal weight or diet-induced obese rats were injected with vehicle (5 µL H_2O, $n = 8$ and $n = 5$, respectively) or nesfatin-1_{30-59} (0.3 nmol/rat in 5 µL H_2O, $n = 8$ and $n = 5$, respectively) at the beginning of the dark phase and 30 min later placed into a novel cage without bedding or enrichment. The latency to approach the food and the intake of a palatable snack was assessed for another 30 min. In normal weight rats, the latency to approach the food did not differ between the nesfatin-1_{30-59} and vehicle animals (**A**), whereas the total food intake (g) was significantly decreased in nesfatin-1_{30-59} injected rats (**B**). In diet-induced obese rats, neither the latency to approach the food (**C**) nor the food intake (**D**) was significantly affected by the nesfatin-1_{30-59} injection (0.3 nmol/rat in 5 µL H_2O) compared to vehicle (5 µL H_2O). Data were normally distributed and are expressed as mean ± sem. Data have been analyzed by *t*-test. Abbreviations: DIO, diet-induced obesity; NW, normal weight. * $p < 0.05$ vs. vehicle.

3.2. Nesfatin-1_{30-59} Injected Intracerebroventricularly Induced Anxious Behavior in Normal Weight but Not DIO Rats

Nesfatin-1_{30-59} injected in normal weight rats ICV at a dose of 0.3 nmol ($n = 12$) significantly reduced the entries into the center zone (20.6 ± 2.0 vs. 37.8 ± 6.1, $p = 0.008$) and reduced the duration spent in this zone (11.5 ± 0.7 vs. 16.3 ± 2.6 s, $p = 0.04$), whereas the average velocity in the open field test was not altered compared to vehicle (7.4 ± 0.4 vs. 7.4 ± 0.5 cm/s, $p = 0.47$; $n = 11$, Figure3). The overall distance crossed was not different between the nesfatin-1_{30-59} and vehicle groups (22.3 ± 1.2 vs. 22.6 ±0.9 m, $p = 0.82$). None of these parameters was significantly altered by 0.3 nmol

nesfatin-1_{30-59} in DIO rats when compared to vehicle (p = 0.47, p = 0.63 and p = 0.50; n = 5/group, Figure3, overall distance crossed: 16.9 ± 1.6 vs. 18.5 ± 0.7 m, p = 0.41).

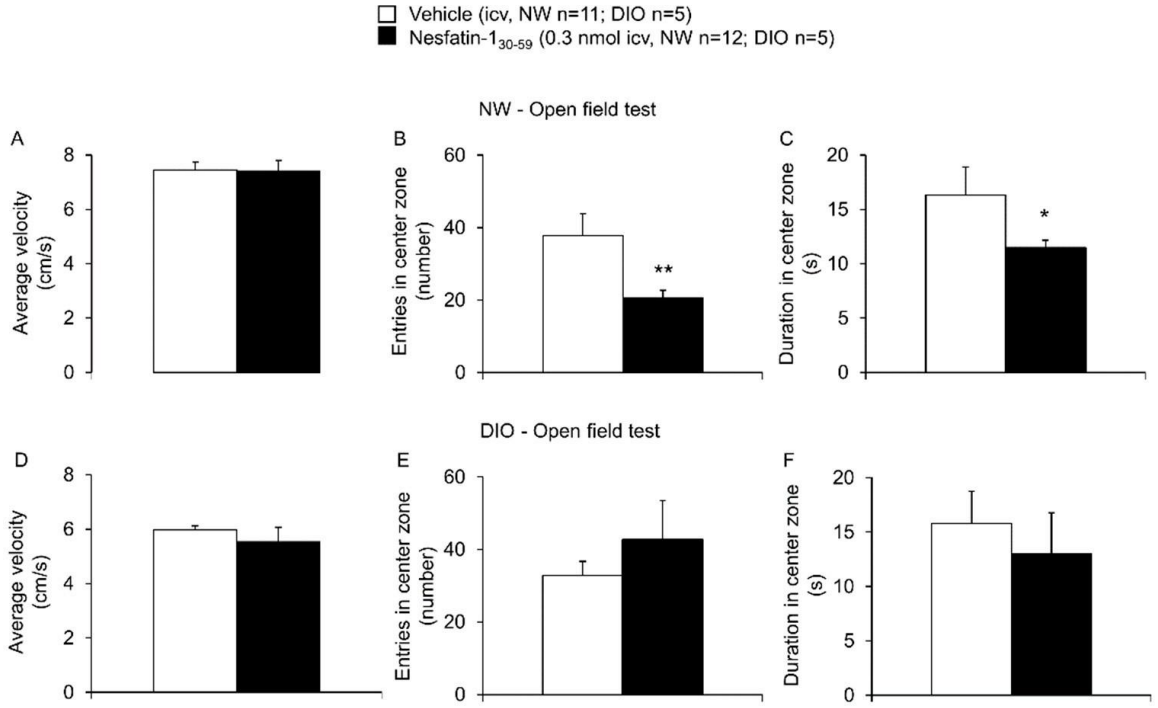

Figure 3. Nesfatin-1_{30-59} induced anxiety in the open field test in normal weight but not in diet-induced obese rats. Intracerebroventricularly cannulated normal weight or diet-induced obese rats were injected with vehicle (5 µL H_2O, n = 11 and n = 5, respectively) or nesfatin-1_{30-59} (0.3 nmol/rat in 5 µL H_2O, n = 12 and n = 5, respectively) at the beginning of the dark phase and 30 min later placed in a box with a center and an outer zone. Behavior including velocity ((**A**): normal weight; (**D**): diet-induced obesity), entries in the center zone ((**B**): normal weight; (**E**): diet-induced obesity) and duration in the center zone ((**C**): normal weight; (**F**): diet-induced obesity) was assessed for 5 min using a computer-supported technique. While the number of entries in center zone and the duration in the center zone were significantly decreased in normal weight nesfatin-1_{30-59} injected rats compared to vehicle, no significant changes were observed in diet-induced obese rats. Data were distributed normally except for the parameter entries in center zone in the normal weight group treated with nesfatin-1_{30-59}. For better comparability, all data are expressed as mean ± SEM. Data were analyzed using the Mann–Whitney-U test (entries in center zone in the normal weight group) or t-test (all other data). Abbreviations: DIO, diet-induced obesity; NW, normal weight. * $p < 0.05$ and ** $p < 0.01$ vs. vehicle.

In the elevated zero maze, nesfatin-1_{30-59} injected ICV at 0.3 nmol/rat in normal weight rats (n = 9) reduced the visits of the open arms (19.8 ± 1.8 vs. 32.5 ± 2.8, p = 0.002) and tended to decrease the time in open arms (92.7 ± 11.9 vs. 128.9 ± 15.6 s, p = 0.05), whereas the overall track length crossed was not different compared to vehicle (25.6± 1.4 vs. 27.9 ± 1.1 m, p = 0.14; n = 8, Figure4). Again, none of these parameters was altered by 0.3 nmol nesfatin-1_{30-59} in DIO rats when compared to vehicle (p = 0.94, p = 0.93 and p = 0.93; n = 5/group, Figure4).

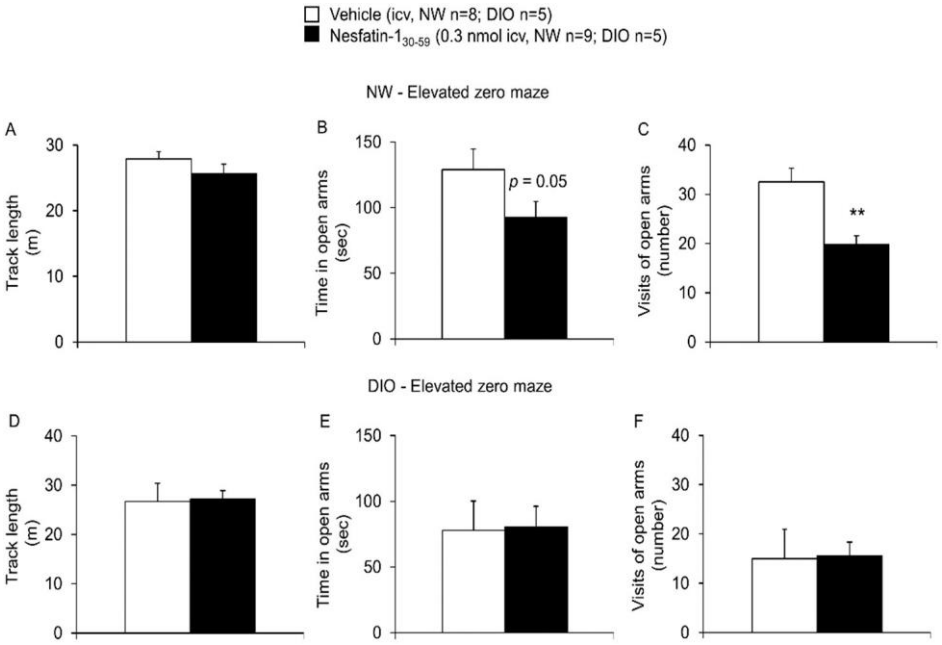

Figure 4. Nesfatin-1$_{30-59}$ induced anxiety in the elevated zero maze in normal weight but not in diet-induced obese rats. Intracerebroventricularly cannulated normal weight or diet-induced obese rats were injected with vehicle (5 µL H_2O, *n* = 8 and *n* = 5, respectively) or nesfatin-1$_{30-59}$ (0.3 nmol/rat in 5 µL H_2O, *n* = 9 and *n* = 5, respectively) at the beginning of the dark phase and 30 min later placed on a zero-shaped, elevated platform with two closed and two open zones. Behavior including visits of open arms ((**A**): normal weight; (**D**): diet-induced obesity), time in open arms ((**B**): normal weight; (**E**): diet-induced obesity) and track length ((**C**): normal weight; (**F**): diet-induced obesity) were assessed for 5 min using a computer-supported technique. While a tendency in the time in open arms ($p = 0.05$) and a significant reduction of the number of visits in open arms was observed in nesfatin-1$_{30-59}$ injected rats compared to vehicle, none of the parameters were affected in diet-induced obese rats. All data were distributed normally except for the time in open arms and visits of open arms in the DIO/vehicle group. For better comparability, all data are expressed as mean ± SEM. Data have been analyzed using the Mann–Whitney-U test (time in open arms and visits of open arms in DIO) or *t*-test (all other data). Abbreviations: DIO, diet-induced obesity; NW, normal weight. ** $p < 0.01$ vs. vehicle.

Lastly, nesfatin-1$_{30-59}$ injected in normal weight rats ICV at a dose of 0.3 nmol (*n* = 7) did not significantly alter the time in dark (381.9 ± 12.2 vs. 413.0 ± 15.9 s, $p = 0.10$), the latency to cross to the dark compartment (21.5 ± 3.2 vs. 32.6 ± 5.6 s, $p = 0.08$), the number of visits of the bright side (12.0 ± 0.9 vs. 11.5 ± 0.6, $p = 0.33$), and the time in the bright side (218.1 ± 12.2 vs. 187.0 ± 15.9 s, $p = 0.10$) during the light/dark box compared to vehicle treated rats (*n* = 8, Figure5). Similarly, none of these parameters was significantly altered in DIO rats by nesfatin-1$_{30-59}$ (0.3 nmol/rat, ICV) compared to vehicle ($p = 0.97$, $p = 0.57$, $p = 0.63$ and $p = 0.97$; *n* = 5/group, Figure5).

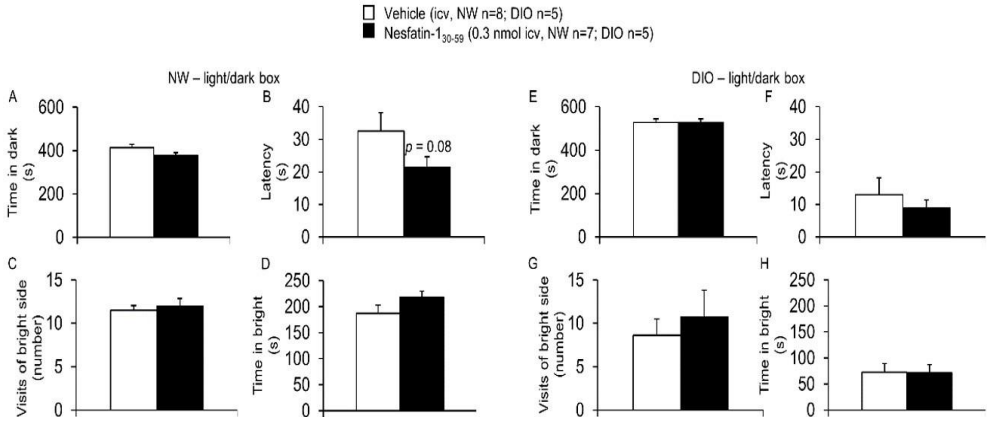

Figure 5. Nesfatin-1$_{30-59}$ neither affected the explorative behavior in the light/dark box in normal weight nor in diet-induced obese rats. Intracerebroventricularly cannulated normal weight or diet-induced obese rats were injected with vehicle (5 µL H_2O, *n* = 8 and *n* = 5, respectively) or nesfatin-1$_{30-59}$ (0.3 nmol/rat in 5 µL H_2O, *n* = 7 and *n* = 5, respectively) at the beginning of the dark phase and 30 min later placed in the bright compartment of the light/dark box. Behavior including time in dark ((**A**): normal weight; (**E**): diet-induced obesity), latency to the first entry into the black compartment ((**B**): normal weight; (**F**): diet-induced obesity), the number of visits of the bright side ((**C**): normal weight; (**G**): diet-induced obesity) and the time in bright side ((**D**): normal weight; (**H**): diet-induced obesity) was assessed for 10 min using a computer-supported technique. None of the parameters were affected by intracerebroventricularly injected nesfatin-1$_{30-59}$ in any group. All data in the normal weight group were distributed normally. In the DIO group, the parameter latency was not distributed normally in the vehicle group. For better comparability, all data are expressed as mean ± SEM. Data have been analyzed using the Mann–Whitney-U test (latency in DIO) or *t*-test (all other data). Abbreviations: DIO, diet-induced obesity; NW, normal weight. $p > 0.05$.

4. Discussion

The current findings indicate that nesfatin-1$_{30-59}$ increases anxiety, depression-like behavior, and anhedonia in normal weight rats. However, these anxiogenic/anhedonic effects could not be observed in DIO animals.

The present study further corroborates the assumption of nesfatin-1$_{30-59}$ being the active core of full-length nesfatin-1 as suggested before for the food intake-regulatory effect [2]. However, it has to be noted that higher doses are necessary for nesfatin-1$_{30-59}$ (1.1 µg) compared to full-length nesfatin-1 (0.24 µg) to exert an anxiogenic action as observed before for the anorexigenic effect [5]. This might be due to differential receptor binding and/or activation, a hypothesis to be further investigated after the identification of the receptor. Moreover, the dose-dependent effect of nesfatin-1$_{30-59}$ on sucrose preference displayed a U-shaped relation with 0.3 nmol/rat being the most effective dose. Whether antagonistic effects or supraphysiological stimulation of the receptor contributes to this U-shaped curve will have to be further investigated.

In the elevated zero maze, in normal weight rats injected with nesfatin-1$_{30-59}$, the number of entries into the open arms was significantly reduced compared to controls, an observation giving rise to anxious behavior. This finding is consistent with a previous study showing that full-length nesfatin-1 reduces the number of entries into the open arms and the percent time spent in open arms of an elevated plus maze [29], thereby extending the finding to the elevated zero maze which has been suggested to be beneficial to assess anxiety as rats [40]. The anxiogenic effect of the peptide was further supported by the behavioral patterns observed in the open field test with lesser entries into the center zone and less time spent in this zone. These observations extend the findings of a previous study where high doses of nesfatin-1 (2, 4, and 8 µg/day) injected daily intraperitoneally over a period of three weeks led to a decreased moving distance, duration in center zone and frequency in rearing and grooming in the open field test [30]. Interestingly, nesfatin-1$_{30-59}$, injected at the beginning of the dark

phase, did not significantly alter behavior in the light/dark box, another well-established test to assess anxious behavior in rats [43]. This discrepancy might be associated with the notion that nesfatin-1 acts in a photosensitive manner with a robust action in the dark photoperiod, whereas no effect was observed during the light phase [4,42]. Moreover, also the initial study on nesfatin-1, although the experiments were conducted between 9 A.M. and 12 P.M., tested the food intake-modulating effects of the peptide under low illumination of 30–40 lx [1]. Lastly, since rodents as nocturnal animals are more active during the dark [45,46], behavioral tests should be performed during this photoperiod under conditions of low illumination. While the light/dark box intrinsically has a brightly lit zone, the other two tests (elevated zero maze and open field test) were performed under dimmed light conditions.

Moreover, nesfatin-$1_{30\text{-}59}$ also induced anhedonic/depression-like behavior as indicated by reduced food intake of a palatable snack under novelty conditions, a.k.a. hyponeophagia, a finding in line with previous data on full-length nesfatin-1 [29]. The novelty-induced hypophagia test is a sensitive tool to evaluate anxiety and depression-like behavior that has been used to describe the anxiogenic or anxiolytic effects of drugs, such as antidepressants [47]. Likewise, nesfatin-$1_{30\text{-}59}$ reduced the amount of sucrose consumed as reflected in a decreased sucrose/water intake ratio, a finding indicative of increased anhedonia characteristics for depressive disorder [48]. Since circulating NUCB2/nesfatin-1 levels have been reported to be elevated in patients with depression [49] or to be correlated with reported levels of depressive behavior [50] along with the finding that also high doses of nesfatin-1 injected intraperitoneally induce anhedonia in rats [51], peripheral nesfatin-1 might well be involved in the development and/or maintenance of depressive symptoms such as reduced appetite [27]. It is unlikely that the effects observed in these tests occur subsequently to the anorexigenic effect of nesfatin-$1_{30\text{-}59}$. First, the number of snacks consumed in the home cage—unlike in the novel cage—was reported before to be not decreased after the injection of full-length nesfatin-1 [29]. Second, the anorexigenic effect was shown to have a delayed onset at 2 or 4 h after ICV injection of full length or mid fragment nesfatin-1, respectively [4,5], while the anhedonic effect was observed within the first-hour post injection. Lastly, the lack of difference in latency to reach for the familiar palatable food observed in the present study may indicate a similar motivation to approach a familiar object although placed in a new environment. Therefore, this effect is considered a specific anhedonic action of the peptide as a part of depression-like behavior, while an anxiogenic component might also contribute to the effect.

Interestingly, in contrast to the observed effects in normal weight rats, nesfatin-$1_{30\text{-}59}$ injected ICV did not induce behavioral alterations in DIO rats in neither of the anxiety—(elevated zero maze, open field, light/dark box) or anhedonia/depression-like—(novelty-induced hypophagia, sucrose preference test) assessing tools. Nesfatin-1 is produced not only centrally but also in peripheral tissues such as adipose tissue [6] with an upregulation under conditions of obesity [6,52]. In DIO rats, the amount of adipose tissue is greatly increased, likely leading to elevated levels of NUCB2/nesfatin-1, a hypothesis corroborated by the correlation of NUCB2/nesfatin-1 with the body mass index in humans [6,53]. Since the gastric expression of NUCB2/nesfatin-1 is elevated also with increasing body mass index, this source is also likely to contribute to the high circulating levels of the peptide. Since nesfatin-1 can cross the blood-brain-barrier [11,12], it can be hypothesized that these higher NUCB2/nesfatin-1 levels can also exert central anxiogenic effects. Noteworthy chronic peripheral injections of nesfatin-1 were also shown to modulate anxiety in rats. However, the hypothesis of central effects of peripherally secreted nesfatin-1 as well as a central desensitization of nesfatin-1 signaling under conditions of obesity should be further investigated.

Centrally, the higher nesfatin-1 levels could stimulate the PVN known to secrete CRF since nesfatin-1 was shown to activate CRF positive neurons in the PVN as assessed using phospho (p)-ERK1/2 [54] likely leading to an upregulated expression of CRF mRNA [55] and CRF protein [56] which subsequently results in elevated circulating levels of adrenocorticotropic hormone (ACTH) and corticosterone [21] and may result in an increased stress response. Whether DIO rats display

chronically elevated NUCB2/nesfatin-1 levels associated with increased corticosterone concentrations warrants further investigation. This chronic stimulation might lead to desensitization towards stressful and anxious situations in line with a study reporting that chronically increased glucocorticoid signaling in the hypothalamus does not induce a hyperactivity of the hypothalamus-pituitary-adrenal axis [57]. Whether a nesfatin-1 desensitization plays a role in the lack of effect of nesfatin-1_{30-59} on anxiety and anhedonia under conditions of DIO will have to be further investigated. Interestingly, the anorexigenic effect of nesfatin-1 [1] and nesfatin-1_{30-59} [2] is exerted in a leptin-independent manner possibly giving rise to different routes of downstream signaling mediating the anorexigenic or anxiogenic effects. Whether different brain areas are recruited by nesfatin-1_{30-59} under conditions of DIO could be investigated by Fos immunohistochemistry in future studies.

In summary, nesfatin-1_{30-59} injected ICV exerts an anxiogenic and anhedonic/depression-like effect under normal weight but not DIO conditions. Further investigations are needed to explore whether a desensitization of the receptor (for the dose tested in normal weight animals) in DIO rats contributes to the missing response or whether a complete resistance exists which should be tested using higher doses. Lastly, an alteration of downstream mediators might play a role in this lack of effect in obese rats which will have to be further investigated.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/10/12/1889/ s1, Figure S1: Open field test, elevated zero maze and light/dark box.

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Central blockage of nesfatin-1 has anxiolytic effects but does not prevent corticotropin-releasing factor-induced anxiety in male rats

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abstract

Nesfatin-1, a pleotropic peptide, was recently implicated in the regulation of anxiety and depression-like behavior in rats. However, the underlying mechanisms remain unclear so far. Thus, this study aimed to investigate the role of endogenous nesfatin-1 in the mediation of anxiety and depression-like behavior induced by corticotropin-releasing factor (CRF). Therefore, normal weight male intracerebroventricularly (icv) cannulated Sprague Dawley rats received two consecutive icv injections of anti-nesfatin-1 antibody or IgG control antibody followed by CRF or saline, before being exposed to a behavioral test. In the elevated zero maze test, assessing anxiety and explorative behavior, blockade of nesfatin-1 using an anti-nesfatin-1 antibody under basal conditions increased the number of entries into the open arms compared to control antibody/vehicle (1.6-fold, $p < 0.05$) and the time in open arms compared to the other groups ($p < 0.05$). Control antibody/CRF-treated animals tended to spend less time in the open arms compared to control antibody/vehicle (0.7-fold, p ¼ 0.17), an effect not altered by the nesfatin-1 antibody (control antibody/CRF-treated animals *vs.* nesfatin-1 antibody/CRF group, p 1.00). In the novelty-induced hypophagia test, assessing anhedonia as part of depression-like behavior, no significant differences were observed between the four groups for the latency to the first bout, number of bouts and the amount of palatable snack eaten ($p > 0.05$). In summary, CRF tended to increase anxiety and explorative behavior an effect not altered by blockade of nesfatin-1, whereas no significant effect of CRF on anhedonia was observed. Blockade of endogenous nesfatin-1 significantly decreased anxiety-like behavior giving rise to a physiological role of brain nesfatin-1 in the mediation of anxiety.

1. Introduction

Nesfatin-1 was discovered in the rat hypothalamus in 2006 [1]. In the brain, nesfatin-1 was e among other nuclei e predominantly expressed in food intake-regulatory nuclei such as the arcuate nucleus (Arc), the paraventricular nucleus (PVN) and the nucleus of the solitary tract (NTS) [2]. In the periphery, higher levels of nucleobindin2 (NUCB2)/nesfatin-1 expression were detected in adipose tissue [3], endocrine pancreatic beta cells [4] and testis [5]. However, its major source are endocrine X/A-like cells of the stomach where NUCB2/nesfatin-1 is co-localized with ghrelin in rats [6] and humans [7]. The polypeptide consists of 82 amino acids, whose central part, nesfatin-1_{30-59}, has been identified as active core able to reduce food intake after intracerebroventricular (icv) injection in mice [8,9] and rats [10]. While the food intake-reducing effect was not consistently observed following peripheral application of nesfatin-1 in rodents [9,11] peripheral nesfatin-1 is likely involved in glucose control [12], inflammation [13], cardiovascular regulation [14] and gastrointestinal motility [15]. An autoradiographic study (the nesgfatin-1 receptor is still unknown) showed

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widespread binding of nesfatin-1 in the periphery throughout the gastrointestinal tract and different endocrine organs [16]; therefore, additional effects of peripheral nesfatin-1 might be suspected. We recently showed an anxiogenic and anhedonic effect of nesfatin-1_{30-59}, injected icv in normal weight but not diet-induced obese rats [17] corroborating former findings after acute central and chronic peripheral administration of nesfatin-1 in rats [18,19]. In humans, a positive association between circulating nesfatin-1 concentrations and perceived anxiety has been described in obese [20] and anorectic women [21], whereas in obese men a negative correlation was observed [22] giving rise to a sex- dependent regulation. Moreover, elevated NUCB2/nesfatin-1 plasma levels were found in patients with major depression [23]. These findings give rise to a role of nesfatin-1 in the mediation of anxiety and depression in animals and humans. However, it re- mains unclear whether endogenous nesfatin-1 is mediating these
effects under basal conditions, and if so by which pathway(s).

Noteworthy, brain NUCB2/nesfatin-1's expression is affected by different stressors including restraint stress [24], abdominal surgery [25] and inflammation [26]. This increase results in higher circulating plasma levels as shown after injection of lipopolysaccharide [27]. Moreover, central nesfatin-1 injection activated corticotropin-releasing hormone (CRF)-positive neurons resulting in elevated circulating adrenocorticotropic hormone (ACTH) and corticosterone levels [28]. Lastly, nesfatin-1's anorexigenic effect was shown to be mediated by downstream CRF_2 receptor signaling [6] pointing towards an intimate interaction of nesfatin-1 and CRF in the response to stress and in the reduction of food intake. The question arises whether nesfatin-1 is also involved in the CRF-mediated behavioral changes such as anxiety [29] and depression-like behavior [30] observed after central injection of CRF.

Thus, in the present study we aimed to investigate whether blockage of central nesfatin-1 signaling using an anti-nesfatin-1 antibody affects anxiety or anhedonia (as hallmark feature of depression-like behavior) using two well-established tests, the elevated zero maze [31,32] and the novelty-induced hypophagia [17,19,33] tests. Moreover, the present study investigated whether CRF-induced behavioral changes are mediated by downstream nesfatin-1 signaling.

2. Material and methods

Animals. Male Sprague Dawley rats (Envigo, Germany) weighing between 200 and 250 g housed in groups of four per cage for a week of acclimatization were subsequently separated into individual cages under controlled conditions at a temperature of 21**e**23 °C, humidity of 45**e**65% and a 12-h dark/light cycle with lights on at 6 am. Except during behavioral experiments, animals had *ad libitum* access to standard rodent diet (D12450B, 3.9 kcal/g, 10% fat, 70% carbohydrates, 20% proteins, Research Diets, Inc., Jules Lane, New Brunswick, NJ, USA) and water. All animals were weighed and handled daily to adapt to the investigators and the experimental handling; additionally, food and water intake were documented daily. The experiments were approved by the state authority for animal research (Landesamt für Gesundheit und Soziales Berlin, Berlin). Institutional ethics guidelines for animal care and experimental procedures were followed accordingly.

Intracerebrovertricular (icv) cannulation. For icv cannulation, rats were anesthetized with a combination of xylazine (10 mg/kg; Rompun™, 2%, Bayer, Leverkusen, Germany) and ketamine (ketamine (100 mg/kg; Ketanest™, Curamed, Karlsruhe, Germany) as described before [34]. After fixation in a stereotactic apparatus, the rat's scalp was incised to uncover the bregma, which served as orientation point for the location of the guide cannula (22-gauge, Plastics One Inc., Roanoke, VA, USA) in the right lateral ventricle. The coordinates 0.8 mm posterior, 1.5 mm right lateral and 3.5 mm ventral from bregma were determined using the rat brain atlas [35]. Four holes were drilled, three for sterile stainless-steel screws (Plastics One Inc.) and one for the guide cannula. The guide cannula was fixed with dental cement (Stoelting Co., Wood Dale, IL, USA). A dummy cannula was inserted into the guide cannula for flexible closing and opening. After surgery, the animals had five days to recover, in which they received buprenorphine (0.03 mg/kg subcutaneously for three days, Essex Pharma GmbH, Munich, Germany) to prevent post-operative pain and enrofloxacin (2.5% ad us. vet. 0.1 ml/l in drinking water, Bayer Vital GmbH, Leverkusen, Germany) as a prophylaxis for infection. After the last experiment, animals were euthanized using a ketamine/xylazine overdose, followed by an icv injection of 10 ml of 0.1% toluidine blue (to verify the correct position of the canula, indicated by dye in the ventricle system) and decapitation. None of the animals had to be excluded retrospectively due to incorrect placement of the cannula.

Intracerebroventricular injections. Nesfatin-1 antibody (kindly provided by Masatomo Mori, Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, Maebashi, Japan) was stored as lyophilized powder at 80 °C. Before the experiment it was dissolved in sterile water. The control antibody, anti-rabbit- IgG antibody (rabbit IgG; Sigma-Aldrich, Darmstadt, Germany) was aliquoted in sterile water and stored at −80 °C. Corticotropin-releasing factor (CRF, catalog no. 019-06, Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) was dissolved in 0.9% sodium chloride and stored at 80 °C.

For the icv experiments, animals received an icv injection of control antibody (8 mg, 5 ml) or anti-nesfatin-1 antibody (8 mg/5 ml) followed by an injection of vehicle (5 ml of 0.9 sterile sodium chloride) or CRF (0.6 mg/5ml) using a 28-gauge cannula (Plastics One Inc.) connected to a 25-ml Hamilton syringe by a PE-50 catheter (Intramedic Polyethylene Tubing, Clay Adams, NJ). After each injection the gauge-cannula was left inserted for 60 s for the injection to drain from the syringe into the ventricle. The doses of the anti-nesfatin-1 antibody and CRF were based on previous publications [1,36,37].

Behavioral experiments. The *elevated zero maze* was used to assess anxious and explorative behavior. It is a further advancement of the elevated plus maze; without the central area the elevated zero maze allows for direct measurement of the time in open space [31]. As described before [17] the tool is a zero-shaped, elevated platform with two open and two closed quarters in which the rat was placed. A camera connected to an analysis software (Biobserve GmbH, Bonn, Germany) captured the total track length as well as time and number of entries into open and closed arms. On the experimental day, rats were manually placed on one open arm (facing one closed arm) 30 min after the double icv injection and behavior was assessed over a period of 5 min. After every usage the apparatus was cleaned with 5% ethanol.

The *novelty-induced hypophagia* test, used to assess a hallmark feature of depression-like behavior [33], was conducted with an automated intake monitoring system (BioDAQ, Research Diets Inc., Jules Lane, New Brunswick, NJ, USA). As described before [17], the system weighs food and fluid placed on the hoppers every second using a microbalance (±0.01 g). Stable weights are interpreted as 'not eating' and weight changes are registered as 'eating'. The system is thus able to determine the microstructure of food intake. Meals comprise one or more bouts separated by an inter-meal interval (15 min with a minimum meal size of 0.1 g) [38]. Bouts, defined as changes in stable weight before and afterwards, were registered with a start time, duration and amount consumed and are separated by an inter-bout interval (IBI). Before testing, rats had five days to accustom to the system which consisted of feeding and

drinking from modules mounted to standard laboratory cages. For the novelty-induced hypophagia test a five-day long training period was performed, where rats received a palatable snack (HoneyMaid™ Graham Cracker Crumbs, Nabisco, East Hanover, NJ, USA) for 30 min at every beginning of the dark phase in addition to water *ad libitum*, aiming to achieve a stable baseline palatable snack intake before the experiment. At the sixth day, rats received two consecutive icv injections and were placed back into their home cage. Thirty minutes later, animals were placed into novel cages without bedding or enrichment (representing novelty) with access to the palatable snack and water *ad libitum* for 30 min, so that the intake during novelty stress (novel environment) was determined using the automated system attached to the cage.

Statistical analysis was performed using SigmaStat 3.1. (Systat Software, San Jose, CA, USA). The Kolmogorov-Smirnov test was used to test the normality of the distribution of the data. Differ- ences between groups were assessed using one-way ANOVA if normally distributed or Kruskal-Wallis analysis if not. The signifi- cance level a was set to 0.05. Data are expressed as mean and standard error of the mean (SEM).

3. Results

Nesfatin-1 antibody significantly decreased anxiety-like behavior in rats under basal conditions but had no effect on CRF-induced anxiety. Icv injection of an anti-nesfatin-1 antibody significantly increased the number of entries into the open arms of the elevated zero maze under basal conditions compared to control antibody/ vehicle (1.6-fold, $p < 0.05$), control antibody/CRF (2.4-fold, $p < 0.001$) and nesfatin-1 antibody/CRF group (2.2-fold, $p < 0.001$; Fig. 1A). Moreover, the nesfatin-1 antibody/control group spent more time in the open arms compared to the control antibody/CRF (1.9-fold, $p < 0.05$) and the nesfatin-1 antibody/CRF group (1.9-fold, $p < 0.05$; Fig. 1B). Track length in the open arms was increased accordingly in the nesfatin-1 antibody/control group compared to the control antibody/CRF (3.0-fold, $p < 0.001$) and the nesfatin-1 antibody/CRF group (2.4-fold, $p < 0.01$; Fig. 1C). Lastly, overall track length (in open and closed arms) was increased in the nesfatin-1 antibody/control group compared to control antibody/ vehicle (1.4-fold, $p < 0.05$), control antibody/CRF (1.9-fold, $p < 0.001$) and the nesfatin-1 antibody/CRF group (2.0-fold, $p < 0.001$; Fig. 1D).

CRF tended to increase anxiety and explorative behavior an effect not altered by blockade of nesfatin-1. In the elevated zero maze, control antibody/CRF-treated animals tended to spend less time in the open arms compared to control antibody/vehicle (0.7-fold, p 0.17), an effect not altered by the nesfatin-1 antibody (p 1.00; Fig. 1B). The number of entries was not different between the control antibody/CRF and the nesfatin-1 antibody/CRF group (p 0.99; Fig. 1A). Track length in the open arms tended to be lower in the control antibody/CRF compared to the control antibody/ vehicle group (0.5-fold, p 0.23; Fig. 1C). Again, this effect was not altered by pre-injection of the nesfatin-1 antibody (p 0.95; Fig. 1C). Lastly, overall track length was not different between the control antibody/CRF and the nesfatin-1 antibody/CRF group (p 1.00; Fig. 1D).

Nesfatin-1 antibody did not alter snack intake in the novelty-induced hypophagia test. Injection of the anti-nesfatin-1 antibody under basal conditions did not significantly alter the latency to the first feeding bout compared to control antibody/vehicle (0.3-fold, $p > 0.05$; Fig. 2A). Similarly, the number of bouts (1.5-fold, Fig. 2B) and the amount of snack consumed (1.0-fold, Fig. 2C) was not significantly different between the nesfatin-1 antibody/vehicle and control antibody/vehicle group ($p > 0.05$).

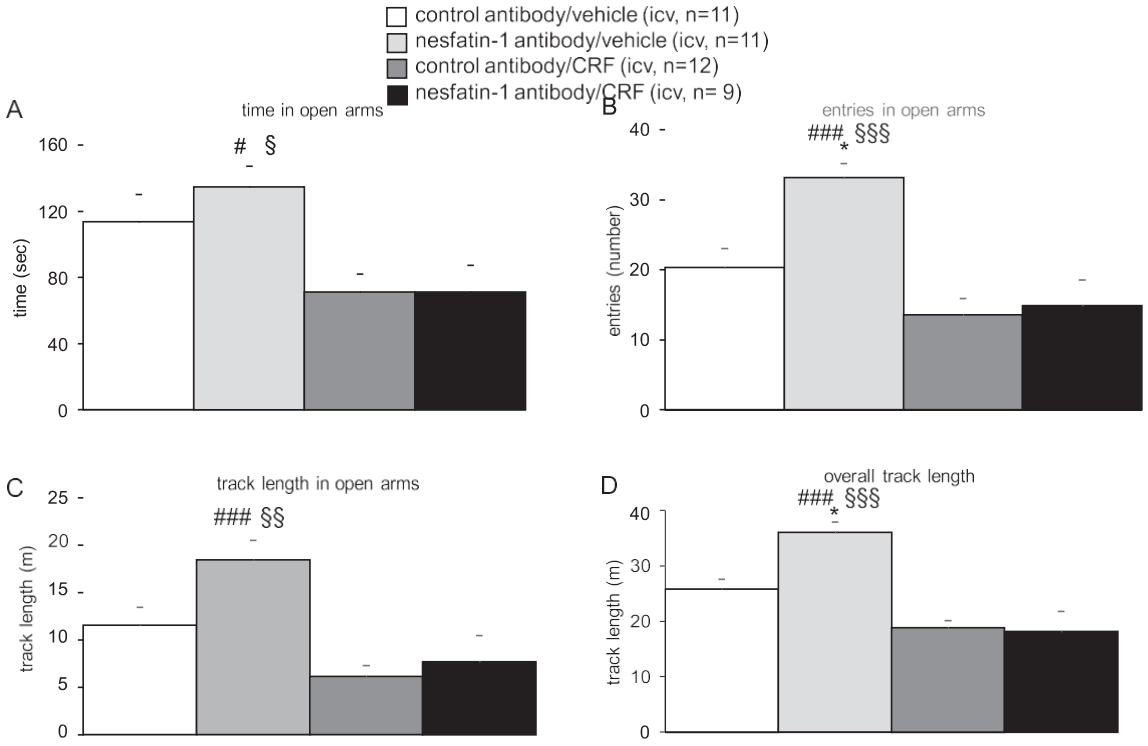

Fig. 1. Blockade of endogenous nesfatin-1 using an anti-nesfatin-1 antibody reduces anxiety in the elevated zero maze test. Icv cannulated rats were injected with control antibody or anti-nesfatin-1 antibody followed by an injection of vehicle or CRF. Thirty minutes later, behavior consisting of time in open arms (A), entries in open arms (B), track length in open arms (C) and overall track length (D) was assessed in the elevated zero maze over a period of 5 min. Data of 9–12 rats/group are presented as mean ± SEM. *$p < 0.05$ *vs.* control antibody/vehicle; #$p < 0.05$ and ###$p < 0.001$ *vs.* control antibody/CRF; x$p < 0.05$, xx$p < 0.01$ and xxx$p < 0.001$ *vs.* nesfatin-1 antibody/CRF.

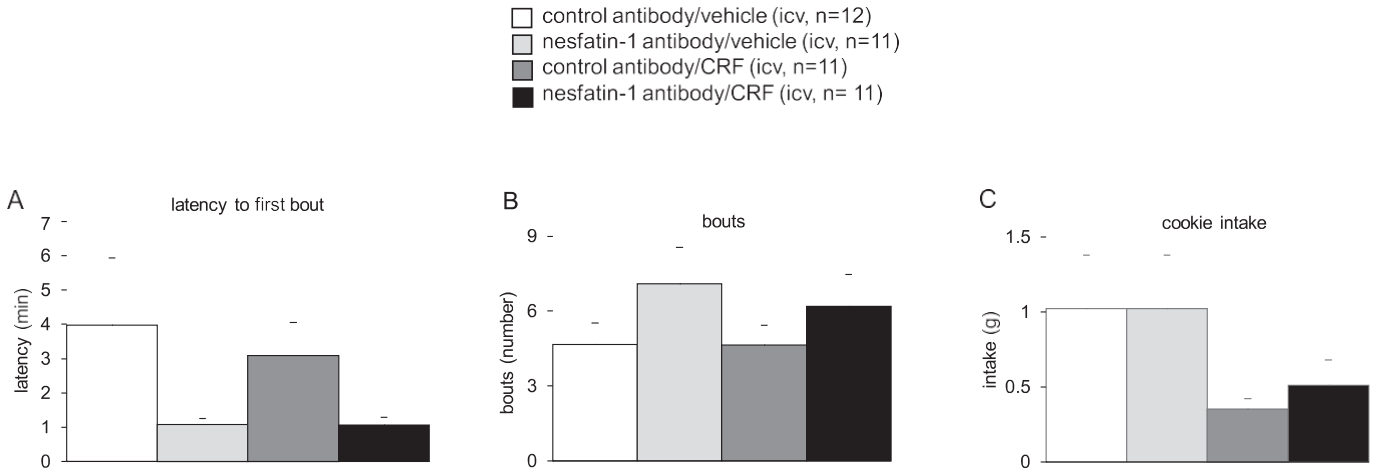

Fig. 2. Blockade of endogenous nesfatin-1 using an anti-nesfatin-1 antibody does not alter anhedonia in the novelty-induced hypophagia test. Icv cannulated rats were injected with control antibody or anti-nesfatin-1 antibody followed by an injection of vehicle or CRF. Thirty minutes later, behavior consisting of latency to first bout (A), number of bouts (B) and snack intake (C) was assessed in the novelty-induced hypophagia test over a period of 30 min. Data of 11e12 rats/group are presented as mean ± SEM. $p > 0.05$.

CRF did not induce anhedonia in the novelty-induced hypophagia test. Icv injection of CRF did not significantly alter the latency to the first feeding bout compared to the control antibody/vehicle group (0.8-fold, $p > 0.05$; Fig. 2A). No significant difference was observed between the control antibody/CRF and the nesfatin-1 antibody/CRF group (2.9-fold, $p > 0.05$; Fig. 2A). Similarly, the control antibody/CRF group did not show an altered number of bouts (1.0-fold, Fig. 2B) or a significantly different amount of snack consumed (0.3- fold, Fig. 2C) compared to control antibody/vehicle ($p > 0.05$). No significant difference was observed for the number of bouts consumed (0.8-fold, Fig. 2B) and the amount of snack intake (0.7-fold, Fig. 2C) between the control antibody/CRF and nesfatin-1 antibody/CRF group ($p > 0.05$).

4. Discussion

The present study showed that blockade of endogenous brain nesfatin-1 using an anti-nesfatin-1 antibody reduces anxiety behavior in the elevated zero maze reflected in more frequent entries into the open arms and an increased overall track length. This finding extends previous studies that reported an anxiogenic effect of peripherally [18] and centrally [17,19] injected nesfatin-1 in rats. While so far it was not possible to distinguish between a physiological and a pharmacological action of the peptide, the current data clearly point towards a physiological anxiogenic effect of endogenous brain nesfatin-1. Injection of CRF tended to decrease the time in the open arms likely reflecting the anxiogenic feature of the peptide. However, this effect was not altered by pretreatment with anti-nesfatin-1 antibody, suggesting that nesfatin-1 does not act downstream of CRF in the mediation of anxiety. Lastly, in the present study neither blockade of nesfatin-1 under basal conditions nor injection of CRF significantly affected anhedonia as part of depression-like behavior.

The absence of significant effects observed in the novelty-induced hypophagia test might be due to different reasons. First, the variation within groups is rather high which might have contributed to the non-significant effects despite considerably large group sizes of 11e12 rats/group. Second, CRF might affect other parts of depression-like behavior such as despair measured as increased immobility in forced swim test [39] but not affect anhedonia in particular; thus the model used might not be suitable. Overall, based on these data endogenous nesfatin-1 does not seem to be involved in the mediation of anhedonia under basal conditions. Whether this changes under conditions of activated nesfatin-1 signaling such as depression will have to be further investigated.

As mentioned before, psychological, physical and immunological stressors [24e26] increase central NUCB2/nesfatin-1 signaling likely resulting in/contributing to increased NUCB2/nesfatin-1 levels in the circulation [27]. Moreover, nesfatin-1_{30-59} injected icv was shown to induce anxiety and depression-like behavior [17]. Similarly, central CRF signaling is induced by stress and increases anxiety and depression [29,30] leading to the hypothesis of a shared path of mediation of nesfatin-1 and CRF. Nesfatin-1 was shown to activate CRF positive neurons resulting in increased ACTH and corticosterone levels [28]. Moreover, nesfatin-1 was shown to mediate its anorexigenic effect *via* downstream CRF_2 signaling [6]. While these convergent data support a role for CRF-CRF_2 signaling in the downstream mediation of nesfatin-1, the current study does not support the hypothesis of a downstream signaling of nesfatin-1 in the mediation of CRF's effects on anxiety. Since nesfatin-1 is a well-established anorexigenic peptide, in the future it should be investigated whether endogenous nesfatin-1 is responsible for anxiety-induced anorexia e.g. using a nesfatin-1 knock-out animal model.

In summary, these findings indicate that endogenous nesfatin-1 is likely involved in the mediation of anxiety but not anhedonia as shown using an anti-nesfatin-1 antibody. Moreover, nesfatin-1 is not involved in the downstream mediation of CRF's anxiogenic property. Further studies are warranted to assess the effects of blockade of endogenous nesfatin-1 under conditions of clinically apparent anxiety or anhedonia.

Declaration of competing interest

A.S. is consultant for a & r Berlin, Boehringer-Ingelheim, Takeda and Dr. Wilmar Schwabe. No conflicts of interest exist.

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Gesamtanzahl: 261 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS NEUROSCIENCE	43,107	33.162	0.068480
2	NATURE NEUROSCIENCE	63,390	21.126	0.164700
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4	BEHAVIORAL AND BRAIN SCIENCES	9,377	17.194	0.010240
5	TRENDS IN COGNITIVE SCIENCES	27,095	16.173	0.040040
6	JOURNAL OF PINEAL RESEARCH	10,695	15.221	0.010560
7	NEURON	95,348	14.403	0.218680
8	TRENDS IN NEUROSCIENCES	20,163	12.314	0.024480
9	Annual Review of Neuroscience	14,042	12.043	0.015020
10	MOLECULAR PSYCHIATRY	20,353	11.973	0.049290
11	BRAIN	52,970	11.814	0.074030
12	BIOLOGICAL PSYCHIATRY	43,122	11.501	0.053320
13	PROGRESS IN NEUROBIOLOGY	12,929	10.658	0.013230
14	Nature Human Behaviour	1,230	10.575	0.006550
15	SLEEP MEDICINE REVIEWS	6,920	10.517	0.010920
16	ANNALS OF NEUROLOGY	37,336	9.496	0.048630
17	Molecular Neurodegeneration	4,248	8.274	0.011350
18	NEUROSCIENCE AND BIOBEHAVIORAL REVIEWS	26,724	8.002	0.051580
19	FRONTIERS IN NEUROENDOCRINOLOGY	4,196	7.852	0.005490
20	Neurology-Neuroimmunology & Neuroinflammation	1,996	7.353	0.008220
21	NEUROPSYCHOPHARMACOLOGY	25,672	7.160	0.039090

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
22	Brain Stimulation	5,457	6.919	0.014470
23	NEUROPATHOLOGY AND APPLIED NEUROBIOLOGY	3,876	6.878	0.006420
24	NEUROENDOCRINOLOGY	5,046	6.804	0.005690
25	NEUROSCIENTIST	4,986	6.791	0.008520
26	BRAIN BEHAVIOR AND IMMUNITY	14,533	6.170	0.025700
27	BRAIN PATHOLOGY	5,263	6.155	0.007880
28	Alzheimers Research & Therapy	3,160	6.142	0.010700
29	JOURNAL OF NEUROSCIENCE	175,046	6.074	0.233460
30	JOURNAL OF CEREBRAL BLOOD FLOW AND METABOLISM	19,766	6.040	0.028050
31	PAIN	38,312	6.029	0.039070
32	CURRENT OPINION IN NEUROBIOLOGY	15,090	6.014	0.033650
33	Acta Neuropathologica Communications	3,063	5.883	0.014190
34	Translational Stroke Research	1,955	5.847	0.004330
35	GLIA	14,003	5.829	0.018760
36	NEUROIMAGE	99,720	5.812	0.132720
37	NEURAL NETWORKS	13,063	5.785	0.016060
38	NEUROPSYCHOLOGY REVIEW	2,971	5.739	0.003940
39	Molecular Autism	2,107	5.712	0.008000
40	Journal of Neuroinflammation	11,767	5.700	0.023240
41	Multiple Sclerosis Journal	11,501	5.649	0.022750
42	Annual Review of Vision Science	458	5.622	0.003300
43	Neurotherapeutics	4,475	5.552	0.009060
44	Translational Neurodegeneration	810	5.534	0.002420

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
45	CEREBRAL CORTEX	30,675	5.437	0.059570
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47	NEUROBIOLOGY OF DISEASE	16,363	5.160	0.026710
48	NEUROINFORMATICS	1,277	5.127	0.002920
49	JOURNAL OF PHYSIOLOGY-LONDON	52,037	4.950	0.041100
50	BIPOLAR DISORDERS	5,143	4.936	0.006760
51	Developmental Cognitive Neuroscience	2,470	4.920	0.009240
52	JOURNAL OF PSYCHIATRY & NEUROSCIENCE	3,293	4.899	0.004540
53	JOURNAL OF NEUROCHEMISTRY	35,902	4.870	0.026140
54	Dialogues in Clinical Neuroscience	3,384	4.867	0.004730
55	Annals of Clinical and Translational Neurology	1,858	4.656	0.008750
56	CURRENT OPINION IN NEUROLOGY	5,290	4.647	0.009650
57	MOLECULAR NEUROBIOLOGY	12,806	4.586	0.027560
58	SLEEP	21,434	4.571	0.024240
59	Current Neuropharmacology	3,508	4.568	0.005650
60	EXPERIMENTAL NEUROLOGY	20,500	4.562	0.023440
61	HUMAN BRAIN MAPPING	22,040	4.554	0.043230
62	Journal of Neural Engineering	7,336	4.551	0.012190
63	EUROPEAN NEUROPSYCHOPHARMACOLOGY	7,488	4.468	0.015500
64	CEPHALALGIA	9,983	4.438	0.014480
65	NEUROBIOLOGY OF AGING	22,409	4.398	0.037090
66	EUROPEAN JOURNAL OF NEUROLOGY	10,488	4.387	0.016970

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Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
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69	Cognitive Computation	1,578	4.287	0.002230
70	CORTEX	10,302	4.275	0.024590
71	Neuroscience Bulletin	2,027	4.246	0.004070
72	JOURNAL OF PSYCHOPHARMACOLOGY	6,460	4.221	0.010120
73	INTERNATIONAL JOURNAL OF NEUROPSYCHOPHARMACOLOGY	6,551	4.207	0.012320
74	JOURNAL OF NEUROSCIENCE RESEARCH	12,976	4.139	0.010060
75	Molecular Brain	2,467	4.051	0.007180
76	PSYCHONEUROENDOCRINOLOGY	16,809	4.013	0.028150
77	NEUROCHEMISTRY INTERNATIONAL	8,775	3.994	0.009020
78	NUTRITIONAL NEUROSCIENCE	1,778	3.950	0.002260
79	Frontiers in Systems Neuroscience	4,801	3.928	0.015360
80	JOURNAL OF HEADACHE AND PAIN	3,308	3.918	0.007210
81	Frontiers in Cellular Neuroscience	9,711	3.900	0.035870
82	Journal of Neuroimmune Pharmacology	2,486	3.870	0.004750
83	ACS Chemical Neuroscience	5,238	3.861	0.013320
84	CELLULAR AND MOLECULAR NEUROBIOLOGY	4,488	3.811	0.005740
85	NEUROGASTROENTEROLOGY AND MOTILITY	8,314	3.803	0.014510
86	JOURNAL OF NEUROTRAUMA	14,754	3.754	0.019770
87	Fluids and Barriers of the CNS	1,127	3.727	0.002650
88	Frontiers in Molecular Neuroscience	4,752	3.720	0.014230

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Restraint stress affects circulating NUCB2/nesfatin-1 and phoenixin levels in male rats

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ABSTRACT

The two peptides phoenixin and nesfatin-1 are colocalized in hypothalamic nuclei involved in the mediation of food intake and behavior. Phoenixin stimulates food intake and is anxiolytic, while nesfatin-1 is an anorexigenic peptide shown to increase anxiety and anhedonia. Interestingly, central activation of both peptides can be stimulated by restraint stress giving rise to a role in the mediation of stress. Thus, the aim of the study was to test whether also peripheral circulating levels of NUCB2/nesfatin-1 and phoenixin are altered by restraint stress. Male *ad libitum* fed Sprague Dawley rats equipped with a chronic intravenous catheter were subjected to restraint stress and plasma levels of NUCB2/nesfatin-1, phoenixin and cortisol were measured over a period of 240 min and compared to levels of freely moving rats. Peripheral cortisol levels were significantly increased in restrained rats at 30, 60, 120 and 240 min compared to controls ($p < 0.05$). In contrast, restraint stress decreased plasma phoenixin levels at 15 min compared to unstressed conditions (0.8-fold, $p < 0.05$). Circulating NUCB2/nesfatin-1 levels were increased only at 240 min in restrained rats compared to those in unstressed controls (1.3-fold, $p < 0.05$). In addition, circulating NUCB2/nesfatin-1 levels correlated positively with phoenixin levels ($r = 0.378$, $p < 0.001$), while neither phoenixin nor nesfatin-1 were associated with cortisol levels ($r = 0.0275$, and r=-0.143, $p > 0.05$). These data suggest that both peptides, NUCB2/nesfatin-1 and phoenixin, are affected by restraint stress, although less pronounced than circulating cortisol.

1. Introduction

Nesfatin-1, a peptide consisting of 82 amino acids, was discovered in the rat hypothalamus (Oh et al., 2006), where it was found to be expressed, among others, in the arcuate nucleus, the paraventricular nucleus and the nucleus of the solitary tract (Goebel et al., 2009a), all implicated in the regulation of food intake. Another more recently identified central expression side is the grey matter of porcine spinal cord (Lepiarczyk et al., 2020). Moreover, nesfatin-1 was detected in various peripheral tissues such as in adipose tissue (Ramanjaneya et al., 2010), endocrine pancreatic beta cells (Foo et al., 2010), testis (García-Galiano et al., 2012) and X/A-like cells of the stomach, where it is co-localized with ghrelin (Stengel et al., 2009, 2013). Although nesfatin-1's receptor is still unknown, its binding pattern, as indicated by an autoradiographic study, was shown to be widespread including the hypothalamus, gastrointestinal tract and different endocrine organs (Prinz et al., 2016).

Unsurprisingly, as indicated by its widespread expression, besides its ability to reduce food intake after intracerebroventricular (icv) as well as peripheral injection in various species (Schalla et al., 2020b), nesfatin-1 has pleiotropic effects (Schalla and Stengel, 2018a) such as inhibitory effects on gastrointestinal motility and glucose homeostasis as well as stimulating actions on thermogenesis and cardiac contractility. We showed before that icv injected nesfatin-1_{30-59}, the active core of nesfatin-1 (Stengel et al., 2012), has anxiogenic and anhedonic effects in rats (Kühne et al., 2018) as suggested before for the non-processed

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peptide (Ge et al., 2015; Merali et al., 2008) and also observed in humans (Weibert et al., 2019). Supporting a physiological role of nesfatin-1 in the mediation of anxious behavior, we recently demon- strated that acute blockade of endogenous nesfatin-1 using an icv injected anti-nesfatin-1 antibody reduced anxiety-like behavior in rats (Schalla et al., 2020a). In humans, a positive association between circulating NUCB2/nesfatin-1 concentrations and perceived anxiety has been described in anorectic (Hofmann et al., 2015a) and obese women (Hofmann et al., 2013), whereas in obese men a negative correlation was observed (Hofmann et al., 2015b), suggesting a sex-dependent modu- lation of nesfatin-1.

Interestingly, nesfatin-1's anorexigenic action is mediated *via* CRF_2 receptor signaling (Stengel et al., 2009) suggesting an interaction of nesfatin-1 and the hypothalamic pituitary adrenal (HPA) axis. Despite the fact that corticotropin-releasing factor (CRF)-induced anxiety was not ameliorated by blockade of endogenous nesfatin-1 (Schalla et al., 2020a), icv injection of nesfatin-1 increased levels of adrenocortico- tropic hormone (ACTH) as well as corticosterone and stimulated CRF immunoreactive neurons of the paraventricular nucleus (Yoshida et al., 2010). Moreover, nesfatin-1's expression in the central nervous system was affected by emotional (Goebel et al., 2009b), physical (Stengel et al., 2010b) and immunological stressors (Bonnet et al., 2009). In addition, lipopolysaccharide injection as immunological stressor also increased circulating nesfatin-1 levels (Stengel et al., 2011). Altogether, the literature clearly points towards a role of nesfatin-1 in the mediation of anxiety and stress. Interestingly, the alteration of peripheral nesfatin-1 levels under conditions of stress is much less studied.

Noteworthy, nesfatin-1 was shown to be colocalized in several hypothalamic nuclei with phoenixin (Pałasz et al., 2015). This peptide with its predominant 20 and 14 amino acid long forms was discovered in 2013 (Yosten et al., 2013). Similar to nesfatin-1, phoenixin is also expressed in many brain areas such as in the central amygdaloid nucleus, spinal trigeminal tract, spinocerebellar tract, bed nucleus of the stria terminalis, area postrema, nucleus of the solitary tract, dorsal motor nucleus of the vagus nerve as well as in the duodenum, jejunum and ileum. (Prinz et al., 2017). Also, similar to nesfatin-1, phoenixin displays pleiotropic effects (Schalla and Stengel, 2018b): it is implicated in reproductive functions (Yosten et al., 2013), the stimulation of food intake (Schalla et al., 2017) and cardioprotection (Rocca et al., 2018). In addition, in mice the icv injection of phoenixin reduced anxiety (Jiang et al., 2015) and in obese men phoenixin levels were negatively asso- ciated with anxiety scores (Hofmann et al., 2017), suggesting a possible counterregulatory effect of phoenixin in states of increased anxiety. Just recently, we demonstrated that brain phoenixin expression was significantly increased after 30 min of restraint stress and showed a positive correlation between restraint stress-induced c-Fos and phoenixin expression (Friedrich et al., 2020). Again, much less is known about the effect of stress on peripheral phoenixin concentrations.

Since nesfatin-1 and phoenixin are co-localized, both implicated in the regulation of behavior and their central expression can be stimulated by restraint stress, in the present study we aimed to investigate whether restraint stress does affect peripheral circulating levels of nesfatin-1 and phoenixin. Additionally, cortisol was investigated as a well-established peripheral hormone of the HPA axis (Michaud et al., 2008).

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (Envigo, Germany) weighing between 200—250 g were housed under controlled conditions at a temperature of 21–23 °C, humidity of 45–65 % and a 12-h dark/light cycle with lights on at 6 a.m.. For acclimatization, animals were housed in groups of four per cage. After one week, rats underwent intravenous (iv) cannulation surgery and were afterwards separated into individual cages. All rats had *ad libitum* access to water and standard rodent chow (ssniff Spezialdiäten GmbH, Soest, Germany) except during the experimental restraint stress procedure. Body weight, food and water intake were documented daily; in addition, all animals were handled daily to adapt to the experimental handling and investigators. The experiments were approved by the state authority for animal research (Landesamt für Gesundheit und Soziales Berlin, Berlin) and institutional ethics guide- lines for animal care were followed.

2.2. Surgery

All animals were iv cannulated as described before (Stengel et al., 2010a; Wang et al., 2006): rats were anesthetized with a mixture of xylazine (10 mg/kg; Rompun™, 2%, Bayer, Leverkusen, Germany) and ketamine (100 mg/kg; Ketanest™, Curamed, Karlsruhe, Germany). In brief, the right external jugular vein was cannulated using a sterile PE-50 tube filled with sterile saline which was then subcutaneously tunneled and exteriorized between the scapulae where it was sewed to the skin. Afterwards, the cannula was filled with heparin solution (200 units/mL) and closed using a wire obturator. After surgery, rats were allowed to recover for three days during which they received enrofloxacin (2.5 % ad us. vet. 0.1 mL/L in drinking water, Bayer Vital GmbH, Leverkusen, Germany) and were accustomed to light hand-restraint.

2.3. Restraint stress

Between 8 a.m. and 2.30 pm animals were placed in a Decapi-Cone (DecapiCones, Braintree Scientific, Inc. Braintree, MA), which had been prepared to allow ventilation and heat exchange as described before (Goebel et al., 2009b). One half of the animals (n 7) was restrained in an immobile position that allowed no movement for 240 min. The other half (n 7) was left undisturbed in their home cages as controls; controls were also used to reduce the effect of circadian rhythm of peptide secretion on the statistical analysis. Blood was withdrawn *via* the iv catheter at 0, 15, 30, 60, 120 and 240 min in restrained and control rats. Afterwards, all animals were euthanized.

2.4. Blood analysis

During withdrawal, blood was collected in pre-cooled tubes con- taining EDTA (7.5 %, 10 µl/0.5 mL blood; Sigma-Aldrich Chemie GmbH, Munich, Germany) and aprotinin (1.2 trypsin inhibitory unit per 1 mL blood; Carl Roth GmbH Co. KG, Karlsruhe, Germany). Immediately afterwards it was placed back on ice and centrifuged at 4 °C for 10 min at 3000 x g to separate plasma, which was stored at −80 °C until further processing. The measurement of plasma levels was conducted using commercial enzyme-linked immunosorbent assays for phoenixin (#EK- 079-01, Phoenix Pharmaceuticals Inc., Burlingame, CA, USA), nesfatin-1 (#EK-003-22, Phoenix Pharmaceuticals Inc.) and cortisol (#KGE008B, R&D Systems® Bio-Techne GmbH Wiesbaden-Nordenstadt, Germany). Since the nesfatin-1 kit also detected non-processed NUCB2 we refer to the analyte as NUCB2/nesfatin-1. The linear detection range of the assays for phoenixin, nesfatin-1, and cortisol was 0.07–2.1 ng/mL, 1.26–17.7 ng/mL and 0.2–10 ng/mL (manufacturer's information), respectively. The intra-assay variability was 9.7 %, 6.0 % and 12.2 % for phoenixin, nesfatin-1 and cortisol, respectively.

2.5. Statistical analysis

Statistical analysis was conducted with SPSS 25 (IBM Corp. 2017, IBM SPSS Statistics for Windows, Version 25.0, Armonk, NY, USA). Before analyses, all data was tested for normality using the Kolmogorov- Smirnov test. *t*-Test or Mann-Whitney-U test was used to compare plasma levels between the restrained and control group at respective time points depending on the distribution. In addition, two way ANOVA was used to analyze the effect of time and treatment. Finally, data was analyzed for correlations using Pearson's analysis. Data is expressed as

mean ± SEM, significance was defined as $p < 0.05$.

3. Results

3.1. Restraint stress increased circulating cortisol levels

Restrained rats displayed significantly increased circulating cortisol levels at 30 (108.86 ± 18.82 *vs*. 66.39 ± 9.84 ng/mL, $p < 0.05$), 60 (118.45 ± 18.74 *vs*. 31.91 ± 6.50 ng/mL, $p < 0.01$), 120 (102.55 ± 20.66 *vs*. 17.56 ± 4.48 ng/mL, $p < 0.01$) and 240 (96.21 ± 20.95 *vs*. 23.39 ± 5.31 ng/mL, $p < 0.01$) min compared to controls at the respective time points (Fig. 1). No differences in plasma cortisol levels were observed between stressed and undisturbed rats at 0 and 15 min ($p > 0.05$; Fig. 1). Two way ANOVA indicated a significant impact of treatment ($F_{(1,79)} = 35.243$, $p < 0.001$), time ($F_{(5,79)} = 2.488$, $p = 0.040$) and an interaction of treatment x time ($F_{(5,79)} = 2.746$, $p = 0.026$).

3.2. Restraint stress reduced circulating phoenixin-1 levels

Restraint stress led to a significant decrease of plasma phoenixin levels at 15 min (0.57 ± 0.03 *vs*. 0.72 ± 0.04 ng/mL, $p < 0.05$) compared to undisturbed controls (Fig. 2). Before and afterwards no significant differences were observed between the two groups ($p > 0.05$; Fig. 2). Two way ANOVA indicated a significant effect of time on phoenixin levels ($F_{(5,78)} = 2.491$, $p = 0.040$).

3.3. Restraint stress increased plasma NUCB2/nesfatin-1 levels

Plasma NUCB2/nesfatin-1 levels were significantly increased due to restraint stress at 240 min in comparison to those in controls (5.30 ± 0.33 *vs*. 3.95 ± 0.32 ng/mL, $p < 0.05$), while at 0, 15, 30, 60 and 120 min no differences were observed between circulating NUCB2/nesfatin-1 levels of stressed and undisturbed rats ($p > 0.05$; Fig. 3). Two way ANOVA indicated a trend towards an effect of time on NUCB2/nesfatin- 1 levels ($F_{(5,78)} = 2.356$, $p = 0.050$).

3.4. Phoenixin correlated positively with NUCB2/nesfatin-1

Circulating phoenixin levels showed a significant correlation with plasma NUCB2/nesfatin-1 concentrations (r 0.378, $p < 0.001$; Fig. 4). However, neither phoenixin nor NUCB2/nesfatin-1 significantly corre- lated with circulating cortisol levels ($r = 0.028$ and $r = -0.143$, respectively, $p > 0.05$; data not shown).

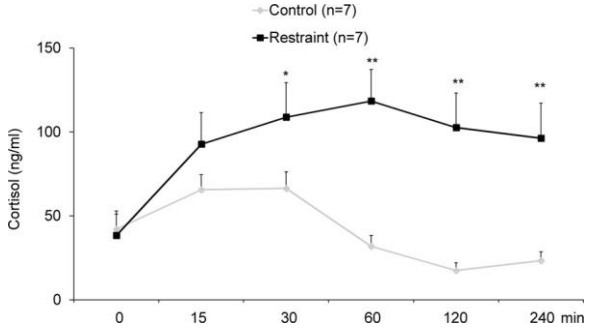

Fig. 1. Restraint stress increased circulating cortisol levels in male rats. Intravenously cannulated male rats were submitted to restraint stress or left undisturbed in their home cage at the beginning of the light phase for 4 h during which blood was withdrawn at 0, 15, 30, 60, 120 and 240 min. Restraint stress showed no effect on circulating cortisol levels at 0 and 15 min ($p > 0.05$) but increased circulating cortisol levels at 30 ($p < 0.05$), 60 ($p < 0.01$), 120 ($p < 0.01$) and 240 ($p < 0.01$) min compared to controls at the respective time points. Data were not normally distributed; thus, data were analyzed using Mann-Whitney-U test comparing the two groups at the respective time points. All data are expressed as mean ± SEM. * $p < 0.05$ and ** $p < 0.01$ *vs*. control.

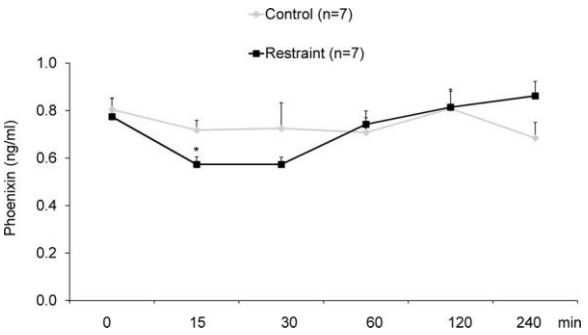

Fig. 2. Restraint stress reduced circulating phoenixin-1 levels in male rats. Intravenously cannulated male rats were submitted to restraint stress or left undisturbed in their home cages at the beginning of the light phase for 4 h during which blood was withdrawn at 0, 15, 30, 60, 120 and 240 min. Restraint stress did not affect phoenixin levels at 0 min but decreased plasma phoenixin levels at 15 min ($p < 0.05$) compared to undisturbed controls. No difference between stressed and undisturbed rats was observed at 30, 60, 120 and 240 min ($p > 0.05$). Data were normally distributed; thus, data were analyzed using t-test comparing the two groups at the respective time points. All data are expressed as mean ± SEM. * $p < 0.05$ *vs*. control.

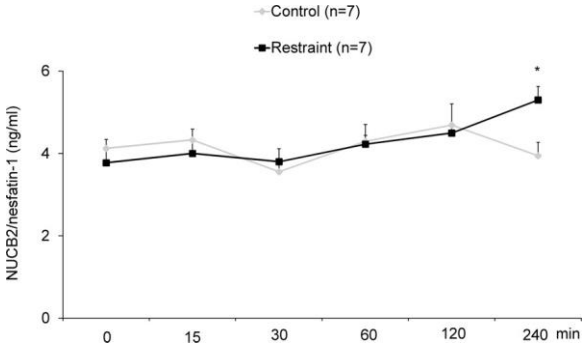

Fig. 3. Restraint stress increased plasma NUCB2/nesfatin-1 levels in male rats. Intravenously cannulated male rats were submitted to restraint stress or left undisturbed in their home cages at the beginning of the light phase for 4 h during which blood was withdrawn at 0, 15, 30, 60, 120 and 240 min. Restraint stress did not affect plasma NUCB2/nesfatin-1 levels at 0, 15, 30, 60 and 120 min ($p > 0.05$). Stressed rats showed increased plasma NUCB2/nesfatin-1 levels at 240 min compared to undisturbed rats ($p < 0.05$). Data were not normally distributed; thus, data were analyzed using Mann-Whitney-U test comparing the two groups at the respective time points. All data are expressed as mean ± SEM. * $p < 0.05$ *vs*. control.

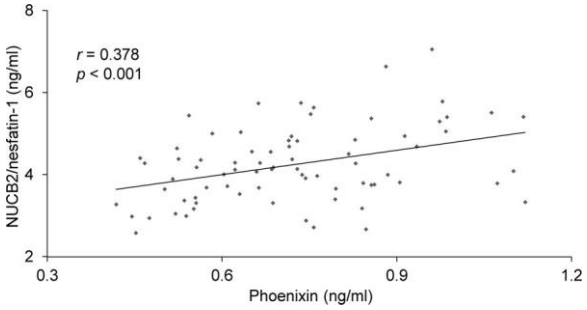

Fig. 4. Phoenixin correlated positively with NUCB2/nesfatin-1. Intravenously cannulated male rats were submitted to restraint stress or left undisturbed in their home cage at the beginning of the light phase for 4 h during which blood was withdrawn at 0, 15, 30, 60, 120 and 240 min. Circu- lating phoenixin concentrations in male rats correlated significantly with plasma NUCB2/nefatin-1 levels ($r = 0.378$, $p < 0.001$).

4. Discussion

In the present study we tested the effect of restraint stress on levels of circulating phoenixin and NUCB2/nesfatin-1. First, we showed that restraint stress in Sprague Dawley rats elicited a robust stress reaction as indicated by a delayed but pronounced increase of cortisol levels in immobilized compared to undisturbed rats corroborating the assumption that restraint stress is a valid model to stimulate the HPA axis. This observation is in line with previous studies showing increased serum cortisol in mice at the first day of repeated restraint stress and during forced swim test (Gong et al., 2015) and elevated cortisol levels in beef cattle enduring restraint isolation (Wagner et al., 2020). Noteworthy, the cortisol levels in controls tended to be higher during the first 60 min compared to afterwards, which could be due to relocation of the animals to the proceeding rooms from their housing room as well as the higher frequency of blood withdrawal accompanied by handling in the beginning of the experiment. Although this is only a trend and not statistically significant, it could be a contributing factor for the indifference of cortisol levels between control and restraint animals in the first 15 min.

Moreover, the present study showed that NUCB2/nesfatin-1 and phoenixin are modulated by restraint stress in a differentiated manner. While phoenixin was decreased shortly after the start of the restraint stress (15 min) and normalized thereafter, NUCB2/nesfatin-1 was similar between stressed and undisturbed rats throughout almost the whole experiment and increased only after 240 min. At what time point between 120 and 240 min exactly circulating nesfatin-1 levels started to increase due to immobilization, however, cannot be answered. Additionally, whether nesfatin-1 would continue to be increased after 240 min or not also remains unanswered; thus, this question warrants further research. It is to note that a longer duration of the stress would not have been feasible since restraint stress is a potent stressor that cannot be applied for too long. Therefore, a milder stressor (*e.g.* water avoidance stress) might be suited in order to investigate the longer-term effects on NUCB2/nesfatin-1. Noteworthy, although the changes in phoenixin and NUCB2/nesfatin-1 were statistically significant, the clinical significance of a change of -21 % and 30 % of circulating phoenixin and NUCB2/ nesfatin-1, respectively should be further investigated. Noteworthy, compared to 120 min there was a slight decline of NUCB2/nesfatin-1 level at 240 min in the control group which could contribute to the statistical difference between control and restraint animals. Therefore, the current data should be interpreted with caution and warrant further replication *e.g.* by another stress model as suggested above.

However, the increase of NUCB2/nesfatin-1 was in accordance with previous data of activation of nesfatin-1 immunopositive hypothalamic nuclei due to different stressors (Bonnet et al., 2009; Goebel et al., 2009b; Stengel et al., 2010b) and increased circulating levels of NUCB2/nesfatin-1 after lipopolysaccharide injection, an immunological stressor (Stengel et al., 2011). Our data is further corroborated by ob- servations showing that acute stress in form of a water avoidance test increased plasma concentrations and hypothalamic mRNA expression of nesfatin-1 (Xu et al., 2015). Noteworthy, the latter study showed no effect of chronic stress on NUCB2/nesfatin-1 levels, while more recently it was observed that chronic immobilization stress for 21 days signifi- cantly increased concentrations of NUCB2/nesfatin-1 in the serum and paraventricular nucleus (Ma et al., 2019). Although there is a clear as- sociation between nesfatin-1 and stress, the precise mechanism and confounders need to be investigated further. Past research pointed to- wards a close relationship between nesfatin-1 and the HPA axis as a possible signaling pathway. Even though there is data demonstrating that nesfatin-1's anorexigenic action is mediated *via* CRF_2 receptor signaling (Stengel et al., 2009) and that nesfatin-1 increased cytosolic Ca^{2+} concentration in CRF immunoreactive neurons thereby increasing plasma levels of ACTH and corticosterone (Yoshida et al., 2010), we did not observe a linear correlation between peripheral nesfatin-1 and cortisol levels. This is in accordance with our previous investigations showing that CRF-induced anxiety does not depend on downstream nesfatin-1 signaling (Schalla et al., 2020a), altogether indicating that although there is a relation between nesfatin-1 and cortisol it should be further investigated especially with regard to brain and peripheral levels and different time axes.

Although the changes in phoenxin-14 levels due to restraint stress were minor, they match previous findings of increased activity of phoenixin immunoreactive hypothalamic nuclei in response to 30 min of restraint stress (Friedrich et al., 2020). In addition, another recent study showed that stress due to construction work in the animal care facility as well as 2-week long corticosterone treatment induced a lack of neuronal responsiveness to phoenixin *in vitro* (Grover et al., 2020). However, the alterations of brain phoenixin observed before were more pronounced than those described in the present study. Whether brain phoenixin levels are correlated with hormones of the HPA axis (in absence of the correlation of the peripheral hormones described here) will have to be further investigated.

Noteworthy, NUCB2/nesfatin-1 and phoenixin were positively associated with each other, which is in accordance with human data showing a negative correlation between perceived anxiety and NUCB2/ nesfatin-1 as well as with phoenixin in obese men (Hofmann et al., 2015b, 2017). However, the association of NUCB2/nesfatin-1 and anx- iety scores was positive in obese and anorectic women (Hofmann et al., 2015a, 2013), suggesting a more complex relation not only between nesfatin-1 and behavior but also between nesfatin-1 and phoenixin. The hypothesis of a complex relationship between both peptides is corrob- orated by observations rather indicating a negative relation between the two peptides: nesfatin-1 has anorexigenic and anxiogenic effects (Schalla and Stengel, 2018a), while phoenixin displays contrasting ac- tions, meaning orexigenic and anxiolytic functions (Schalla and Stengel, 2018c). Thus, there is a need for further research to characterize the underlying mechanisms inducing changes in nesfatin-1 and phoenixin levels in the brain and the periphery in response to stress along with their possible functional interaction.

In summary, restraint stress modulates NUCB2/nesfatin-1 and phoenixin levels in a differentiated manner, changes that were less pronounced than those of cortisol and did not seem to – at least directly – be related to the HPA axis.

Declaration of competing interest

No conflicts of interest exist.

Acknowledgements

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Curriculum vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

List of publications

*shared first authorship

Original articles

1. Schalla MA*, Prinz P*, Friedrich T, Scharner S, Kobelt P, Goebel-Stengel M, Rose M, Stengel A., Phoenixin-14 injected intracerebroventricularly but not intraperitoneally stimulates food intake in rats., Peptides (2017);96:53-60. (IF 2.535 in 2015)
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