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DISSERTATION

Activity-based anorexia is an animal model for anorexia nervosa in rats

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1. Table of abbreviations

10N ..... **dorsal motor nucleus of the vagus nerve**

ABA ..... **activity-based anorexia**

ABC ..... **avidin-biotin-peroxidase complex**

AC ..... **activity animal group**

AL ..... **ad libitum animal group**

AN ..... **anorexia nervosa**

ANOVA ..... **analysis of variance**

Arc ..... **arcuate nucleus**

CRF ..... **corticotropin-releasing factor**

D receptor ..... **dopamine receptor**

DAB ..... **diaminobenzidine tetrachloride**

DMH ..... **dorsomedial hypothalamic nucleus**

DR ..... **dorsal raphe nuclei**

EW ..... **Edinger-Westphal nucleus**

FAA ..... **food-anticipatory activity**

GOAT ..... **ghrelin-O-acyltransferase**

LC ..... **locus coeruleus**

LHA ..... **lateral hypothalamic area**

LS ..... **lateral septal nucleus**

NAcc ..... **nucleus accumbens**

NTS ..... **nucleus of the solitary tract**

NUCB2 ..... **nucleobindin 2**

PBS ..... **phosphate-buffered saline**

PVN ..... **paraventricular nucleus**

Rpa ..... **raphe pallidus**

RF ..... **restricted feeding animal group**

Sch ..... **suprachiasmatic nucleus**

SEM ..... **standard error of the mean**

SON ..... **supraoptic nucleus**

SSRI ..... **selective serotonin reuptake inhibitor**

VTA ..... **ventral tegmental area**

2.1. ABSTRACT [English]

Anorexia nervosa (AN) in an eating disorder diagnosed by the occurrence of the ensuing symptoms: a strong wish to lose body weight, restricted caloric intake, a distorted body image, and hyperactivity. Despite the well-defined symptomatology of the disease, the pathophysiology still remains poorly explained. Moreover, a specific pharmacological treatment is lacking. In order to better characterize pathophysiological alterations occurring under disease conditions, animal models can be useful. The activity-based anorexia (ABA) model for rats mimics the disease by combining the two factors of restricted feeding (access to food: 1.5h/day) with the possibility of voluntary exercise in a running wheel installed in the rats' cage. The aim of the studies was to investigate the modulation of food intake and body weight under conditions of ABA as well as to describe neuronal changes possibly underlying the observed alterations. Female Sprague-Dawley rats were used and randomly assigned to one of four groups: *ad libitum* (AL, *ad libitum* food, no running wheel, n=9), activity (AC, *ad libitum* food and running wheel, n=9), restricted feeding (RF, food restriction, no running wheel, n=12) and activity-based anorexia (ABA, food restriction and running wheel, n=11). Following validation of an automated food intake-monitoring system for the use in rats, food intake microstructure was assessed under conditions of ABA. ABA resulted in a pronounced body weight loss of -22% compared to the first day of food restriction ($p<0.001$) and compared to the other groups (RF -13%, AC +10% and AL +13%, $p<0.001$). However, the food intake microstructure (different continuously measured parameters including meal size, frequency and duration) and the activity pattern did not differ from the respective control groups (ABA *vs*. RF and AC *vs*. AL, $p>0.05$). An analysis of the neuronal changes showed that ABA leads to an activation of distinct brain nuclei involved in the regulation of food intake (LS, LHA, Arc, DMH, NTS), gastrointestinal motility (LHA, NTS, 10N), thermoregulation (DMH), circadian rhythm (DMH), stress (PVN, LC), memory (hippocampus) and depressiveness/anxiety (SON, PVN, DR, Rpa). Immunohistochemical doublestaining for c-Fos and the anorexigenic peptide nesfatin-1 -indicated by a significant increase of nesfatin-1 immunoreactive cells in PVN, DMH, Arc, LC and NTS in ABA rats ($p<0.05$)- suggested that nesfatin-1 might play a role in the development/maintenance of ABA and potentially in patients with AN too. In summary, although cautious interpretation is necessary, ABA might be a useful tool to investigate pathophysiological alterations occurring also in AN.

2.2. ABSTRACT [German]

Anorexia nervosa (AN) ist eine Essstörung mit folgenden Symptomen: starker Wunsch Gewicht abzunehmen, Kalorienrestriktion, Körperschemastörung und, bei vielen Patienten, Hyperaktivität. Wenn auch das klinische Erscheinungsbild gut beschrieben ist, ist die Pathophysiologie der Erkrankung noch weitgehend ungeklärt und es gibt bisher keine spezifische Pharmakotherapie. Tiermodelle von Erkrankungen können hier manchmal einen hilfreichen Beitrag zur besseren Charakterisierung von pathophysiologischen Veränderungen leisten. Das Aktivitäts-basierte Anorexie-Modell (ABA) für Ratten stellt die Erkrankung nach, in dem es die zwei Faktoren, eingeschränkten Zugang zu Nahrung auf 1.5h täglich und die Möglichkeit sich in einem im Käfig befindenden Laufrad zu bewegen, kombiniert. Das Ziel der Experimente war erstens, die Veränderungen von Nahrungsaufnahme und Körpergewicht bei ABA zu untersuchen, und zweitens, die neuronalen Veränderungen, die den untersuchten Parametern eventuell zugrunde liegen, zu beschreiben. Zu diesem Zweck wurden weibliche Sprague-Dawley Ratten zufällig einer von vier Gruppen zugeteilt: *ad libitum* (AL, kein Laufrad, n=9), Aktivität (AC, *ad libitum* Futter und Laufrad, n=9), Futterrestriktion (RF, kein Laufrad, n=12) und Aktivitäts-basierte Anorexie (ABA, Futterrestriktion und Laufrad, n=11). Zuerst wurde ein automatisches Nahrungsaufnahme-Messsystem für die Anwendung bei Ratten validiert und anschließend damit die Mikrostruktur der Nahrungsaufnahme unter ABA-Bedingungen erhoben. Verglichen mit dem ersten Tag der Nahrungsrestriktion verloren ABA-Tiere -22% ihres Körpergewichts ($p<0.001$, im Vergleich zu den anderen Gruppen: RF -13%, AC +10% und AL +13%, $p<0.001$). Dennoch gab es weder einen Unterschied in der Mikrostruktur der Nahrungsaufnahme (ABA *vs*. RF und AC *vs*. AL $p>0.05$), noch in der Laufradaktivität (ABA *vs*. AC, $p>0.05$). Die Analyse der neuronalen Veränderungen ergab, dass ABA zu einer Aktivierung von Gehirngebieten führt, die in die Regulation von Nahrungsaufnahme (LS, LHA, Arc, DMH, NTS), gastrointestinaler Motilität (LHA, NTS, 10N), Thermoregulation (DMH), zirkadiane Rhythmen (DMH), Stress (PVN, LC), Gedächtnis (Hippocampus) und Depressivität/Angst (SO, PVN, DR, Rpa) involviert sind. Immunhistochemische Doppelfärbungen von c-Fos und dem anorexigenen Peptid Nesfatin-1 gaben Anlass zu der Vermutung, dass Nesfatin-1 eine Rolle bei ABA und eventuell auch bei Patienten mit AN spielt, denn Nesfatin-1 immunreaktive Zellen waren bei ABA-Tieren im PVN, DMH, Arc, LC und NTS ($p<0.05$) vermehrt nachweisbar. Zusammengefasst stellt ABA ein potentiell hilfreiches Modell zur Untersuchung von pathophysiologischen Veränderungen bei AN dar, auch wenn die Tiermodell-Daten vorsichtig interpretiert werden sollten.

3. INTRODUCTION

The eating disorder anorexia nervosa (AN) is most prevalent in women and is defined by a caloric intake reduction driven by a predominant desire to lose body weight (1). Further symptoms are a distorted body image and an increased eagerness to exercise often resulting in hyperactivity (2,3). AN has a prevalence of 0.9% in European women (4). Many patients of this disease suffer from comorbidities (5), mainly depression, anxiety and insomnia but also somatic complications of the body weight loss such as cardiac arrhythmias, osteoporosis and gastrointestinal dysfunction (6–8). Pharmacological treatment symptomatically targets the associated comorbidities, whereas no specific drug treatment is available for AN itself (9). Identification of these drugs is hampered by the fact that the pathophysiology of AN is only poorly understood. Animal models often represent a suited tool to get further insight into the pathophysiology of a respective disease.

The activity-based anorexia (ABA) paradigm in rats is the most established animal model for AN (10). Already in 1967 Routtenberg and Kuznesof observed that a time-restricted access to food combined with 24h access to a running wheel led to severe body weight loss initially described as “self-starvation” (11). Subsequently, the term “activity-based anorexia” was established and the alterations were further studied. A feeding schedule with access to food for 1.5h a day and the use of adolescent rats weighing around 150-180g has been shown to reproducibly induce a pronounced body weight loss of -20% (12,13). The advantage of this model is that it mimics the main features of AN (3), namely decreased caloric intake and hyperactivity (14), the latter one being displayed by 31-80% of patients suffering from AN (2).

For the examination of food intake, it is important to employ a tool which measures not only the total amount of food ingested, but which also provides detailed information on the underlying food intake microstructure. This can be achieved *via* an automated food intake-monitoring system which has been previously validated for the use in mice (15). Therefore, in the first study this system was established for the use in rats (16). In the second study, the food intake microstructure was investigated under conditions of ABA (17).

As a further step, the neuronal changes underlying the behavioral changes observed in the ABA model were investigated by c-Fos immunohistochemistry, which is a well-established marker for neuronal activity (18). A semi-quantitative brain mapping of nuclei activated in ABA rats compared to AL rats was conducted (17).

Various aspects of the ABA model have been characterized in the past. ABA rats show an increased activity of the hypothalamus-pituitary-adrenal axis (19), disrupted dopamine (20,21) and endocannabinoid signaling (22), along with an altered pattern of several food intake-regulatory hormones (10), e.g. ghrelin (23) and leptin (24). Since nesfatin-1 is an anorexigenic peptide (25,26) also strongly linked to the stress response *via* the hypothalamus-pituitary-adrenal axis (26–28), nesfatin-1 may play a role in the development of ABA as well. The decrease of circulating NUCB2/nesfatin-1 in patients of AN (29) further suggests the involvement of altered nesfatin-1 signaling under these conditions. In order to examine a possible role of nesfatin-1 in ABA, the third study investigated the activation of nesfatin-1 immunoreactive cells in different brain nuclei under conditions of ABA (30).

4. METHODS

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

Monitoring of food intake

Adult male Sprague Dawley rats (Harlan-Winkelmann Co., Borchen, Germany) weighing 220-300g upon arrival were used. In order to monitor food intake manually, the rats' food intake was assessed by providing rats with pre-weighed rat chow (standard rodent chow, Altromin™, Lage, Germany) from the top of the cage and weighing the remaining food every 12h (6 am and 6 pm). Subsequently, the BioDAQ episodic food intake-monitoring system (BioDAQ, Research Diets, Inc., New Brunswick, NJ, USA), an automated food intake-monitoring system, was evaluated for the use in rats (BioDAQ, Research Diets, Inc.). This system allows the continuous monitoring of solid rat chow without human interference. Rats were housed in regular single housing rat cages that were placed adjacently to each other to allow sight, odor and acoustic contact, and which provided bedding material and environmental enrichment. Additionally, a low spill food hopper which was placed on an electronic balance was mounted on the cages. The food intake assessment system weighs the filled hopper every second (±0.01g). Time periods -during which changes in weight are detected- are registered as "eating". Thereby, every interaction with the hopper is recorded in detail (start, duration, amount of the food ingested). The food intake microstructure and associated meal parameters (including meal size and duration, time spent in meals and rate of ingestion) for any period of interest can be extracted from the software (BioDAQ Monitoring Software 2.3.07) afterwards.

Statistical analysis

Data were expressed as mean ± SEM. Distribution of the data was determined by the Kolmogorov-Smirnov test and the data were further analyzed by the t-test or one-way ANOVA followed by the Tukey *post hoc* test, or by two-way ANOVA followed by the Holm-Sidak method. Differences were considered significant when $p<0.05$ (SigmaStat 3.1., Systat Software, San Jose, CA, USA).

4.2. Publication: "Activity-based anorexia reduces body weight without inducing a separate food intake microstructure or activity phenotype in female rats – mediation via an activation of distinct brain nuclei"

Animals and groups

Female Sprague-Dawley rats weighing 150-180g upon arrival were used. After one week of acclimatization during which the rats were housed in groups, they were randomly assigned to

one of four groups: a) control group with *ad libitum* food (AL), b) control group with *ad libitum* food and access to a running wheel (activity, AC), c) control group with restricted feeding regimen (RF) and d) activity-based anorexia group with restricted feeding regimen and activity (ABA).

Activity-based anorexia (assessed parameters)

According to their group, animals were either housed in standard single housing cages or cages with or without a running wheel, all equipped with the automated food intake-monitoring system (for a description see publication 1). Wheel rotations were assessed electronically (Campden Instruments Ltd., Loughborough, UK). After one week of habituation to the running wheel (ABA and AC group) and to the daily interaction with the investigator for body weight measurement (8 am - 9 am), food restriction conditions started and ABA and RF animals solely had access to food from 9 to 10:30 am. Body weight, food intake and the underlying microstructure were assessed over a period of two weeks. If body weight loss exceeded -25% of the initial body weight, animals were removed from the experiment. Statistical analysis was performed as described above.

c-Fos immunohistochemistry

During the last feeding period, food restricted animals (ABA and RF) received only a fixed amount of 1.5g in order to avoid brain signals derived from great distention of the stomach or nausea. Afterwards, the rats were deeply anesthetized and after thoracotomy, they were transcardially perfused by a 1-min flush of sodium chloride (0.9%) followed by 500ml of fixative (4% paraformaldehyde and 14% saturated picric acid in 0.1M phosphate buffer). Then, brains were removed and further processed for c-Fos immunohistochemistry (postfixation and cryoprotection with 10% sucrose, then, snap-frozen). Lastly, the whole brain was cut into coronal 25µm sections using a cryostat. Every third section was used for immunohistochemistry applying the free-floating technique and every step of the protocol was followed by a 3 x 15min washing step with phosphate-buffered saline (PBS). The protocol started with treatment of the sections with 0.3% H_2O_2, followed by 2% normal goat serum. Next, the sections were incubated with the primary antibody anti-c-Fos (1:20.000, ABE457, Merck Millipore, Darmstadt, Germany) overnight at 4°C. On the second day, sections were incubated with biotinylated secondary goat anti-rabbit IgG (1:1000, 111-065-144, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2h, followed by avidin-biotin-peroxidase complex (ABC, 1:500, Vector Laboratories, Burlingame, CA, USA) in 0.3% Triton-PBS for 1h. Staining was visualized with diaminobenzidine tetrachloride (DAB) and nickel ammonium sulfate. Immunoreactivity of the sections was examined under a light microscope and the density was

described semi-quantitatively as – no; +, low (~ 1-10); ++, medium (~10-20) and +++, high (>20 c-Fos positive cells in a 100 x 100µm area of an ocular grid with a 10x objective). Brain nuclei were identified according to the rat brain atlas by Paxinos and Watson (31). ABA animals were compared to controls (AL, n=3/group) in a descriptive manner.

4.3. Publication: "Activity-based anorexia activates nesfatin-1 immunoreactive neurons in distinct brain nuclei of female rats"

c-Fos and Nesfatin-1 immunohistochemistry

As mentioned above, animals were exposed to the ABA regimen or one of three control groups. Rat brains from all four groups were processed for immunohistochemistry (n=6/group; n=24 in total). After performing the two-day c-Fos immunohistochemical protocol as described above, sections were incubated with the second primary antibody anti-nesfatin-1 (1:20.000, H-003-22, Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) overnight at 4°C. On the third day of the protocol all steps were conducted as described above; visualization was performed using DAB only. Statistical analysis followed the steps described above.

5. RESULTS

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

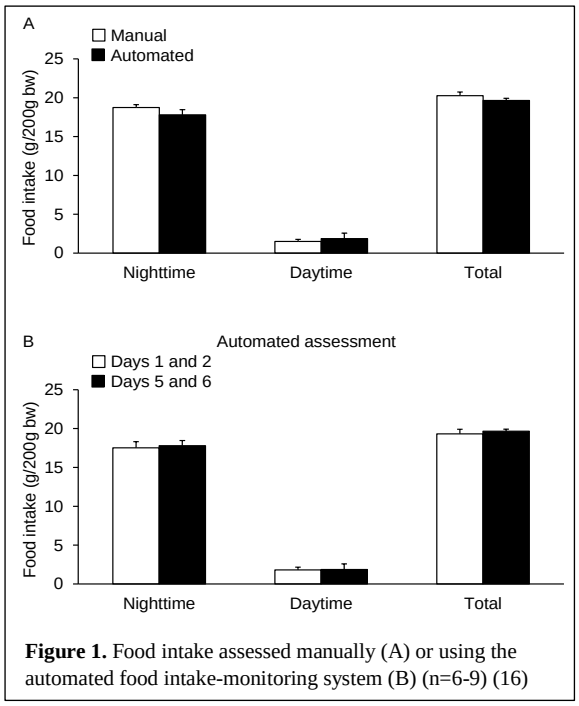

Figure 1. Food intake assessed manually (A) or using the automated food intake-monitoring system (B) (n=6-9) (16)

The BioDAQ episodic food intake-monitoring system allows for the automated assessment of the physiological food intake microstructure in undisturbed rats

Rats quickly adapted to eating from the food hopper of the food intake-monitoring system and did not show any differences in food intake compared to the standard feeding method where the food was placed on top of the cage (Fig.1). A physiological behavioral satiety sequence was detected under these conditions (Fig.2).

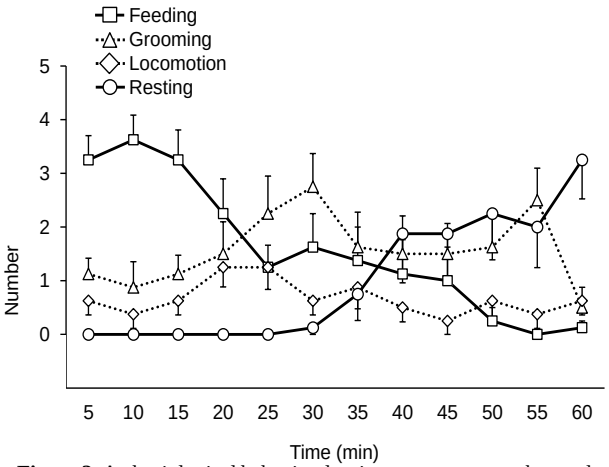

Figure 2. A physiological behavioral satiety sequence was observed with a decrease of dark phase feeding behavior and an increase in grooming, locomotion and particularly resting. The rats were single-housed in the automated feeding monitoring system cages. Each line represents the mean ± SEM of 8 rats/group. (16)

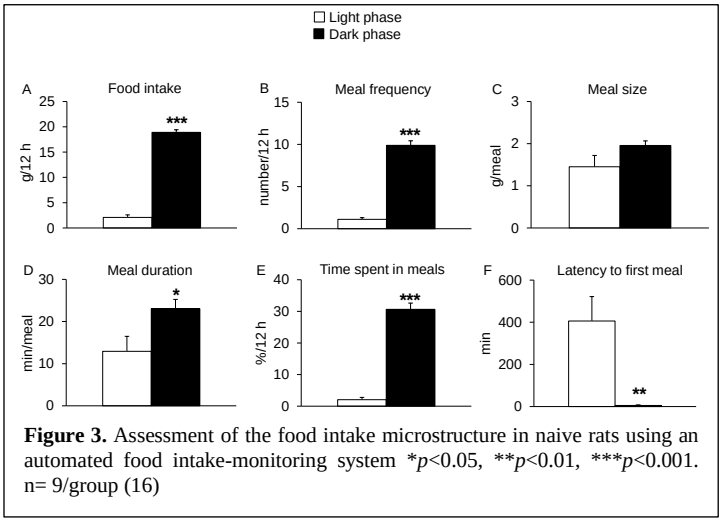

Figure 3. Assessment of the food intake microstructure in naive rats using an automated food intake-monitoring system *p<0.05, **p<0.01, ***p<0.001. n= 9/group (16)

An analysis of the food intake microstructure shows greater food intake at night due to a higher meal frequency and a longer duration of meals during the dark phase compared to the light phase (Fig.3).

5.2. Publication: "Activity-based anorexia reduces body weight without inducing a separate food intake microstructure or activity phenotype in female rats – mediation via an activation of distinct brain nuclei"

ABA rats show the greatest body weight loss

ABA rats showed a significantly higher reduction in body weight of -22% (p<0.001) compared to the RF group, which reached a body weight loss of -13%. Thus, the ABA group exhibited an additional body weight loss of -9% in comparison to the RF group (p<0.001), and compared to the AC and AL groups, which both gained body weight (+10% and +13% respectively; p<0.001; Fig.4).

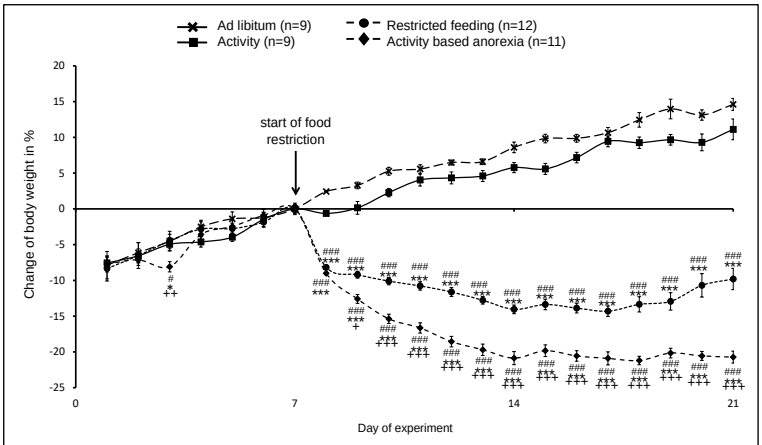

Figure 4. ABA reduces body weight more prominently than RF. +p<0.05, ++p<0.01, +++p<0.001 *vs*. RF; *p<0.05, ***p<0.001 *vs*. AL; #p<0.05, ### p<0.001 *vs*. AC. n= 9-12/group (17)

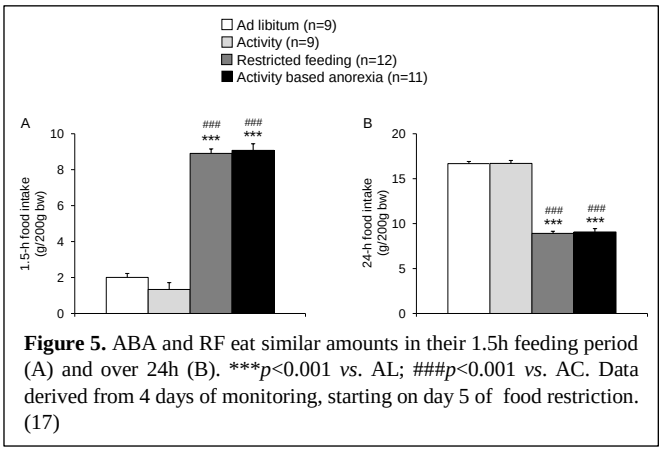

Figure 5. ABA and RF eat similar amounts in their 1.5h feeding period (A) and over 24h (B). ***p<0.001 *vs*. AL; ###p<0.001 *vs*. AC. Data derived from 4 days of monitoring, starting on day 5 of food restriction. (17)

ABA and RF rats show a similar reduction in food intake

No significant differences in the reduction of food intake were observed between ABA and RF rats (p>0.05). Significant differences in food intake were detected between the two food restricted (ABA and RF) and the two *ad libitum* fed (AC and AL) groups (Fig.5).

ABA does not induce a distinct feeding or activity phenotype

Analysis of the food intake microstructure indicated similar changes in ABA and RF rats in 24h (data not shown) and in the 1.5h feeding period compared to the two control groups (AL, AC, Fig.6).

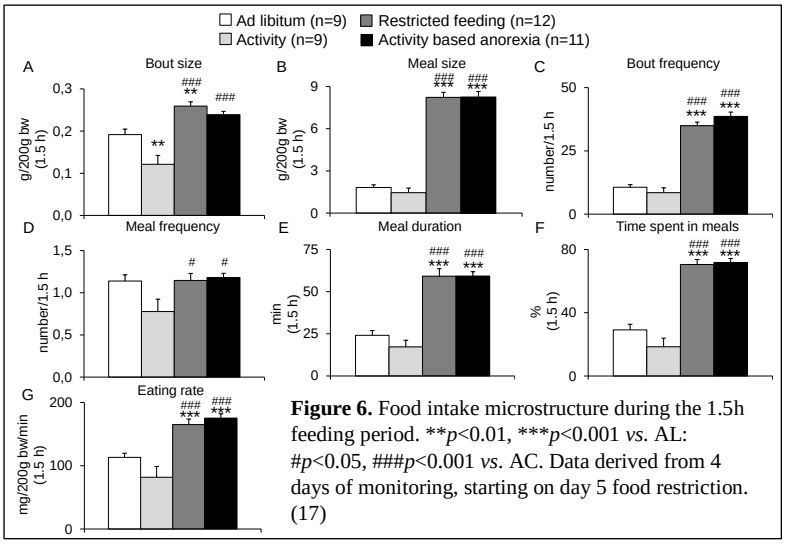

Figure 6. Food intake microstructure during the 1.5h feeding period. **p<0.01, ***p<0.001 *vs*. AL: #p<0.05, ###p<0.001 *vs*. AC. Data derived from 4 days of monitoring, starting on day 5 food restriction. (17)

Similarly, no differences were observed between the number of daily running wheel rotations of ABA and AC rats (p>0.05, data not shown).

ABA leads to an activation of distinct brain nuclei

In a further step, brain activity of ABA rats as opposed to AL rats was investigated using c-Fos immunohistochemistry. ABA rats showed higher c-Fos activation levels in several different brain nuclei compared to AL rats (Table 1).

Table 1. Neuronal activation in distinct brain nuclei of ABA compared to AL rats.

Area	**Brain structure**	**Ad libitum**	**Activity based anorexia**
Forebrain	Piriform cortex	++	+++
	Cingulate cortex	+	++
	Somatomotor cortex	+	+++
	Lateral septal nucleus	+ - ++	+++
	Caudate putamen	++	++ - +++
	Amygdala (central, medial and basolateral)	-	-
	Hippocampus	+ - ++	+++
Thalamus	Paraventricular thalamic nucleus, anterior part	+	++
	Lateral habenula	+ - ++	+ - ++
Hypothalamus	Suprachiasmatic nucleus	++	++ - +++
	Supraoptic nucleus	-	+++
	Anterior hypothalamic area	+	++
	Paraventricular nucleus, magnocellular part	+	++
	Lateral hypothalamic area	++	+++
	Ventromedial hypothalamic nucleus	++	++
	Dorsomedial hypothalamic nucleus	-	++
	Arcuate nucleus	+	+++
Midbrain	Edinger-Westphal nucleus	++	++
	Dorsal raphe nuclei	+	++
	Locus coeruleus	-	+
Medulla	Raphe pallidus	+	++
	Area postrema	-	+ - ++
	Nucleus of the solitary tract	-	+ - ++
	Dorsal motor nucleus of the vagus nerve	-	- - +

– no; +, low (~1-10); ++, medium (~10-20); +++, high (>20 c-Fos positive cells in a 100 x 100µm area of an ocular grid with a 10x objective) n=3/group (17)

5.3. Publication: "Activity-based anorexia activates nesfatin-1 immunoreactive neurons in distinct brain nuclei in female rats"

ABA induces an increase of nesfatin -1 immunoreactive neurons

ABA significantly increased the number of nesfatin-1 immunoreactive cells in the paraventricular nucleus (PVN), dorsomedial hypothalamic nucleus (DMH), arcuate nucleus (Arc), and locus coeruleus (LC) in comparison to AL and AC animals, and in the nucleus of the solitary tract (NTS) in comparison to AL, AC and RF animals (Table 2)

Table 2: ABA leads to an increase in nesfatin-1, c-Fos and double-labeled cells in distinct brain nuclei.

	Nesfatin -1				**c-Fos**				**c-Fos + Nesfatin -1**			
Brain structure	AL	AC	RF	ABA	AL	AC	RF	ABA	AL	AC	RF	ABA
SON	102.97 ± 11.96	90.08 ± 6.29	114.55 ± 6.65	109.19 ± 5.97	0.04 ± 0.04	0.08 ± 0.08	1.32 ± 0.56	**11.76 ± 4.71 ** # +**	0.04 ± 0.04	0.08 ± 0.08	1.30 ± 0.57	**11.75 ± 4.70 * # +**
PVN	74.10 ± 14.94	65.28 ± 9.20	106.60 ± 7.24	**137.74 ± 7.57 ** ###**	1.75 ± 0.99	1.21 ± 0.46	4.37 ± 1.51	**7.83 ± 1.75 * ##**	0.55 ± 0.34	0.69 ± 0.25	2.79 ± 0.97	**4.00 ± 0.76 ** ##**
DMH	18.25 ± 2.02	15.58 ± 1.36	**30.05 ± 3.34 #**	**41.90 ± 5.94 ** ###**	2.48 ± 1.24	0.97 ± 0.62	10.67 ± 2.42	**15.36 ± 4.11 ** ##**	0.62 ± 0.36	0.53 ± 0.32	2.39 ± 0.63	**6.21 ± 1.90 ** ##**
Arc	35.24 ± 4.89	26.61 ± 6.19	48.71 ± 6.43	**66.36 ± 11.42 * #**	0.00 ± 0.00	0.01 ± 0.01	13.04 ± 3.66	**26.76 ± 8.63 ** ##**	0.00 ± 0.00	0.01 ± 0.01	9.07 ± 4.27	**21.79 ± 9.19 * #**
EW	21.14 ± 2.95	19.60 ± 3.20	17.97 ± 0.88	19.70 ± 3.00	1.33 ± 0.62	0.70 ± 0.25	**7.14 ± 1.94 * ##**	3.84 ± 0.92	0.62 ± 0.34	0.38 ± 0.21	1.82 ± 0.65	2.21 ± 0.76
DR	73.36 ± 17.83	63.59 ± 8.90	5.65 ± 10.03	70.79 ± 10.72	3.03 ± 1.32	3.06 ± 1.64	15.02 ± 6.43	**20.19 ± 4.65 * ##**	2.63 ± 1.12	2.37 ± 1.07	10.72 ± 4.17	**13.49 ± 2.11 * #**
LC	80.75 ± 7.93	79.18 ± 4.75	102.24 ± 7.71	**117.24 ± 5.03 * #**	1.05 ± 0.55	1.11 ± 0.43	0.59 ± 0.21	1.35 ± 0.52	1.05 ± 0.55	1.11 ± 0.43	0.59 ± 0.21	1.35 ± 0.52
Rpa	20.73 ± 3.05	15.57 ± 3.03	18.03 ± 3.41	19.29 ± 2.43	1.13 ± 0.19	1.50 ± 0.65	4.78 ± 2.38	6.69 ± 1.90	0.97 ± 0.18	0.68 ± 0.18	3.30 ± 1.54	**5.80 ± 1.88 * #**
NTS	22.34 ± 2.09	19.23 ± 3.63 *	24.73 ± 1.61	**31.20 ± 2.97 #**	0.00 ± 0.00	0.00 ± 0.00	0.19 ± 0.14	0.63 ± 0.32	0.00 ± 0.00	0.00 ± 0.00	0.19 ± 0.14	0.63 ± 0.32

*p<0,05, **p<0,01, ***p<0,001 *vs*. AL; #p<0,05, ##p<0,01, ###p<0,001 *vs*. AC; +p<0,05 *vs*. RF. n=6/group (30)

ABA leads to an increase in nesfatin-1/c-Fos double-labeled neurons

Moreover, ABA led to a significant increase in nesfatin-1 and c-Fos double-labeled cells in the supraoptic nucleus (SON) (Fig.7) compared to all three other rat groups (p<0.05), and in the PVN, DMH, Arc, dorsal raphe nuclei (DR) and raphe pallidus nucleus (Rpa) in comparison to AL and AC rats (p<0.05) (Table 2).

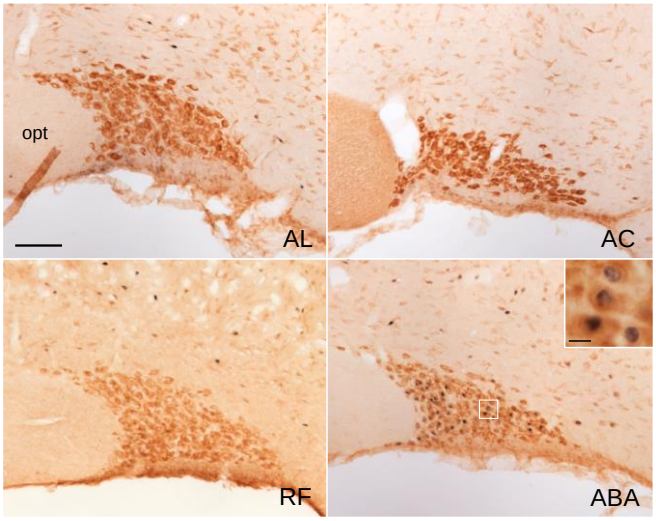

Figure 7. ABA induces an increase in nesfatin-1/c-Fos double-labeled and the number of c-Fos positive neurons in the supraoptic nucleus (SON). Representative microphotographs. Scale bar 100µm, in insert 10µm. opt, optic tract. n=6/group (30)

6. DISCUSSION

In the first publication, an automated food intake-monitoring system was established for the use in experiments with rats. In the next step, the system was used to examine the food intake pattern of rats under conditions of ABA. In the third study, neuronal changes induced by the ABA model were assessed using c-Fos and nesfatin-1 immunohistochemistry.

Compared to conventional manual assessment of food intake, an automated analysis of food intake has two main advantages: Firstly, the system allows a notably more detailed analysis of the food intake microstructure and secondly, the rats are not disturbed by the investigator. Parameters of the food intake microstructure encompass the latency period of the first meal, meal size, meal duration, eating rate, inter-meal intervals and the satiety ratio, information largely lacking in conventional manual assessment of food intake. Using these parameters, two main features of food intake-modulating conditions/hormones can be defined: satiation (mechanisms causing meal termination (32)) and satiety (mechanisms causing a later onset of the next meal after one meal is completed (33)). The first publication showed that rats quickly adapt to the food intake-monitoring system and display a similar overall food intake compared to rats assessed under conventional/manual conditions. Moreover, rats housed in the automated food intake-monitoring system displayed a physiological behavioral satiety sequence, a well-established physiological postprandial behavior in rats (34,35).

Furthermore, the monitoring system was utilized to investigate the food intake patterns observable in rats under the condition of ABA. The model ABA in rats combines a time-restricted access to food with voluntary physical activity in a running wheel and therefore mimics conditions also relevant in human AN. Analysis of body weight showed that all four experimental groups displayed a linear body weight gain during the first week of acclimatization. The two *ad libitum* fed animal groups (AL and AC) continued to constantly gain weight until the end of the experiment indicating a physiological growth under conditions of housing in the food intake-monitoring cages. After two weeks, AL rats reached a body weight gain of +13%, while AC rats gained +10% compared to the first day. In the two food restricted animal groups (RF and ABA), a body weight loss was observed starting on the second day of limited access to food. Both food restricted groups continued to lose weight for about one week followed by a stabilization period. After two weeks ABA rats showed a body weight loss of -22%, whereas rats of the RF group lost -13%. ABA and RF rats had access to food during the early light phase, a period where rats usually do not eat (36), a finding also shown in the present study in the AL and AC groups. Although RF and ABA rats increased their 1.5h food intake

from days one to ten, they did not reach the levels consumed by the other groups within 24h and therefore showed a food intake reduction by -38% and -41% respectively, data consistent with previous studies (12,37,38).

A detailed analysis of the 1.5h food intake microstructure indicated an increase in the eating rate and meal size of ABA and RF rats -both food restricted groups- in comparison to the *ad libitum* fed groups, AL and AC. The meal duration and time spent eating meals was also significantly increased, indicating that the animals adapt to the restricted feeding schedule by eating a large amount of food during a short period of time. However, it should be noted that there were no significant differences between the two food restricted or the two *ad libitum* fed groups, respectively, in terms of food intake microstructure, arguing against a distinct food intake phenotype induced under conditions of ABA.

Behavioral changes have been described in ABA rats with a considerable shift in running wheel activity from the dark phase, which is the physiological activity phase in rats, to the four to five hours before the feeding period and have been termed food-anticipatory activity (FAA) (39,40). This shift of circadian rhythm might be associated with the elevated c-Fos signal displayed in the DMH and the suprachiasmatic nucleus (Sch) -both being involved in regulation of circadian rhythm (41,42)- of ABA rats. Despite the fact that ABA rats showed robust physical activity during the light phase, similar to the food intake pattern no distinct activity phenotype was observed when compared to AC.

Based on the food intake and running activity data it can be suggested that the combination of the food restriction regimen with the possibility of exercising in a running wheel is necessary in order to induce the additional body weight loss of ABA in comparison to RF. However, the food restriction seems to exert the predominant influence on body weight compared to the running wheel since the AC group did not show a decrease in body weight.

The present data confirmed that ABA mimics three essential symptoms of AN, namely body weight loss, caloric restriction and activity and therefore might be a suited model to study pathophysiological alterations also occurring in AN. Nevertheless, several important limitations have to be taken into consideration. Firstly, rats do not voluntarily lose body weight, and when they are given more/longer access to food, they regain their body weight (37,43). Secondly, rats lose body weight very rapidly with an average loss of body weight of -22% within two weeks. Therefore, ABA represents a subacute rather than a chronic model and it does not reflect the chronic character of the eating disorder AN (44). Thirdly, it is to note that some established factors contributing to the development of human AN (e.g. genetic susceptibility (45) and

psychosocial aspects (9)) are not mimicked by this model. Lastly, analysis of the food intake microstructure indicated that ABA rats display an eating behavior with a greatly increased meal size, meal duration and eating rate also occurring in anorexia patients of the binge-purging type (1), while the restrictive subtype of AN seems to be less reflected in this model.

Despite these important limitations, ABA is considered the most established and -so far- best animal model for human AN (13). Although it does not mimic all aspects of AN, it seems to be suited to further characterize the hitherto poorly understood pathophysiology of the disease. However, it is important to note that data derived from this model have to be interpreted with caution and cannot be directly transferred to humans.

As a further step, we focused on neuronal changes that could potentially underlie the behavioral changes observed under conditions of ABA. c-Fos immunohistochemical analyses of brain sections showed that ABA rats display a greater level of activation in neurons of distinct brain nuclei distributed throughout the whole brain. In comparison to AL rats ABA rats showed a stronger activation of brain nuclei involved in the mediation of food intake, (e.g. in the lateral septal nucleus (LS) (46), and lateral hypothalamic area (LHA) (47)) and in areas involved in olfaction such as the piriform cortex (48) which probably reflects the drive to eat and the occupation with food. In ABA rats, there was also increased activity in areas involved in the mediation of physical activity, namely the somatomotor cortex (49). Moreover, ABA rats displayed higher neuronal activity in areas involved in thermoregulation (DMH) (50), circadian rhythms (namely Sch and DMH (42)), emotional processing (areas with mainly oxytocinergic and serotonergic neurons) (51–54), sleep (50) and stress (areas with CRF-containing noradrenergic neurons) (55), which may play a role in the pathophysiology and symptomatology of AN.

Based on the results of this mapping experiment, nine distinct brain nuclei were selected for a detailed analysis of all four animal groups and phenotyping using c-Fos/nesfatin-1 double labeling immunohistochemistry. ABA induced an increase of nesfatin-1 immunoreactive cells in five of the nine analyzed nuclei. Nesfatin-1 expression was increased in nuclei involved in food intake regulation, stress response and emotional processing, which is important to note given that depression and anxiety are common comorbidities in AN (5,6). In line with the results of this study, nesfatin-1 was previously shown to play a role in the stress response (56), anxiety (56–58) and depression (59) in humans. Interestingly, significant differences were mainly observable between ABA and the two *ad libitum* fed animal groups (AC and AL rats). One may speculate that central nesfatin-1 immunoreactivity is inversely correlated with body weight,

whereas NUCB2/nesfatin-1 plasma levels were shown to correlate positively with body weight in humans (29). We detected elevated levels of immunoreactive nesfatin-1 neurons in many brain areas involved in the regulation of food intake, namely the SON, PVN, Arc and DMH, which is especially interesting because the anorexigenic nesfatin-1 was shown to play an already well-established function in homeostatic feeding (25,26). Nesfatin-1 is also expressed in reward-related areas and, therefore, one may speculate that nesfatin-1 might play a role in hedonic feeding, as well (60,61). Further analyses of the effects of nesfatin-1 on food intake under reward inducing conditions would prove helpful to address this hypothesis.

The increase of nesfatin-1 immunoreactive neurons was more prominent in ABA rats than in RF rats but reached significance only in one analyzed nucleus (namely the SON), pointing towards restricted feeding as the main contributing effect. The SON consists of neurons prominently expressing oxytocin, a hormone well known for its effects in the mediation of social memory and cognition, aggression and anxiety (51). Interestingly, besides the findings that AN patients display lower oxytocin plasma levels (62) and an association of AN with polymorphisms of the oxytocin receptor (63), further data give rise to the speculation that altered oxytocin signaling is involved in the development and/or maintenance of AN symptoms (64,65). Whether the observed increase of double-labeled c-Fos/nesfatin-1 immunoreactive neurons in the SON leads to altered oxytocin signaling warrants further investigation.

With regards to the motivation to exercise in a running wheel despite the caloric deficit one might assume that the reward system and associated dopamine signaling (66) play an important role. It has been shown that a non-selective dopamine antagonist, cis-flupenthixol, inhibits anorectic behavior in ABA (20). Furthermore, the antipsychotic drug olanzapine increases survival in ABA mice, while the anti-depressive selective serotonin reuptake inhibitor (SSRI), fluoxetine had no effect (67,68). Olanzapine exerts its effect not only by a blockade of the dopaminergic D2 and D4 receptors, but also influences other receptors such as the 5HT-2A receptor (69). The effect of olanzapine is associated with a decrease in hyperactivity in ABA rats, which probably contributes to the prolonged survival (70). In line with the data of the ABA model, not only fluoxetine and other SSRIs are commonly prescribed to AN patients, probably treating mainly the comorbid depression (71,72), but also olanzapine has shown to display beneficial effects on AN patients (73–75). However, it is not yet defined which subgroups of AN patients benefit the most of an olanzapine treatment (76).

The nucleus accumbens (Nacc) is one of the major brain areas involved in reward and reward-motivated behavior (77,78). Analysis of the Nacc in ABA rats shows that dopamine

release was increased during feeding, however, during food-anticipatory behavior neither dopamine nor serotonin release were elevated (21). Selective activation of the mesolimbic reward pathway firing from the ventral tegmental area (VTA) to the Nacc, mainly consisting of dopaminergic neurons (66), drastically increased survival of ABA rats associated with increased daily food intake (79). Moreover, an activation of the VTA-Nacc projections helped reverse ABA in rats that already fully developed the ABA phenotype (79). In the present experiment, an elevated neuronal activation in the striatum of ABA compared to AL rats was detected, as assessed by c-Fos immunohistochemistry. Whether a blockade of this pathway would exert beneficial effects on the ABA phenotype will have to be further investigated.

Considering the results of neuroimaging studies in patients suffering from AN, it should be noted that altered central reward processing seems to be involved in the development and the maintenance of the eating disorder (80–82). Moreover, a decreased endogenous dopamine concentration in the striatum was found in AN patients compared to healthy controls (83), indicating that a disrupted dopaminergic signaling might play an important role. In line with that assumption, several dopamine receptor polymorphisms affecting the dopamine receptor D2 transcription efficiency, such as 141 C/- insertion/deletion (-141 Indel), are more frequent in patients suffering from AN (84).

In summary, the three studies presented show that the automated food intake-monitoring system previously established in mice is also suited for the use in rats. It is a necessary tool in order to study food intake of rats in more detail and continuously without disturbance. Using this system, the food intake microstructure underlying the reduced food intake in rats developing ABA has been investigated. Although ABA seems to be a suited model to study pathophysiological alterations possibly also occurring in human AN, this model does not show a distinct food intake or activity phenotype. A whole brain mapping indicated that ABA rats showed higher activation of several distinct brain nuclei involved in the modulation of food intake, thermogenesis, stress and emotional processing. ABA also induces a robust activation of nesfatin-1 immunoreactive neurons giving rise to a possible role of nesfatin-1 in the development or maintenance of ABA.

7. REFERENCES

1. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5). Fifth Edition. United States; 2013. 947 p.
2. Davis C, Katzman DK, Kaptein S, Kirsh C, Brewer H, Kalmbach K, et al. The prevalence of high-level exercise in the eating disorders: etiological implications. Compr Psychiatry. 1997; 38:321–6.
3. Treasure J, Zipfel S, Micali N, Wade T, Stice E, Claudino A, et al. Anorexia nervosa. Nat Rev Dis Primer. 2015; 1:15074.
4. Preti A, Girolamo G de, Vilagut G, Alonso J, Graaf R de, Bruffaerts R, et al. The epidemiology of eating disorders in six european countries: results of the ESEMeD-WMH project. J Psychiatr Res. 2009; 43:1125–32.
5. Keski-Rahkonen A, Mustelin L. Epidemiology of eating disorders in europe: prevalence, incidence, comorbidity, course, consequences, and risk factors. Curr Opin Psychiatry. 2016; 29:340–5.
6. Miniati M, Benvenuti A, Bologna E, Maglio A, Cotugno B, Massimetti G, et al. Mood spectrum comorbidity in patients with anorexia and bulimia nervosa. Eat Weight Disord EWD. 2016 Oct 20;
7. Erdur L, Kallenbach-Dermutz B, Lehmann V, Zimmermann-Viehoff F, Köpp W, Weber C, et al. Somatic comorbidity in anorexia nervosa: first results of a 21-year follow-up study on female inpatients. Biopsychosoc Med. 2012; 6:4.
8. Westmoreland P, Krantz MJ, Mehler PS. Medical complications of anorexia nervosa and bulimia. Am J Med. 2016; 129:30–7.
9. Zipfel S, Giel KE, Bulik CM, Hay P, Schmidt U. Anorexia nervosa: aetiology, assessment, and treatment. Lancet Psychiatry. 2015; 2:1099–111.
10. Méquinion M, Chauveau C, Viltart O. The use of animal models to decipher physiological and neurobiological alterations of anorexia nervosa patients. Front Endocrinol. 2015; 6:68.
11. Routtenberg A, Kuznesof AW. Self-starvation of rats living in activity wheels on a restricted feeding schedule. J Comp Physiol Psychol. 1967; 64:414–21.
12. Carrera O, Fraga Á, Pellón R, Gutiérrez E. Rodent model of activity-based anorexia. Curr Protoc Neurosci. 2014; 67:9.47.1-11.
13. Gutierrez E. A rat in the labyrinth of anorexia nervosa: contributions of the activity-based anorexia rodent model to the understanding of anorexia nervosa. Int J Eat Disord. 2013; 46:289–301.
14. Casper RC, Sullivan EL, Tecott L. Relevance of animal models to human eating disorders and obesity. Psychopharmacology. 2008; 199:313–29.
15. Stengel A, Goebel M, Wang L, Rivier J, Kobelt P, Mönnikes H, et al. Activation of brain somatostatin 2 receptors stimulates feeding in mice: analysis of food intake microstructure. Physiol Behav. 2010; 101:614–22.
16. Teuffel P, Wang L, Prinz P, Goebel-Stengel M, Scharner S, Kobelt P, et al. Treatment with the ghrelin-O-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats. J Physiol Pharmacol. 2015; 66:493–503.
17. Scharner S, Prinz P, Goebel-Stengel M, Kobelt P, Hofmann T, Rose M, et al. Activity-based anorexia reduces body weight without inducing a separate food intake microstructure or activity phenotype in female rats-mediation via an activation of distinct brain nuclei. Front Neurosci. 2016; 10:475.
18. Sagar SM, Sharp FR, Curran T. Expression of c-fos protein in brain: metabolic mapping at the cellular level. Science. 1988; 240:1328–31.
19. Burden VR, White BD, Dean RG, Martin RJ. Activity of the hypothalamic-pituitary-adrenal axis is elevated in rats with activity-based anorexia. J Nutr. 1993; 123:1217–25.
20. Verhagen LAW, Luijendijk MCM, Hillebrand JJG, Adan RAH. Dopamine antagonism inhibits anorectic behavior in an animal model for anorexia nervosa. Eur Neuropsychopharmacol. 2009; 19:153–60.
21. Verhagen LAW, Luijendijk MCM, Korte-Bouws GAH, Korte SM, Adan RAH. Dopamine and serotonin release in the nucleus accumbens during starvation-induced hyperactivity. Eur Neuropsychopharmacol. 2009; 19:309–16.
22. Casteels C, Gérard N, van Kuyck K, Pottel L, Nuttin B, Bormans G, et al. Small animal PET imaging of the type 1 cannabinoid receptor in a rodent model for anorexia nervosa. Eur J Nucl Med Mol Imaging. 2014; 41:308–21.
23. Legrand R, Lucas N, Breton J, Azhar S, do Rego J-C, Déchelotte P, et al. Ghrelin treatment prevents development of activity based anorexia in mice. Eur Neuropsychopharmacol. 2016; 26:948–58.
24. Hillebrand JJG, Koeners MP, de Rijke CE, Kas MJH, Adan RAH. Leptin treatment in activity-based anorexia. Biol Psychiatry. 2005; 58:165–71.
25. Oh-I S, Shimizu H, Satoh T, Okada S, Adachi S, Inoue K, et al. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. Nature. 2006; 443:709–12.

26. Stengel A, Goebel M, Wang L, Rivier J, Kobelt P, Mönnikes H, et al. Central nesfatin-1 reduces dark-phase food intake and gastric emptying in rats: differential role of corticotropin-releasing factor2 receptor. Endocrinology. 2009; 150:4911–9.
27. Gotoh K, Masaki T, Chiba S, Ando H, Shimasaki T, Mitsutomi K, et al. Nesfatin-1, corticotropin-releasing hormone, thyrotropin-releasing hormone, and neuronal histamine interact in the hypothalamus to regulate feeding behavior. J Neurochem. 2013; 124:90–9.
28. Price CJ, Hoyda TD, Samson WK, Ferguson AV. Nesfatin-1 influences the excitability of paraventricular nucleus neurones. J Neuroendocrinol. 2008; 20:245–50.
29. Ogiso K, Asakawa A, Amitani H, Nakahara T, Ushikai M, Haruta I, et al. Plasma nesfatin-1 concentrations in restricting-type anorexia nervosa. Peptides. 2011; 32:150–3.
30. Scharner S, Prinz P, Goebel-Stengel M, Lommel R, Kobelt P, Hofmann T, et al. Activity-based anorexia activates nesfatin-1 immunoreactive neurons in distinct brain nuclei of female rats. Brain Res. 2017; Epub Sept 24.
31. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 6th ed. Academic Press/Elsevier. 2007; 1 p.
32. Strubbe JH, Woods SC. The timing of meals. Psychol Rev. 2004; 111:128–41.
33. Fekete EM, Inoue K, Zhao Y, Rivier JE, Vale WW, Szücs A, et al. Delayed satiety-like actions and altered feeding microstructure by a selective type 2 corticotropin-releasing factor agonist in rats: intra-hypothalamic urocortin 3 administration reduces food intake by prolonging the post-meal interval. Neuropsychopharmacol. 2007; 32:1052–68.
34. Antin J, Gibbs J, Holt J, Young RC, Smith GP. Cholecystokinin elicits the complete behavioral sequence of satiety in rats. J Comp Physiol Psychol. 1975; 89:784–90.
35. Smith GP, Gibbs J. Postprandial satiety. Prog Psychobiol Physiol Psychol. 1979; 179–242.
36. Rosenwasser AM, Boulos Z, Terman M. Circadian organization of food intake and meal patterns in the rat. Physiol Behav. 1981; 27:33–9.
37. Dixon DP, Ackert AM, Eckel LA. Development of, and recovery from, activity-based anorexia in female rats. Physiol Behav. 2003; 80:273–9.
38. Barbarich-Marsteller NC, Underwood MD, Foltin RW, Myers MM, Walsh BT, Barrett JS, et al. Identifying novel phenotypes of vulnerability and resistance to activity-based anorexia in adolescent female rats. Int J Eat Disord. 2013; 46:737–46.
39. Wu H, van Kuyck K, Tambuyzer T, Luyten L, Aerts J-M, Nuttin B. Rethinking food anticipatory activity in the activity-based anorexia rat model. Sci Rep. 2014; 4:3929.
40. Adan R a. H, Hillebrand JJG, Danner UN, Cardona Cano S, Kas MJH, Verhagen L a. W. Neurobiology driving hyperactivity in activity-based anorexia. Curr Top Behav Neurosci. 2011; 6:229–50.
41. Verhagen LAW, Luijendijk MCM, de Groot J-W, van Dommelen LPG, Klimstra AG, Adan RAH, et al. Anticipation of meals during restricted feeding increases activity in the hypothalamus in rats. Eur J Neurosci. 2011; 34:1485–91.
42. Acosta-Galvan G, Yi C-X, van der Vliet J, Jhamandas JH, Panula P, Angeles-Castellanos M, et al. Interaction between hypothalamic dorsomedial nucleus and the suprachiasmatic nucleus determines intensity of food anticipatory behavior. Proc Natl Acad Sci USA. 2011; 108:5813–8.
43. Ratnovsky Y, Neuman P. The effect of pre-exposure and recovery type on activity-based anorexia in rats. Appetite. 2011; 56:567–76.
44. Steinhausen H-C. The outcome of anorexia nervosa in the 20th century. Am J Psychiatry. 2002; 159:1284–93.
45. Clarke TK, Weiss ARD, Berrettini WH. The genetics of anorexia nervosa. Clin Pharmacol Ther. 2012; 91:181–8.
46. Mitra A, Lenglos C, Timofeeva E. Inhibition in the lateral septum increases sucrose intake and decreases anorectic effects of stress. Eur J Neurosci. 2015; 41:420–33.
47. Bernardis LL, Bellinger LL. The lateral hypothalamic area revisited: neuroanatomy, body weight regulation, neuroendocrinology and metabolism. Neurosci Biobehav Rev. 1993; 17:141–93.
48. Roullet F, Liénard F, Datiche F, Cattarelli M. Fos protein expression in olfactory-related brain areas after learning and after reactivation of a slowly acquired olfactory discrimination task in the rat. Learn Mem Cold Spring Harb N. 2005; 12:307–17.
49. Elias CF, Sita LV, Zambon BK, Oliveira ER, Vasconcelos L a. P, Bittencourt JC. Melanin-concentrating hormone projections to areas involved in somatomotor responses. J Chem Neuroanat. 2008; 35:188–201.
50. Gooley JJ, Schomer A, Saper CB. The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms. Nat Neurosci. 2006; 9:398–407.
51. Caldwell HK, Aulino EA, Freeman AR, Miller TV, Witchey SK. Oxytocin and behavior: lessons from knockout mice. Dev Neurobiol. 2017; 77:190–201.
52. Kirby LG, Rice KC, Valentino RJ. Effects of corticotropin-releasing factor on neuronal activity in the serotonergic dorsal raphe nucleus. Neuropsychopharmacology. 2000; 22:148–62.

53. Kataoka N, Hioki H, Kaneko T, Nakamura K. Psychological stress activates a dorsomedial hypothalamus-medullary raphe circuit driving brown adipose tissue thermogenesis and hyperthermia. Cell Metab. 2014; 20:346–58.
54. Michelsen KA, Prickaerts J, Steinbusch HWM. The dorsal raphe nucleus and serotonin: implications for neuroplasticity linked to major depression and Alzheimer's disease. Prog Brain Res. 2008; 172:233–64.
55. Aston-Jones G, Waterhouse B. Locus coeruleus: from global projection system to adaptive regulation of behavior. Brain Res. 2016; 1645:75–8.
56. Hofmann T, Stengel A, Ahnis A, Bußе P, Elbelt U, Klapp BF. NUCB2/nesfatin-1 is associated with elevated scores of anxiety in female obese patients. Psychoneuroendocrinology. 2013; 38:2502–10.
57. Hofmann T, Ahnis A, Elbelt U, Rose M, Klapp BF, Stengel A. NUCB2/nesfatin-1 is associated with elevated levels of anxiety in anorexia nervosa. PloS One. 2015; 10:e0132058.
58. Hofmann T, Elbelt U, Ahnis A, Rose M, Klapp BF, Stengel A. Sex-specific regulation of NUCB2/nesfatin-1: differential implication in anxiety in obese men and women. Psychoneuroendocrinology. 2015; 60:130–7.
59. Ari M, Ozturk OH, Bez Y, Oktar S, Erduran D. High plasma nesfatin-1 level in patients with major depressive disorder. Prog Neuropsychopharmacol Biol Psychiatry. 2011; 35:497–500.
60. Goebel-Stengel M, Wang L, Stengel A, Taché Y. Localization of nesfatin-1 neurons in the mouse brain and functional implication. Brain Res. 2011; 1396:20–34.
61. Dore R, Levata L, Lehnert H, Schulz C. Nesfatin-1: functions and physiology of a novel regulatory peptide. J Endocrinol. 2017; 232:R45–65.
62. Rutigliano G, Rocchetti M, Paloyelis Y, Gilleen J, Sardella A, Cappucciati M, et al. Peripheral oxytocin and vasopressin: Biomarkers of psychiatric disorders? A comprehensive systematic review and preliminary meta-analysis. Psychiatry Res. 2016; 241:207–20.
63. Acevedo SF, Valencia C, Lutter M, McAdams CJ. Severity of eating disorder symptoms related to oxytocin receptor polymorphisms in anorexia nervosa. Psychiatry Res. 2015; 228:641–8.
64. Afinogenova Y, Schmelkin C, Plessow F, Thomas JJ, Pulumo R, Micali N, et al. Low fasting oxytocin levels are associated with psychopathology in anorexia nervosa in partial recovery. J Clin Psychiatry. 2016; 77:e1483–90.
65. Kasperek-Zimowska BJ, Zimowski JG, Biernacka K, Kucharska-Pietura K, Rybakowski F. Impaired social cognition processes in Asperger syndrome and anorexia nervosa. In search for endophenotypes of social cognition. Psychiatr Pol. 2016; 50:533–42.
66. Björklund A, Dunnett SB. Dopamine neuron systems in the brain: an update. Trends Neurosci. 2007; 30:194–202.
67. Klenotich SJ, Seiglie MP, McMurray MS, Roitman JD, Le Grange D, Dugad P, et al. Olanzapine, but not fluoxetine, treatment increases survival in activity-based anorexia in mice. Neuropsychopharmacol. 2012; 37:1620–31.
68. Klenotich SJ, Ho EV, McMurray MS, Server CH, Dulawa SC. Dopamine D2/3 receptor antagonism reduces activity-based anorexia. Transl Psychiatry. 2015 ;5:e613.
69. McCormick PN, Kapur S, Graff-Guerrero A, Raymond R, Nobrega JN, Wilson AA. The antipsychotics olanzapine, risperidone, clozapine, and haloperidol are D2-selective ex vivo but not in vitro. Neuropsychopharmacology. 2010; 35:1826–35.
70. Hillebrand JJG, van Elburg AA, Kas MJH, van Engeland H, Adan RAH. Olanzapine reduces physical activity in rats exposed to activity-based anorexia: possible implications for treatment of anorexia nervosa? Biol Psychiatry. 2005; 58:651–7.
71. de Vos J, Houtzager L, Katsaragaki G, van de Berg E, Cuijpers P, Dekker J. Meta analysis on the efficacy of pharmacotherapy versus placebo on anorexia nervosa. J Eat Disord. 2014; 2:27.
72. Aigner M, Treasure J, Kaye W, Kasper S, WFSBP Task Force On Eating Disorders. World Federation of Societies of Biological Psychiatry (WFSBP) guidelines for the pharmacological treatment of eating disorders. World J Biol Psychiatry. 2011; 12:400–43.
73. Bissada H, Tasca GA, Barber AM, Bradwejn J. Olanzapine in the treatment of low body weight and obsessive thinking in women with anorexia nervosa: a randomized, double-blind, placebo-controlled trial. Am J Psychiatry. 2008; 165:1281–8.
74. Brambilla F, Garcia CS, Fassino S, Daga GA, Favaro A, Santonastaso P, et al. Olanzapine therapy in anorexia nervosa: psychobiological effects. Int Clin Psychopharmacol. 2007; 22:197–204.
75. Treasure J, Claudino AM, Zucker N. Eating disorders. Lancet Lond Engl. 2010; 375:583–93.
76. Lebow J, Sim LA, Erwin PJ, Murad MH. The effect of atypical antipsychotic medications in individuals with anorexia nervosa: a systematic review and meta-analysis. Int J Eat Disord. 2013; 46:332–9.
77. Der-Avakian A, Markou A. The neurobiology of anhedonia and other reward-related deficits. Trends Neurosci. 2012; 35:68–77.
78. Berridge KC, Kringelbach ML. Affective neuroscience of pleasure: reward in humans and animals. Psychopharmacology. 2008; 199:457–80.

79. Foldi CJ, Milton LK, Oldfield BJ. The role of mesolimbic reward neurocircuitry in prevention and rescue of the activity-based anorexia (ABA) phenotype in rats. Neuropsychopharmacol. 2017 Apr 19;
80. O'Hara CB, Campbell IC, Schmidt U. A reward-centred model of anorexia nervosa: a focussed narrative review of the neurological and psychophysiological literature. Neurosci Biobehav Rev. 2015; 52:131–52.
81. Frank GKW, Reynolds JR, Shott ME, Jappe L, Yang TT, Tregellas JR, et al. Anorexia nervosa and obesity are associated with opposite brain reward response. Neuropsychopharmacol. 2012; 37:2031–46.
82. Frank GKW, Shott ME, Riederer J, Pryor TL. Altered structural and effective connectivity in anorexia and bulimia nervosa in circuits that regulate energy and reward homeostasis. Transl Psychiatry. 2016; 6:e932.
83. Frank GK, Bailer UF, Henry SE, Drevets W, Meltzer CC, Price JC, et al. Increased dopamine D2/D3 receptor binding after recovery from anorexia nervosa measured by positron emission tomography and [11c]raclopride. Biol Psychiatry. 2005; 58:908–12.
84. Bergen AW, Yeager M, Welch RA, Haque K, Ganjei JK, van den Bree MBM, et al. Association of multiple DRD2 polymorphisms with anorexia nervosa. Neuropsychopharmacol. 2005; 30:1703–10.

8. Affidavit

I, Sophie Scharner, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic **"Activity-based anorexia is an animal model for anorexia nervosa in rats".** I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

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

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

Date _____ Sophie Scharner

Declaration of any eventual publications

Sophie Scharner had the following share in the following publications:

Publication 1:
Teuffel P, Wang L, Prinz P, Goebel-Stengel M, **Scharner S**, Kobelt P, Hofmann T, Rose M, Klapp BF, Reeve JR Jr, Stengel A. Treatment with the ghrelin-O-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats. J Physiol Pharmacol. 2015; 66:493-503.

Contribution in detail:
Performance of animal studies (GOAT injection, daily maintenance of animals and automated food monitoring system, measurement of food intake, behavioral measurements)

Publication 2:
Scharner S, Prinz P, Goebel-Stengel M, Kobelt P, Hofmann T, Rose M, Stengel A. Activity-based anorexia reduces body weight without inducing a separate food intake microstructure or activity phenotype in female rats – mediation via an activation of distinct brain nuclei. Front Neurosci. 2016; 25;10:475.

Contribution in detail:
Contribution to the planning of the experiments, performance of animal studies (daily maintenance of animals and automated food monitoring system, measurement of food intake, characterization of activity-based anorexia, perfusion of the animals), contribution to data analysis, immunohistochemical staining and assessment of immunopositive cells, writing of the first draft of the paper

Publication 3:
Scharner S, Prinz P, Goebel-Stengel M, Lommel R, Kobelt P, Hofmann T, Rose M, Stengel A. Activity-based anorexia activates nesfatin-1 immunoreactive neurons in distinct brain nuclei of female rats. Brain Res. 2017; Epub Sept 24.

Contribution in detail:
Contribution to the planning of the experiments, performance of animal studies (daily maintenance of animals and automated food monitoring system, measurement of food intake, perfusion of the animals), immunohistochemical staining and assessment of immunopositive cells, contribution to data analysis, writing of the first draft of the paper, finalization of the paper

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Sophie Scharner

Date

9. Print copies of the selected publications

Publication 1:
Teuffel P, Wang L, Prinz P, Goebel-Stengel M, **Scharner S**, Kobelt P, Hofmann T, Rose M, Klapp BF, Reeve JR Jr, Stengel A. Treatment with the ghrelin-O-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats. J Physiol Pharmacol. 2015; 66:493-503. (Open access)

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Original articles

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TREATMENT WITH THE GHRELIN-*O*-ACYLTRANSFERASE (GOAT) INHIBITOR GO-COA-TAT REDUCES FOOD INTAKE BY REDUCING MEAL FREQUENCY IN RATS

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The ghrelin acylating enzyme ghrelin-*O*-acyltransferase (GOAT) was recently identified and implicated in several biological functions. However, the effects on food intake warrant further investigation. While several genetic GOAT mouse models showed normal food intake, acute blockade using a GOAT inhibitor resulted in reduced food intake. The underlying food intake microstructure remains to be established. In the present study we used an automated feeding monitoring system to assess food intake and the food intake microstructure. First, we validated the basal food intake and feeding behavior in rats using the automated monitoring system. Afterwards, we assessed the food intake microstructure following intraperitoneal injection of the GOAT inhibitor, GO-CoA-Tat (32, 96 and 288 µg/kg) in freely fed male Sprague-Dawley rats. Rats showed a rapid habituation to the automated food intake monitoring system and food intake levels were similar compared to manual monitoring (P = 0.43). Rats housed under these conditions showed a physiological behavioral satiety sequence. Injection of the GOAT inhibitor resulted in a dose-dependent reduction of food intake with a maximum effect observed after 96 µg/kg (–27%, P = 0.03) compared to vehicle. This effect was delayed in onset as the first meal was not altered and lasted for a period of 2 h. Analysis of the food intake microstructure showed that the anorexigenic effect was due to a reduction of meal frequency (–15%, P = 0.04), whereas meal size (P = 0.29) was not altered compared to vehicle. In summary, pharmacological blockade of GOAT reduces dark phase food intake by an increase of satiety while satiation is not affected.

Key words: *automated food intake monitoring system, behavior, behavioral satiety sequence, food intake pattern, ghrelin,*

INTRODUCTION

Ghrelin was discovered more than a decade ago and is the endogenous ligand of the growth hormone secretagogue receptor 1a (GHS-R1a) (1), later renamed ghrelin receptor (2). Ghrelin is predominantly produced in the stomach (1, 3) and so far the only known peripherally produced and centrally acting hormone that stimulates food intake (4, 5). In addition, ghrelin is involved in several local effects directly in the stomach such as mucosal healing (6) and may also play a role in gastric carcinogenesis (7). A unique feature of ghrelin is the fatty acid residue on the third amino acid, a prerequisite for binding to the ghrelin receptor (1). The enzyme that catalyzes this acylation was unknown for a long time but identified in 2008 as member of the membrane-bound *O*-acyltransferases (MBOATs) by two independent groups and named ghrelin-*O*-acyltransferase (GOAT) (8, 9). GOAT protein was detected in ghrelin-containing cells of the rodent stomach (10) but also in the peripheral circulation of rodents (10) and humans (11). This may point towards an acylation of ghrelin outside of the stomach.

Several effects of GOAT have been reported, namely an involvement in glucose homeostasis (12), bile acid reabsorption (13) and responsiveness for salty and lipid taste (14). However, only few studies have investigated an effect of GOAT on food intake. GOAT seems to be involved in the hedonic aspect of feeding as mice lacking GOAT show a reduced hedonic feeding response compared to their wild type littermates (15). Interestingly, mice overexpressing ghrelin and GOAT showed an increase in body weight when fed a medium-chain triglyceride-enriched diet while food intake was not altered (16). Similarly, mice lacking GOAT also did not display alterations in food intake (12, 16). One study in Siberian hamsters reported that intraperitoneal (i.p.) injection of the GOAT inhibitor, GO-CoA-Tat reduced food intake, food foraging and hoarding compared to vehicle (17). These partly inconsistent findings may be due to the time course of the studies with compensatory mechanisms becoming more important over time but may also be related to the assessment of overall food intake, while a detailed analysis of the food intake microstructure is lacking.

The food intake microstructure encompasses parameters such as latency to a meal, eating rate, meal frequency, meal size, meal duration and the inter-meal interval. These parameters can be used to distinguish two major characteristics of a condition or a compound influencing food intake: satiation (mechanisms

causing meal termination) and satiety (mechanisms causing a later onset of the next meal after one meal is completed) (18, 19).

In the present study we used an automated episodic food intake monitoring device that allows for continuous monitoring of food intake and the food intake microstructure in undisturbed rats (20-22) and mice (23). Although this system has been validated for mice (24), the validation is still lacking for rats. Therefore, we first validated this system for rats under different experimental conditions. We also manually monitored the behavioral satiety sequence (a progression of behaviors following food intake in rats encompassing 'feeding' itself, 'grooming' and exploration/'locomotion' towards 'resting' (25)) to assess the occurrence of physiological behavior under these conditions. Afterwards, we investigated whether the GOAT inhibitor, GO-CoA-Tat alters food intake and the food intake microstructure in *ad libitum* fed rats during the dark phase, the photoperiod when rats show their greatest food intake (26). We also investigated whether inhibition of GOAT would affect circulating ghrelin levels and alter behavior in addition to food intake.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (Harlan-Winkelmann Co., Borchen, Germany and Harlan, San Diego, CA, USA) weighing 220 – 300 g were group housed under controlled illumination (6:00 AM to 6:00 PM) and temperature (21 – 23°C). Animals had free access to standard rodent diet (Altromin™, Lage, Germany) unless otherwise specified, and tap water. Animal care and experimental procedures followed institutional ethic guidelines and conformed to the requirements of the state authority for animal research (#G 0131/11 and #01001-13).

Compound

The GOAT inhibitor, GO-CoA-Tat (Peptides International Inc., Louisville, KY, USA) was kept in powder form at –80°C and dissolved in pyrogen-free saline before the experiments.

Monitoring

1. Manual food intake monitoring

Rats were handled daily to become accustomed to the investigators and the experimental procedures. This included removal of the rat from the cage to measure food intake and light hand restraint for body weight monitoring. This daily routine was performed at the same time each day. Food intake was monitored by providing rats with pre-weighed rat chow and weighing of food after defined time intervals (directly after lights on and off, respectively). Food intake was corrected for spillage and expressed as g/200 g body weight (b.w.).

2. Automated food intake monitoring

The microstructural analysis of feeding behavior was conducted using the BioDAQ episodic food intake monitoring system for rats (BioDAQ, Research Diets, Inc., New Brunswick, NJ, USA), which allows for continuous monitoring of meal patterns in undisturbed rats with minimal human interference as recently described for the use in mice (24). The system consists of a low spill food hopper placed on an electronic balance. Both are mounted on a regular rat single housing cage containing environmental enrichment and bedding material. Water was provided *ad libitum* from regular water bottles. Rats were kept on regular rodent diet unless otherwise specified since it did not cause much spillage. The "bridging phenomenon", that occurs when a pile of retained food spillage underneath the gate can cause erroneous measurements, was observed very rarely.

The food intake monitoring system weighs the hopper with food (± 0.01 g) second by second and detects 'not eating' as weight stable and 'eating' as weight unstable. Every interaction of the rat with the food hopper is recorded. Feeding 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), and meals consist of one or more bouts separated by an inter-meal interval (IMI). The minimum IMI was defined as 15 min, the minimum meal amount as 0.1 g as described in our previous study (21). Based on this definition, food intake was considered as one meal when the feeding bouts occurred within 15 min of the previous response and their sum was equal to or greater than 0.1 g. When bouts of feeding were longer than 15 min apart, they were considered as a new meal. Meal parameters extracted from the software (BioDAQ Monitoring Software 2.3.07) for these studies encompassed the latency to the first meal, meal frequency, meal size, meal duration, inter-meal interval, time spent in meals and the rate of ingestion. Since food intake data were collected continuously, periods of interest could be chosen freely afterwards for the data analysis. Data could be viewed either in the Data Viewer (BioDAQ Monitoring Software 2.3.07) or Excel (Microsoft) for analysis.

3. Behavioral monitoring of satiety sequence

Rats were acclimated to the BioDAQ system for 1 week. The behavior was monitored in the 1st hour of the dark phase under conditions of dimmed red light by two experienced investigators and consisted of feeding (biting and chewing food), grooming (scratching, licking or biting the fur, limbs or genitals), locomotion (movements involving all four limbs; walking, jumping or circling) and resting (sitting or lying in a relaxed position) as described before (27). Eight rats were monitored at the same time once per min and 5 s per rat. The behavior counts were grouped in 12 × 5 min time bins.

4. Behavioral monitoring following treatment

Rats were acclimated to the BioDAQ system for 1 week. *Ad libitum* fed rats were treated with vehicle or GOAT inhibitor directly before the onset of the dark phase as described below and placed in their home cage with a paper grid under the cage divided into six equal squares. Behavior was monitored during the 2nd hour post injection during the dark phase. Behavior was assessed manually and simultaneously in 3 rats/investigator as described in our previous studies using a time-sampling technique (21, 28). Briefly, during the 2nd hour post injection behaviors including eating (eating as well as food approach consisting of sniffing and licking food), drinking (drinking and water approach), grooming (washing, licking, and scratching) and locomotor activity (defined as at least one rat paw crossing the boundary of one square, the total number of squares crossed was counted) were assessed by two investigators who sat motionless in front of the cages with a dim light for a period of 1 h. Each behavior was counted again when it lasted > 5 s. Food intake was assessed at the same time. In pilot experiments we established that the inter-investigator variability was < 5%.

Measurement of acyl and total ghrelin levels

Group housed rats were handled for a period of 1 week. *Ad libitum* fed rats were treated with vehicle or GOAT inhibitor

directly before the onset of the dark phase as described below and food was removed. Blood was obtained at 0 h (before injection) or 1, 2 or 3 h post injection by cardiac puncture. Therefore, rats were anesthetized with a mixture of ketamine (75 mg/kg i.p.; Fort Dodge Laboratories, Fort Dodge, IA, USA) and xylazine (5 mg/kg i.p.; Mobay, Shawnee, KS, USA). Afterwards, the thoracic cavity was quickly opened and 1 ml of cardiac blood was collected in chilled syringes rinsed with ethylene diamine tetraacetic acid (EDTA) and transferred into cooled tubes containing 10 µl EDTA (7.5%, Sigma, St. Louis, MO, USA) and aprotinin (1.2 Trypsin Inhibitory Unit per 1 ml blood; ICN Pharmaceuticals, Costa Mesa, CA, USA) for peptidase inhibition. Tubes were placed back on ice and immediately (within 3 min) centrifuged at 4°C for 10 min at 3000 × g. Plasma was separated and stored at −80°C until further processing.

Rat acyl (# EZRGRA-90K, Millipore, Billerica, MA, USA) and total (#EZRGRT-91K, Millipore) ghrelin levels were assessed using commercial ELISA kits following the manufacturer's instructions. Desacyl ghrelin was calculated as the difference of total minus acyl ghrelin for each individual sample. All samples were processed in one batch. The intra-assay variability was < 5% for acyl and < 2% for total ghrelin.

Experimental protocols

1. Habituation to automated food intake monitoring system and comparison with manual assessment

After an initial habituation period of seven days, rats continued to be group-housed (3 – 4/cage) and food intake and body weight were monitored daily. After five days, rats were separated into single housing cages which were placed adjacent to each other so the animals could stay in eye and odor contact. Food was provided from the top of the cage and the manual monitoring of food intake and body weight was continued. After another three days, food was provided from the hopper and food intake measured by the automated food intake monitoring system. Body weight was monitored daily throughout this period. Food intake assessed by the automated food intake monitoring system was compared between different time points of the habituation period (days 1 and 2 versus days 5 and 6) and also to the manual assessment. The food intake microstructure was compared between the light and the dark phase.

2. Monitoring of behavior in the automated food intake monitoring system

To assess the occurrence of physiological behavior in rats single housed in cages connected to the automated food intake monitoring system, the behavior was monitored manually in *ad libitum* fed naïve rats during the first hour of the dark phase.

3. Food intake microstructure in rats injected intraperitoneally with ghrelin-O-acyltransferase inhibitor

Ad libitum fed naïve rats were habituated to the system and injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor GO-CoA-Tat (32, 96 or 288 µg/kg in 300 µl saline) directly at the beginning of the dark phase and food intake was monitored using the automated food intake monitoring system. The medium dose was based on a recent study investigating the effect of GOAT inhibition on the hypothalamic-pituitary-adrenal axis in rats (29). The dose inducing the most pronounced reduction in food intake was selected for analysis of the food intake microstructure.

4. Acyl and desacyl ghrelin levels in rats injected intraperitoneally with ghrelin-O-acyltransferase inhibitor

Ad libitum fed naive rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor GO-CoA-Tat (96 µg/kg in 300 µl saline, the dose that induced the most pronounced reduction of food intake) directly at the beginning of the dark phase. Food was removed and blood obtained before injection (0 h) or at 1, 2 and 3 h post injection and acyl as well as total ghrelin levels assessed by ELISA. Desacyl ghrelin was calculated as the difference of total minus acyl ghrelin.

5. Monitoring of behavior in rats injected intraperitoneally with ghrelin-O-acyltransferase inhibitor

Ad libitum fed naive rats were habituated to the system and on the day of the experiment the amount of bedding was reduced and a paper grid dividing the cage into 6 squares was placed underneath the cage. Directly before the dark phase started rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor GO-CoA-Tat (96 µg/kg in 300 µl saline, the dose that induced the most pronounced reduction of food intake). Behavior was monitored during the 2nd h post injection, the period when GOAT inhibition showed the maximum reduction of food intake.

Statistical analysis

Data are expressed as mean ± S.E.M. Distribution of the data was determined by using the Kolmogorov-Smirnov test. Differences between two groups were assessed using the t-test, one-way ANOVA followed by all pair-wise multiple comparison procedures (Tukey post hoc test) or two-way ANOVA followed by Holm-Sidak method. Differences were considered significant when $P < 0.05$ (SigmaStat 3.1., Systat Software, San Jose, CA, USA).

RESULTS

Rats show normal body weight gain when housed individually and quickly adapt to the automated food intake monitoring system

Naive, group-housed rats showed a linear body weight gain during the first four days (3.1 ± 1.5 g/day, *Fig. 1*). On the day of separation, there was a slight decrease in body weight (−1.5 ± 0.8 g). This quickly faded and rats housed individually and fed from the cage tops again showed a linear body weight gain of 3.6 ± 1.3 g/day (*Fig. 1*). After providing food from the food hopper instead of the top of the cage, the linear body weight gain was also observed (2.7 ± 0.1 g/day; P = 0.71 compared to previous time points; *Fig. 1*).

We next compared the food intake of naive rats housed in individual cages and assessed manually with food intake assessed by the automated food intake monitoring system. Neither the dark phase (18.8 ± 0.4 vs. 17.8 ± 0.7 g/200 g b.w.), light phase (1.5 ± 0.3 vs. 1.9 ± 0.7 g/200 g b.w.) nor the total 24-h food intake (20.3 ± 0.5 vs. 19.7 ± 0.3 g/200 g b.w.) differed between the two methods of assessment (P = 0.43). Likewise, when assessed at different time points after providing food from the feeding hopper (days 1 and 2 compared to days 5 and 6 of the habituation period), no differences of dark phase (17.5 ± 0.7 vs. 17.8 ± 0.7 g/200 g b.w., P = 0.79), light phase (1.8 ± 0.4 vs. 1.9 ± 0.7 g/200 g b.w., *P* = 0.94) and total 24-h food intake (19.3 ± 0.5 vs. 19.7 ± 0.3 g/200 g b.w., P = 0.59) were observed.

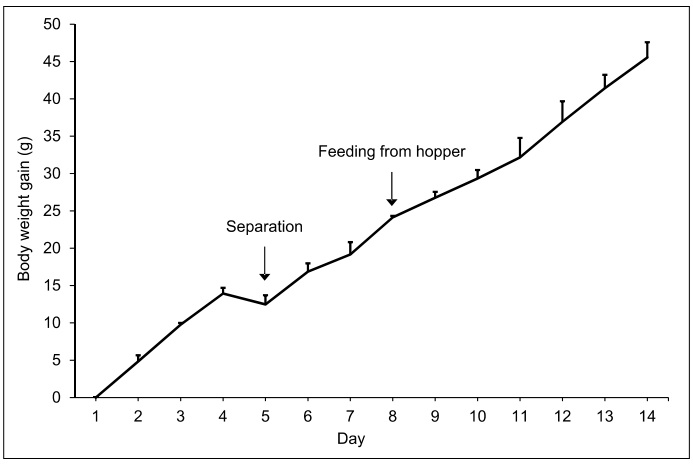

Fig. 1. Body weight gain in rats before and after separation. Rats were housed in groups of three and then on day five separated in single housing cages with eye and odor contact. Food was provided from the top of the cage and on day eight from the hopper of the automated feeding monitoring system. Body weight was assessed daily and expressed as body weight gain. Data are presented as mean ± S.E.M., n = 6.

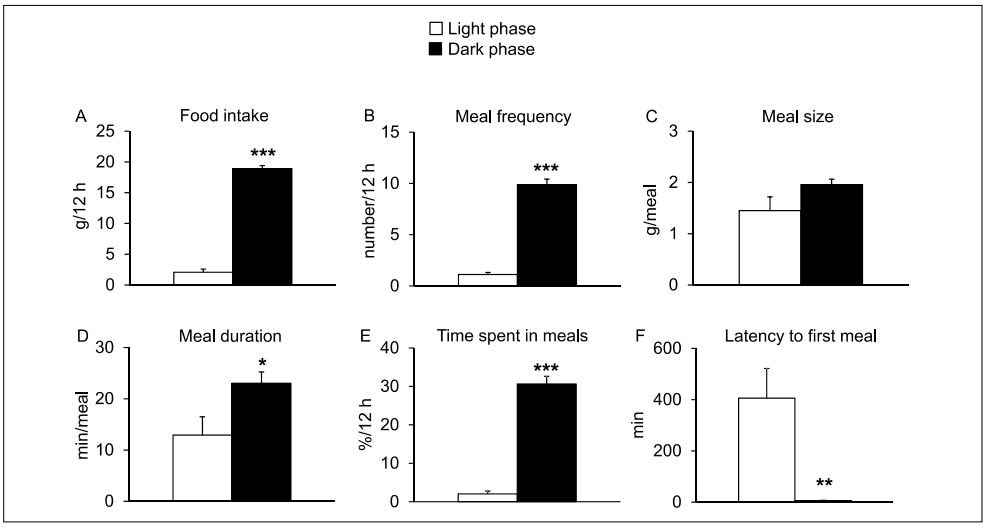

Fig. 2. Food intake microstructure during the light and dark photoperiod. Food intake (A) and the underlying food intake microstructure encompassing meal frequency (B), meal size (C), meal duration (D), time spent in meals (E) and the latency to the first meal (F) were assessed over a period of 24 h and the parameters compared for light (6:00 AM to 6:00 PM) versus dark phase (6:00 PM to 6:00 AM). Each bar represents the mean ± S.E.M. of 9 rats/group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. light phase.

Undisturbed rats show a greater food intake at night compared to the light phase which is associated with a higher meal frequency and longer duration but not meal size

We investigated the food intake microstructure for dark and light phase meals in individually housed undisturbed rats fed normal rat chow and habituated to the food intake monitoring system. At night, rats showed a 9.1-times greater food intake compared to light phase intake ($P < 0.001$; *Fig. 2A*). This increase was associated with a higher meal frequency (8.9-times, $P < 0.001$; *Fig. 2B*), longer meal duration (1.8-times, $P < 0.05$; *Fig. 2D*) and more time spent in meals (15.0-times, $P < 0.001$; *Fig. 2E*), whereas the meal size was not significantly larger compared to the light phase (1.3-times, $P = 0.13$; *Fig. 2C*). Also the latency to the first meal was shorter (75-times) in the dark compared to the light phase ($P < 0.01$; *Fig. 2F*).

A physiological behavioral satiety sequence is observed in rats housed in automated food intake monitoring cages

The behavioral satiety sequence was investigated manually at the beginning of the dark phase in rats housed in cages of the automated food intake monitoring system. Feeding behavior initially increased up to a maximum

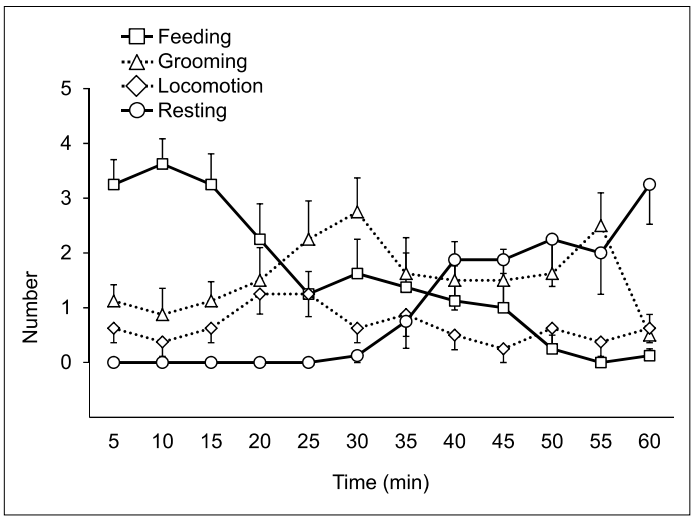

Fig. 3. The behavioral satiety sequence observed in rats housed in cages of the automated feeding monitoring system. Rats were single-housed in regular cages connected to the automated food intake monitoring system. While food intake was measured automatically, the behavior consisting of feeding, grooming, locomotion and resting was monitored manually at the beginning of the dark phase (6:00 PM to 7:00 PM) over a period of one hour. The physiological behavioral satiety sequence was observed with a decrease of dark phase feeding behavior and an increase in grooming, locomotion and particularly resting. Each line represents the mean ± S.E.M. of 8 rats/group.

Figure 4

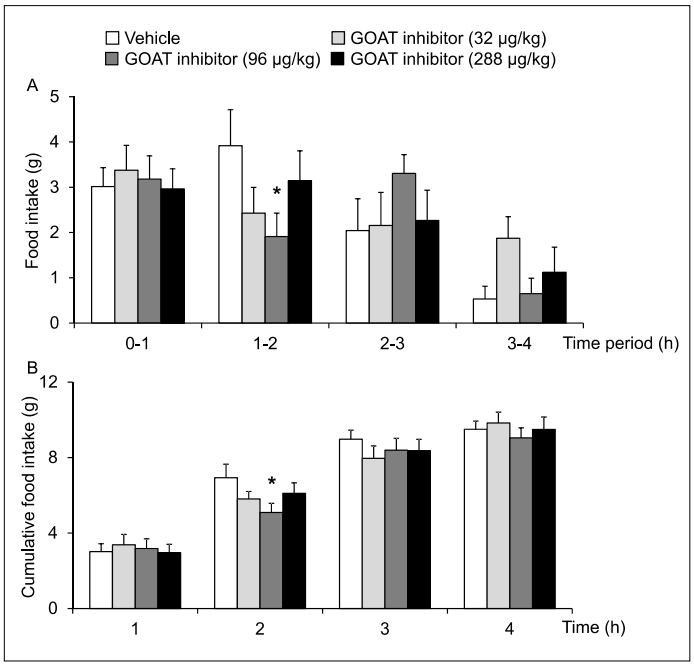

Fig. 4. Dark phase food intake in rats intraperitoneally injected with the GOAT inhibitor. *Ad libitum* fed rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor, GO-CoA-Tat (32, 96 or 288 µg/kg in 300 µl saline) directly at the beginning of the dark phase and food intake was monitored using the automated food intake monitoring system and expressed as hourly (A) or cumulative (B) food intake. Each bar represents the mean ± S.E.M. of 9 – 11 rats/group. * $P < 0.05$ vs. vehicle.

observed at 10 min (3.6 ± 0.5) and then gradually decreased reaching a nadir at 60 min (0.1 ± 0.1; *Fig. 3*). Grooming behavior showed the opposite pattern with low values at the beginning (1.1 ± 0.3) and a gradual increase until 30 min (2.8 ± 0.6). Afterwards, a temporary decrease was observed at 35 min (1.6 ± 0.7) followed by an increase reaching 2.5 ± 0.6 at 55 min and a decrease at 60 min (0.5 ± 0.4, *Fig. 3*). Locomotion remained fairly stable over the 1-h observation period (e.g. 30 min: 0.6 ± 0.3, *Fig. 3*). Resting behavior was absent at the beginning (5 min: 0.0 ± 0.0) and gradually increased reaching a maximum at 60 min (3.3 ± 0.7, *Fig. 3*). The lines of feeding and resting behavior crossed between 35 and 40 min (*Fig. 3*). No abnormal behavior was observed during this experiment.

Table 1. Food intake in rats fed *ad libitum* and injected with vehicle or GOAT inhibitor intraperitoneally before the dark phase.

Food intake (g)	Group Vehicle (n = 10)	GOAT inhibitor (32 µg/kg, n = 11)	GOAT inhibitor (96 µg/kg, n = 9)	GOAT inhibitor (288 µg/kg, n = 10)
Food intake per period				
0–4 h	9.5 ± 0.4	9.8 ± 0.6	9.0 ± 0.5	9.5 ± 0.7
4–8 h	7.6 ± 0.7	6.1 ± 0.6	8.3 ± 0.5	6.6 ± 0.6
8–12 h	3.7 ± 0.9	3.4 ± 0.9	2.1 ± 0.7	3.8 ± 0.7
12–16 h	0.5 ± 0.3	0.4 ± 0.3	0.4 ± 0.3	0.3 ± 0.2
16–20 h	0.3 ± 0.2	0.5 ± 0.2	0.1 ± 0.1	0.4 ± 0.3
20–24 h	2.7 ± 0.3	2.3 ± 0.4	3.3 ± 0.4	2.2 ± 0.4
Cumulative food intake				
4 h	9.5 ± 0.4	9.8 ± 0.6	9.0 ± 0.5	9.5 ± 0.7
8 h	17.1 ± 0.8	16.0 ± 0.7	17.3 ± 0.7	16.1 ± 0.6
12 h	20.9 ± 0.6	19.4 ± 0.5	19.4 ± 0.9	19.8 ± 0.6
16 h	21.3 ± 0.5	19.8 ± 0.5	19.8 ± 0.7	20.1 ± 0.5
20 h	21.6 ± 0.4	20.3 ± 0.4	19.9 ± 0.7	20.5 ± 0.5
24 h	24.4 ± 0.5	22.6 ± 0.6	23.3 ± 0.6	22.7 ± 0.5

Mean ± S.E.M. No significant differences were observed.

Table 2. Food intake microstructure of the first meal in rats fed *ad libitum* and injected with vehicle or GOAT inhibitor intraperitoneally before the dark phase.

Parameter	Vehicle (n = 10)	GOAT inhibitor (96 µg/kg, n = 9)
Latency to first meal (min)	4.0 ± 1.1	4.9 ± 1.3
Size of first meal (g)	2.8 ± 0.4	2.7 ± 0.3
Duration of first meal (min)	25.9 ± 5.3	21.2 ± 4.8
Eating rate of first meal (mg/min)	38.3 ± 5.7	28.6 ± 3.3
Inter-meal interval (min)	**52.4 ± 6.9**	**76.9 ± 5.9***
Satiety ratio after first meal (min/g food eaten)	**21.8 ± 3.6**	**30.3 ± 3.1***

Mean ± S.E.M. Significant differences are shown in bold. * P < 0.05.

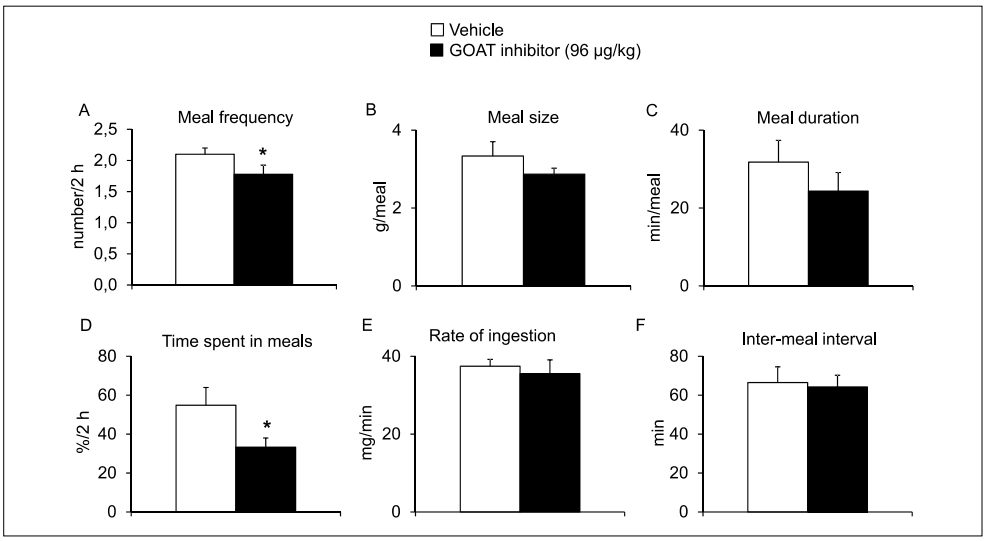

Fig. 5. Food intake microstructure in rats intraperitoneally injected with the GOAT inhibitor. *Ad libitum* fed rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor, GO-CoA-Tat (96 µg/kg in 300 µl saline) directly at the beginning of the dark phase and food intake microstructure encompassing meal frequency (A), meal size (B), meal duration (C), time spent in meals (D), rate of ingestion (E) and inter-meal interval (F) was assessed using the automated food intake monitoring system and analyzed for the first 2 h post injection. Each bar represents the mean ± S.E.M. of 9 – 10 rats/group. * P < 0.05 vs. vehicle.

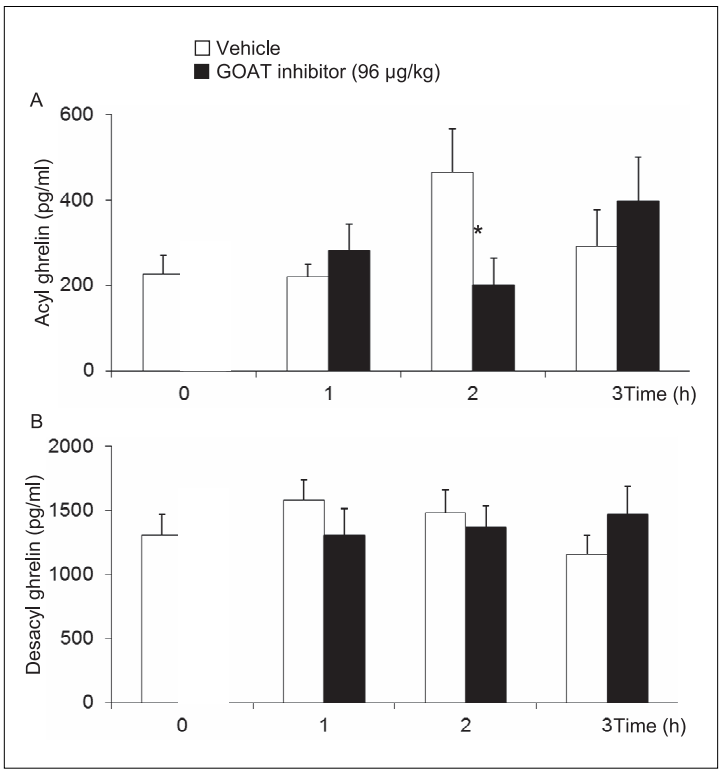

Fig. 6. Circulating acyl and desacyl ghrelin levels in rats intraperitoneally injected with the GOAT inhibitor. *Ad libitum* fed rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor, GO-CoA-Tat (96 µg/kg in 300 µl saline) directly at the beginning of the dark phase. Food was removed but rats had access to water. Blood was obtained at 0, 1, 2 or 3 h post injection and acyl as well as total ghrelin levels measured by ELISA. Desacyl ghrelin levels were calculated by subtracting total minus acyl ghrelin levels for each rat. Each bar represents the mean ± S.E.M. of 5 – 6 rats/group. * P < 0.05 vs. vehicle.

The ghrelin-O-acyltransferase inhibitor GO-CoA-Tat reduces dark phase food intake by a reduction of meal frequency while meal size is not altered

Injection of the GOAT inhibitor at the beginning of the dark phase led to a dose dependent reduction of food intake compared to vehicle (*Fig. 4A*). The reduction was delayed in onset and observed during the second hour post injection, and the dose response of the GOAT inhibitor seems to be U-shaped with a maximum effect at 96 µg/kg (–27%, P = 0.03; *Fig. 4A*). This resulted in a reduction of the 2-h cumulative food intake (P = 0.03; *Fig. 4B*). Two way ANOVA indicated a significant influence of time ($F_{3,159}$ = 10.7, P < 0.001). After 4 h, no significant differences were observed between rats injected with GOAT inhibitor or vehicle (P > 0.05; *Table 1*).

Based on these data the dose of 96 µg/kg and the period of 2 h were used for the analysis of the food intake microstructure. The GOAT inhibitor led to a reduction of meal frequency (–15%, P = 0.04; *Fig. 5A*) and the time spent in meals (–39%, P = 0.03; *Fig. 5D*), whereas meal size (P = 0.29; *Fig. 5B*), meal duration (P = 0.33; *Fig. 5C*), rate of ingestion (P = 0.63; *Fig. 5E*) and the inter-meal interval (P = 0.83; *Fig. 5F*) were not altered during the 2-h period compared to vehicle. However, when analyzing the food intake microstructure of the first meal, the interval following the first meal was prolonged after injection of the GOAT inhibitor (+47%, P = 0.02) leading to an increased satiety ratio compared to vehicle (+39%, P < 0.05; *Table 2*).

The ghrelin-O-acyltransferase inhibitor GO-CoA-Tat prevents the increase of acyl ghrelin levels during the dark phase while desacyl ghrelin is not altered

Baseline levels of acyl ghrelin at the beginning of the dark phase were 226.2 ± 43.8 pg/ml (*Fig. 6A*). At 1 h post injection, no significant differences were observed between rats injected with vehicle vs. the GOAT inhibitor group (P = 0.39; *Fig. 6A)*. At 2 h post injection, rats injected with GOAT inhibitor displayed a –57% reduction of acyl ghrelin levels compared to vehicle injected rats (P = 0.03), while after 3 h no significant difference was observed (P = 0.45; *Fig. 6A)*. Two way ANOVA indicated a significant interaction of treatment × time ($F_{(2,29)}$ = 3.6, P = 0.04).

Baseline levels of desacyl ghrelin at the beginning of the dark phase were 1305.9 ± 160.1 pg/ml (*Fig. 6B*). No significant differences were observed at either time point between rats injected with vehicle or GOAT inhibitor (P > 0.27; *Fig. 6B)*. Two way ANOVA indicated no significant impact of treatment ($F_{(1,30)}$ = 0.03, P = 0.88), time ($F_{(2,30)}$ = 0.24, P = 0.78) or an interaction of treatment × time ($F_{(2,30)}$ = 1.1, P = 0.34).

The ghrelin-O-acyltransferase inhibitor GO-CoA-Tat reduces grooming behavior while locomotion is not altered

Rats injected with the GOAT inhibitor, GO-CoA-Tat showed a –21% reduction of 2-h food intake compared to vehicle treated rats (data not shown). Behavioral assessment during the 2nd h post injection, the period where rats had shown the maximum

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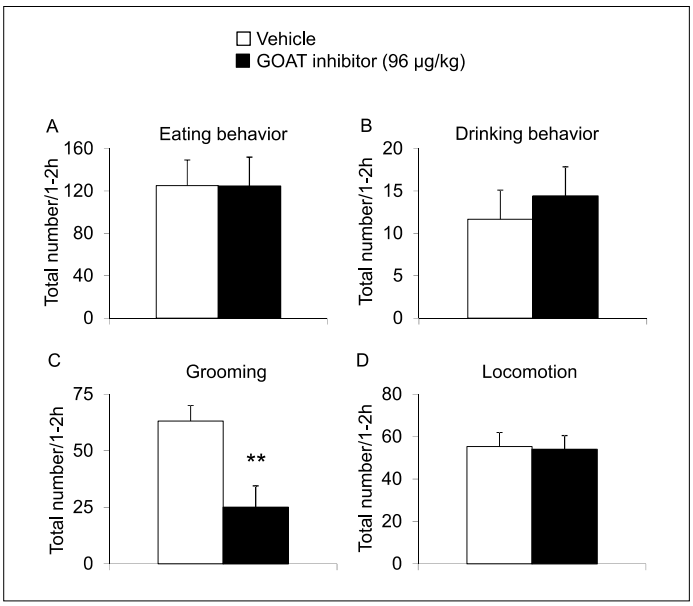

Fig. 7. Behavior in rats intraperitoneally injected with the GOAT inhibitor. *Ad libitum* fed rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor, GO-CoA-Tat (96 µg/kg in 300 µl saline) directly at the beginning of the dark phase. Single housed rats with paper divided into six equal squares that was placed under their home cage had *ad libitum* access to food and water throughout the experiment. During the 2nd hour post injection behaviors, including eating (including food approach, A), drinking (including water approach, B), grooming behavior (washing, licking, and scratching; C) and locomotor activity (total number of squares crossed; D) were monitored manually for 1 h by two observers. Each behavior was counted again when lasting > 5 s. Bars indicate means ± S.E.M. of 6 rats/group. ** $P < 0.01$ vs. vehicle.

(including food approach, *Fig. 7A*) and drinking behavior (including water approach, *Fig. 7B*) were not different between the two groups. Injection of the GOAT inhibitor reduced grooming behavior (–60%, $P < 0.01$; *Fig. 7C*), while locomotor activity was not altered compared to vehicle (–2.4%, $P = 0.89$; *Fig. 7D*). No signs of abnormal behavior were observed following treatment with GO-CoA-Tat (data not shown).

DISCUSSION

Using an automated food intake monitoring device in the present study we show that the GOAT inhibitor, GO-CoA-Tat reduces early dark phase food intake. By analyzing the underlying food intake microstructure, this reduction is due to a decrease in meal frequency, while meal size is not significantly altered.

Food intake is often assessed in animal experiments and the interest is steadily growing in light of the increasing prevalence of human obesity (30, 31) and the consecutive need for a better understanding of the mechanisms regulating hunger and satiety. The manual measurement of food intake is the classical approach; however, this assessment might disturb the animals and does not provide information on the underlying food intake microstructure. Early on, measurement techniques were developed to gain insight into the food intake microstructure including the measurement of consumed liquid (32, 33), powder (34, 35) or micropelleted food (36, 37). However, all these formulations of food do not represent the physiological type of food used in most studies where food intake is assessed manually. Therefore, systems for the assessment of the food intake microstructure using regular solid rat chow have been developed (38, 39). In the present study we used an automated episodic food intake monitoring device to monitor the food intake microstructure of solid food in undisturbed rats. Although

nd validated for

mice (24), the validation was lacking for rats. Therefore, the first step was to validate the system.

Rats showed a rapid habituation to the episodic food intake monitoring system as indicated by the linear continuation of body weight gain despite the single housing and feeding out of a food hopper. Moreover, the system shows good concordance to manual food intake monitoring providing the same amounts of food ingested in either photoperiod. In addition, the system allows for assessment of the underlying food intake microstructure which provides detailed insight into the mechanisms involved in the modulation of food intake under the respective experimental condition without any disturbance of the animals by the investigator or a light source.

It is important to note that rats maintained in the BioDAQ system showed a physiological behavior following food intake, which was assessed using the behavioral satiety sequence, a parameter established several decades ago (25, 40). The behavioral satiety sequence represents a consecutive progression of behaviors following food intake in rats encompassing feeding itself, grooming, exploration and resting. The behavioral satiety sequence is considered physiological if two major requirements are met: the final item ‘resting’ is observed and there is a lack of abnormal behavior during the test (41). In the present study we assessed the occurrence of the behavioral satiety sequence manually in rats housed in cages of the automated food intake monitoring device and observed an initial surge of feeding behavior, a period of grooming and a transition towards a predominant occurrence of resting behavior. The lines of feeding and resting behavior crossed between 35 and 40 min indicating the occurrence of satiety around that time as described before (42-45). No abnormal behavior or signs of sickness were observed. These findings indicate the occurrence of physiological satiety under the present housing conditions.

After these initial experiments we investigated the modulation of food intake using the GOAT inhibitor, GO-CoA-Tat that was introduced by Barnett and colleagues showing an inhibition of GOAT in cell lines stably expressing GOAT and preproghrelin as well as *in vivo* in mice (46). Intraperitoneal injection of the GOAT inhibitor reduced dark phase food intake in freely fed rats. Interestingly, this dose-dependent reduction showed a U-shaped relationship with a maximum effect at 96 µg/kg. Whether higher doses have additional agonistic or unspecific effects needs to be further investigated. The reduction of food intake by GO-CoA-Tat was delayed in onset and observed mainly in the second hour post injection. This is likely due to the fact that circulating ghrelin is already up-regulated at the beginning of the dark phase (47), the phase rats usually eat (26). Considering the half-life of ghrelin of around 30 min (48), an inhibition of GOAT should result in measurable effects of reduced ghrelin signaling with a lag phase in line with the delay observed in the present study. The effect on food intake was short lasting and only observed during the first 2 h, likely due to the clearance of the GOAT inhibitor, GO-CoA-Tat. These hypotheses are corroborated by the alterations of acyl ghrelin observed. While no change of acyl ghrelin levels is detected at 1 h post injection, treatment with GO-CoA-Tat prevents the dark phase related increase of acyl ghrelin which results in a more than 50% difference compared to saline treated rats at 2 h likely underlying the reduction of food intake observed. Interestingly, no modulation of desacyl ghrelin is observed giving rise to a specific effect on the acylation of ghrelin.

Analysis of the food intake microstructure of the first 2 h post injection showed that inhibition of GOAT decreases food intake by a reduction of meal frequency and a prolongation of the interval after the first meal, while meal size is not altered. In addition, the satiety ratio was also increased following inhibition of GOAT. These data give rise to an induction of satiety (mechanisms causing a later onset of the next meal after one meal is completed) (18, 19), while satiation (mechanisms causing meal termination) is not affected. Partly corresponding to these data, Tabarin *et al*. reported an increase of meal size and meal frequency in mice following intraperitoneal injection of the ghrelin agonist, BIM-28131 (49). The differential effects of GOAT inhibition in the present (alteration of satiety while satiation is not affected) and stimulation of ghrelin signaling in the study using the ghrelin agonist, BIM-28131 (alteration of satiation and satiety) may be due to species differences (rats *versus* mice), the assessment method of food intake (micropellet *versus* regular solid rat chow) or reflect additional pharmacological properties of the ghrelin agonist, BIM-28131.

To exclude unspecific effects of GOAT inhibition on behavior and to investigate additional behavioral alterations besides food intake, these were measured manually. Interestingly, although inhibition of GOAT in this experiment reduced food intake by 21% in the first 2 h post injection, behavioral analysis during the 2nd hour, the period when the greatest reduction of food intake was observed before, showed that eating behavior which included eating itself but also food approach (sniffing and licking food) was not different between the two groups. This indicates that, although food intake is reduced, the overall interaction with the food is not altered by GOAT inhibition. Whether this is due to an incomplete blockade of ghrelin acylation or a compensatory effect of other hormones will have to be further investigated. Similar to the effect on eating behavior, also drinking behavior (including water approach) was not different between the two groups. Also locomotor activity was not reduced pointing towards the absence of unspecific sickness and nausea induced by the compound. Interestingly, GOAT inhibition reduced grooming behavior compared to vehicle which may be a subsequent effect due to the reduced food intake as the physiological satiety sequence progresses from food intake to grooming behavior (25, 40). On the other hand, it may also indicate a direct effect as acyl ghrelin was shown to increase grooming behavior in rats (50). Overall, injection of the GOAT inhibitor does not seem to induce sickness or abnormal behaviors, further pointing towards a specific effect on ghrelin acylation.

In summary, in the present study we validated an automated food intake monitoring system for the assessment of food intake microstructure of regular rat chow in undisturbed rats. Importantly, rats housed in these cages show a normal feeding behavior as indicated by a physiological behavioral satiety sequence. Using this system we showed that pharmacological peripheral inhibition of GOAT *via* a reduction of acyl ghrelin levels reduces dark phase food intake with a delayed onset and short duration by an increase of satiety, while satiation is not affected.

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Conflict of interests: None declared.

REFERENCES

1. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999; 402: 656-660.
2. Davenport AP, Bonner TI, Foord SM, *et al*. International Union of Pharmacology. LVI. Ghrelin receptor nomenclature, distribution, and function. *Pharmacol Rev* 2005; 57: 541-546.
3. Jeon TY, Lee S, Kim HH, *et al*. Changes in plasma ghrelin concentration immediately after gastrectomy in patients with early gastric cancer. *J Clin Endocrinol Metab* 2004; 89: 5392-5396.
4. Wren AM, Small CJ, Ward HL, *et al*. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* 2000; 141: 4325-4328.
5. Druce MR, Wren AM, Park AJ, *et al*. Ghrelin increases food intake in obese as well as lean subjects. *Int J Obes* (*Lond*) 2005; 29: 1130-1136.
6. Warzecha Z, Ceranowicz P, Dembinski M, *et al*. Involvement of cyclooxygenase-1 and cyclooxygenase-2 activity in the therapeutic effect of ghrelin in the course of ethanol-induced gastric ulcers in rats. *J Physiol Pharmacol* 2014; 65: 95-106.
7. Rau TT, Sonst A, Rogler A, *et al*. Gastrin mediated down regulation of ghrelin and its pathophysiological role in atrophic gastritis. *J Physiol Pharmacol* 2013; 64: 719-725.
8. Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* 2008; 132: 387-396.
9. Gutierrez JA, Solenberg PJ, Perkins DR, *et al*. Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc Natl Acad Sci USA* 2008; 105: 6320-6325.
10. Stengel A, Goebel M, Wang L, Tache Y, Sachs G, Lambrecht NW. Differential distribution of ghrelin-O-acyltransferase (GOAT) immunoreactive cells in the mouse and rat gastric oxyntic mucosa. *Biochem Biophys Res Commun* 2010; 392: 67-71.
11. Goebel-Stengel M, Hofmann T, Elbelt U, *et al*. The ghrelin activating enzyme ghrelin-O-acyltransferase (GOAT) is

present in human plasma and expressed dependent on body mass index. *Peptides* 2013; 43: 13-19.
12. Zhao TJ, Liang G, Li RL, *et al*. Ghrelin O-acyltransferase (GOAT) is essential for growth hormone-mediated survival of calorie-restricted mice. *Proc Natl Acad Sci USA* 2010; 107: 7467-7472.
13. Kang K, Schmahl J, Lee JM, *et al*. Mouse ghrelin-O-acyltransferase (GOAT) plays a critical role in bile acid reabsorption. *FASEB J* 2012; 26: 259-271.
14. Cai H, Cong WN, Daimon CM, *et al*. Altered lipid and salt taste responsivity in ghrelin and GOAT null mice. *PLoS One* 2013; 8: e76553.
15. Davis JF, Perello M, Choi DL, *et al*. GOAT induced ghrelin acylation regulates hedonic feeding. *Horm Behav* 2012; 62: 598-604.
16. Kirchner H, Gutierrez JA, Solenberg PJ, *et al*. GOAT links dietary lipids with the endocrine control of energy balance. *Nat Med* 2009; 15: 741-745.
17. Teubner BJ, Garretson JT, Hwang Y, Cole PA, Bartness TJ. Inhibition of ghrelin O-acyltransferase attenuates food deprivation-induced increases in ingestive behavior. *Horm Behav* 2013; 63: 667-673.
18. Strubbe JH, Woods SC. The timing of meals. *Psychol Rev* 2004; 111: 128-141.
19. Fekete EM, Inoue K, Zhao Y, *et al*. Delayed satiety-like actions and altered feeding microstructure by a selective type 2 corticotropin-releasing factor agonist in rats: intra-hypothalamic urocortin 3 administration reduces food intake by prolonging the post-meal interval. *Neuropsychopharmacology* 2007; 32: 1052-1068.
20. Wellman PJ, Bellinger LL, Cepeda-Benito A, Susabda A, Ho DH, Davis KW. An inexpensive food cup for use in a commercially available food monitoring system. *Physiol Behav* 2004; 83: 525-530.
21. Goebel-Stengel M, Stengel A, Wang L, Ohning G, Tache Y, Reeve JR, Jr. CCK-8 and CCK-58 differ in their effects on nocturnal solid meal pattern in undisturbed rats. *Am J Physiol Regul Integr Comp Physiol* 2012; 303: R850-R860.
22. Farley C, Cook JA, Spar BD, Austin TM, Kowalski TJ. Meal pattern analysis of diet-induced obesity in susceptible and resistant rats. *Obes Res* 2003; 11: 845-851.
23. Goebel M, Stengel A, Wang L, Tache Y. Central nesfatin-1 reduces the nocturnal food intake in mice by reducing meal size and increasing inter-meal intervals. *Peptides* 2011; 32: 36-43.
24. Stengel A, Goebel M, Wang L, *et al*. Activation of brain somatostatin(2) receptors stimulates feeding in mice: analysis of food intake microstructure. *Physiol Behav* 2010; 101: 614-622.
25. Antin J, Gibbs J, Holt J, Young RC, Smith GP. Cholecystokinin elicits the complete behavioral sequence of satiety in rats. *J Comp Physiol Psychol* 1975; 89: 784-790.
26. Rosenwasser AM, Boulos Z, Terman M. Circadian organization of food intake and meal patterns in the rat. *Physiol Behav* 1981; 27: 33-39.
27. Verbaeys I, Tolle V, Swennen Q, *et al*. Scheduled feeding results in adipogenesis and increased acylated ghrelin. *Am J Physiol Endocrinol Metab* 2011; 300: E1103-E1111.
28. Stengel A, Goebel M, Wang L, *et al*. Selective central activation of somatostatin2 receptor increases food intake, grooming behavior and rectal temperature in rats. *J Physiol Pharmacol* 2010; 61: 399-407.
29. Rucinski M, Ziolkowska A, Szyszka M, Hochol A, Malendowicz LK. Evidence suggesting that ghrelin O-acyl transferase inhibitor acts at the hypothalamus to inhibit hypothalamo-pituitary-adrenocortical axis function in the rat. *Peptides* 2012; 35: 149-159.
30. Kelly T, Yang W, Chen CS, Reynolds K, He J. Global burden of obesity in 2005 and projections to 2030. *Int J Obes* (*Lond*) 2008; 32: 1431-1437.
31. Flegal KM, Carroll MD, Kit BK, Ogden CL. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. *JAMA* 2012; 307: 491-497.
32. Overduin J, Gibbs J, Cummings DE, Reeve JR, Jr. CCK-58 elicits both satiety and satiation in rats while CCK-8 elicits only satiation. *Peptides* 2014; 54C: 71-80.
33. Stratford TR, Gibbs J, Smith GP. Microstructural analysis of licking behavior following peripheral administration of bombesin or gastrin-releasing peptide. *Peptides* 1995; 16: 903-909.
34. Smith JC. Microstructure of the rat's intake of food, sucrose and saccharin in 24-hour tests. *Neurosci Biobehav Rev* 2000; 24: 199-212.
35. Melhorn SJ, Krause EG, Scott KA, *et al*. Meal patterns and hypothalamic NPY expression during chronic social stress and recovery. *Am J Physiol Regul Integr Comp Physiol* 2010; 299: R813-R822.
36. Inoue K, Valdez GR, Reyes TM, *et al*. Human urocortin II, a selective agonist for the type 2 corticotropin-releasing factor receptor, decreases feeding and drinking in the rat. *J Pharmacol Exp Ther* 2003; 305: 385-393.
37. Burton MJ, Cooper SJ, Popplewell DA. The effect of fenfluramine on the microstructure of feeding and drinking in the rat. *Br J Pharmacol* 1981; 72: 621-633.
38. Koehnle TJ, Stephens AL, Gietzen DW. Threonine-imbalanced diet alters first-meal microstructure in rats. *Physiol Behav* 2004; 81: 15-21.
39. Erecius LF, Dixon KD, Jiang JC, Gietzen DW. Meal patterns reveal differential effects of vagotomy and tropisetron on responses to indispensable amino acid deficiency in rats. *J Nutr* 1996; 126: 1722-1731.
40. Smith GP, Gibbs J. Postprandial satiety. In: Progress in Psychobiology and Physiological Psychology, Sprague J, Epstein A, (eds.), New York, Academic 1979. pp. 179-242.
41. Geary N, Smith GP. Pancreatic glucagon and postprandial satiety in the rat. *Physiol Behav* 1982; 28: 313-322.
42. Oliveira Ldos S, da Silva LP, da Silva AI, Magalhaes CP, de Souza SL, de Castro RM. Effects of early weaning on the circadian rhythm and behavioral satiety sequence in rats. *Behav Processes* 2011; 86: 119-124.
43. Ishii Y, Blundell JE, Halford JC, Rodgers RJ. Effects of systematic variation in presatiation and fasting on the behavioural satiety sequence in male rats. *Physiol Behav* 2003; 79: 227-238.
44. Verbaeys I, Leon-Tamariz F, De Buyser K, *et al*. Dose-response effects of PEGylated cholecystokinin on the behavioral satiety sequence. *Physiol Behav* 2009; 98: 198-204.
45. Tallett AJ, Blundell JE, Rodgers RJ. Night and day: diurnal differences in the behavioural satiety sequence in male rats. *Physiol Behav* 2009; 97: 125-130.
46. Barnett BP, Hwang Y, Taylor MS, *et al*. Glucose and weight control in mice with a designed ghrelin O-acyltransferase inhibitor. *Science* 2010; 330: 1689-1692.
47. Sanchez J, Oliver P, Pico C, Palou A. Diurnal rhythms of leptin and ghrelin in the systemic circulation and in the gastric mucosa are related to food intake in rats. *Pflugers Arch* 2004; 448: 500-506.
48. Tolle V, Bassant MH, Zizzari P, *et al*. Ultradian rhythmicity of ghrelin secretion in relation with GH, feeding behavior, and sleep-wake patterns in rats. *Endocrinology* 2002; 143: 1353-1361.
49. Tabarin A, Diz-Chaves Y, Consoli D, *et al*. Role of the corticotropin-releasing factor receptor type 2 in the control

of food intake in mice: a meal pattern analysis. *Eur J Neurosci* 2007; 26: 2303-2314.
50. Szentirmai E, Hajdu I, Obal F, Krueger JM. Ghrelin-induced sleep responses in ad libitum fed and food-restricted rats. *Brain Res* 2006; 1088: 131-140.

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Activity-Based Anorexia Reduces Body Weight without Inducing a Separate Food Intake Microstructure or Activity Phenotype in Female Rats—Mediation via an Activation of Distinct Brain Nuclei

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Anorexia nervosa (AN) is accompanied by severe somatic and psychosocial complications. However, the underlying pathogenesis is poorly understood, treatment is challenging and often hampered by high relapse. Therefore, more basic research is needed to better understand the disease. Since hyperactivity often plays a role in AN, we characterized an animal model to mimic AN using restricted feeding and hyperactivity. Female Sprague-Dawley rats were divided into four groups: no activity/ad libitum feeding (ad libitum, AL, n = 9), activity/ad libitum feeding (activity, AC, n = 9), no activity/restricted feeding (RF, n = 12) and activity/restricted feeding (activity-based anorexia, ABA, n = 11). During the first week all rats were fed ad libitum, ABA and AC had access to a running wheel for 24 h/day. From week two ABA and RF only had access to food from 9:00 to 10:30 a.m. Body weight was assessed daily, activity and food intake monitored electronically, brain activation assessed using Fos immunohistochemistry at the end of the experiment. While during the first week no body weight differences were observed ($p > 0.05$), after food restriction RF rats showed a body weight decrease: −13% vs. day eight ($p < 0.001$) and vs. AC (−22%, $p < 0.001$) and AL (−26%, $p < 0.001$) that gained body weight (+10% and +13%, respectively; $p < 0.001$). ABA showed an additional body weight loss (−9%) compared to RF ($p < 0.001$) reaching a body weight loss of −22% during the 2-week restricted feeding period ($p < 0.001$). Food intake was greatly reduced in RF (−38%) and ABA (−41%) compared to AL ($p < 0.001$). Interestingly, no difference in 1.5-h food intake microstructure was observed between RF and ABA ($p > 0.05$). Similarly, the daily physical activity was not different between AC and ABA ($p > 0.05$). The investigation of Fos expression in the brain showed neuronal activation in several brain nuclei such as the supraoptic nucleus, arcuate nucleus, locus coeruleus and nucleus of the solitary tract of ABA compared to AL rats. In conclusion,

ABA combining physical activity and restricted feeding likely represents a suited animal model for AN to study pathophysiological alterations and pharmacological treatment options. Nonetheless, cautious interpretation of the data is necessary since rats do not voluntarily reduce their body weight as observed in human AN.

Keywords: anorexia nervosa, body weight, brain-gut axis, eating disorder, Fos, psychosomatic, wheel running

INTRODUCTION

Anorexia nervosa (AN) is an eating disorder characterized by the desire to lose body weight or to maintain body weight at a lower level than normal for age and height. Moreover, patients suffer from an intense fear of gaining weight and a body image disturbance (American Psychiatric Association, 2013). AN has a high prevalence in adolescent girls and young women (Nagl et al., 2016); the lifetime prevalence for AN in European women was reported to be 0.9% (Preti et al., 2009), similar levels were reported for the United States (Hudson et al., 2007). The treatment of AN is challenging and mostly comprised of structured care and psychotherapy (Zipfel et al., 2014); however, treatment is hampered by a high relapse rate (Herzog et al., 1997; Zipfel et al., 2015). While only about half of the patients recover, one third improves but continues to have symptoms and 20% remain severely chronically ill (Steinhausen, 2002). Lastly, AN has a considerable weighted mortality rate (deaths per 1000 person-years) of 5.1 (Arcelus et al., 2011). It is to note that although AN is clinically well characterized, the pathogenesis underlying the disease is still not well established. Moreover, no specific pharmacological treatment is available. Therefore, more research is needed to better characterize the disease and to identify possible new treatment targets.

Progress in medical research is often achieved by establishing an animal model of a disease that can help to investigate the underlying pathophysiology. It was already in 1967 when Routtenberg and Kuznesof observed that rodents tend to self-starvation when exposed to a time-restricted feeding schedule and given the possibility of voluntary physical activity in a running wheel (Routtenberg and Kuznesof, 1967). As hyperactivity can be observed in a considerable subset (ranging from 31 to 80%) of patients with AN (Davis et al., 1997), animal models using physical activity mimic this condition. The combination of a restricted feeding schedule and the access to physical exercise using a running wheel has been used to mimic features of human AN; the model was termed activity-based anorexia (Casper et al., 2008).

Subsequently, the model has been largely characterized and several alterations observed such as an increased brain γ-aminobutyric acid (GABA) (Aoki et al., 2012) and endocannabinoid signaling (Casteels et al., 2014), disturbances in food-anticipatory dopamine and serotonin release (Verhagen et al., 2009) along with an involvement of several food intake-regulatory hormones, e.g., ghrelin (Legrand et al., 2016) and leptin (Hillebrand et al., 2005b) and lastly, an activation of the hypothalamus-pituitary-adrenal axis (Burden et al., 1993), changes that might play a role in human AN as well. These alterations are likely to be involved in several changes observed: besides a reduction in food intake and body weight also an intestinal barrier dysfunction (Jésus et al., 2014), a disruption of neural development in the hippocampus (Chowdhury et al., 2014) and an impairment of memory function (Paulukat et al., 2016), increased anxiety (Kinzig and Hargrave, 2010) and the development of stress ulcers (Doerries et al., 1991), features also observed (Kline, 1979; Ghadirian et al., 1993; Swinbourne and Touyz, 2007; Huber et al., 2015; Kjaersdam Telleus et al., 2015) or suspected in patients with AN. Taken together, activity-based anorexia—despite the major limitation of being an animal model merely mimicking features of a disease—is likely a suited tool to study aspects of the pathogenesis of human AN.

The aim of the present study was first to establish the model of activity-based anorexia in our laboratory investigating food intake, running wheel activity and body weight in female rats. Only female rats were used due to the higher prevalence of anorexia in females compared to males (Steinhausen and Jensen, 2015). Next, we investigated the food intake microstructure underlying the reduction in food intake in this animal model using an automated food intake monitoring system recently established for the use in rats (Teufel et al., 2015). To further characterize possible underlying alterations in brain activity we used the neuronal expression marker Fos and performed a brain mapping in rats subjected to activity-based anorexia.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (Harlan-Winkelmann Co., Borchen, Germany) weighing 150–180 g upon their arrival were housed in groups under conditions of controlled illumination (12:12 h light/dark cycle, lights on/off: 06:00 a.m./06:00 p.m.) and temperature (21–23°C). Rats were fed with standard rat chow (ssnif Spezialdiäten GmbH, Soest, Germany) and tap water ad libitum unless otherwise specified. This study was carried out in accordance with the recommendation of the institutional guidelines; the protocol was approved by the state authority for animal research (#G 0117/14).

Activity-Based Anorexia

After an initial acclimatization period of 7 days, rats (total n = 44) were randomly assigned to one of four groups: (a) ad libitum group: no extra activity + ad libitum feeding schedule, (b) activity group: voluntary activity in a running wheel + ad libitum feeding schedule, (c) restricted feeding group: no extra activity + restricted feeding schedule, and (d) activity-based anorexia group: voluntary activity in a running wheel + restricted feeding schedule.

During the first week of the experiment, all rats were fed ad libitum and separated into single housing cages which were placed adjacent to each other to provide sight, acoustic and odor contact. Rats of the activity and activity-based anorexia group had access to a running wheel inside the cage for 24 h/day, while the sedentary groups (ad libitum and restricted feeding group) were housed without running wheel under otherwise identical conditions. All cages contained environmental enrichment and bedding material. Rats were acclimated to their new cages for 1 week and handled daily to become accustomed to the interaction with the investigator. This included daily removal of the rat from the cage to measure body weight. The daily routine was performed between 08:00 and 09:00 a.m.

Food restriction conditions started on day eight of the experiment. Rats of the restricted feeding as well as activity-based anorexia group received food from 09:00 to 10:30 a.m. (the 90-min feeding period during the light phase was based on Luyten et al., 2009; Wu et al., 2014), while the other two groups (ad libitum and activity group) continued to have access to food for 24 h/day. Body weight, food intake and activity were monitored over a period of 21 days. The experiment was discontinued and animals euthanized when the body weight loss exceeded 25%.

MEASUREMENTS

Monitoring of Body Weight

Rats were weighed daily between 08:00 and 09:00 a.m. Body weight and body weight changes were calculated for the whole 21-day experimental period (1 week of ad libitum food intake and 2 weeks of restricted feeding).

Monitoring of Food Intake and Food Intake Microstructure

The microstructural analysis of feeding behavior was conducted using the BioDAQ episodic food intake monitoring system for rats (BioDAQ, Research Diets, Inc., New Brunswick, NJ, USA) which allows the continuous monitoring of solid chow food intake in undisturbed rats as recently reported (Teufel et al., 2015). The system contains a food hopper placed on an electronic microbalance, both are mounted on a regular rat single housing cage. Food intake parameters are measured continuously and can be extracted from the software (BioDAQ Monitoring Software 2.3.07); periods of interest can be chosen freely afterwards for data analysis. Every interaction of the rat with the food hopper is registered as a "bout." A meal is defined as food intake of at least 0.01 g, when feeding bouts occur after an interval of ≥15 min this is considered a new meal. Meal parameters extracted from the software include bout size, meal size, bout frequency, meal frequency, meal duration, time spent in meals and eating rate. The food intake microstructure was analyzed starting at 4 days after food restriction over a period of 4 days (expressed as mean value of 4 days/animal).

Monitoring of Physical Activity

Physical activity in the running wheel was assessed electronically using the software provided by the manufacturer (Campden Instruments Ltd., Loughborough, UK) and expressed as wheel rotations per day as described before (Wu et al., 2014). Here, the activity system was combined with the cages for automated food intake monitoring. Pilot studies did not indicate any deleterious interference between the two measurements (data not shown).

The estimation of energy consumption was based on an earlier study that determined oxygen consumption of rats running at a constant speed (Shepherd and Gollnick, 1976). A respiratory exchange ratio of 1.0 was assumed based on carbohydrates as largest component in the standard rat chow used (58% of calories from carbohydrates, manufacturer's information).

c-Fos Immunohistochemistry

At the end of the observation period, brain activation was assessed using c-Fos immunohistochemistry in ad libitum and activity-based anorexia rats (n = 3/group). In order to avoid signals from overfeeding and great distention of the stomach, food intake was restricted to 1.5 g in this last 1.5-h feeding period in the activity-based anorexia group. Directly after this feeding period, animals were perfused and brains processed for Fos immunohistochemistry as described before (Wang et al., 2011). Briefly, rats were deeply anesthetized by an intraperitoneal injection of 100 mg/kg ketamine (Ketanest™, Curamed, Karlsruhe, Germany) and 10 mg/kg xylazine (Rompun™ 2%, Bayer, Leverkusen, Germany). Transcardial perfusion was performed as described before (Stengel et al., 2009). After thoracotomy a cannula was inserted into the ascending aorta via the left heart ventricle. Perfusion consisted of a 1-min flush with sodium chloride (0.9%) followed by 500 ml of fixative (4% paraformaldehyde and 14% saturated picric acid in 0.1 M phosphate buffer, pH adjusted to 7.4). Afterwards, brains were removed and postfixed overnight in the same fixative at 4°C followed by a cryoprotection in 10% sucrose for 24 h. Lastly, brains were snap-frozen in dry ice-cooled 2-methylbutane (Carl Roth GmbH, Karlsruhe, Germany) and then stored at −80°C until further processing.

Rat brains from the two groups were processed in parallel to ensure similar conditions. Whole brains were cut into coronal sections (25 µm) from prefrontal forebrain to the caudal medulla using a cryostat (CryoStar NX70, Thermo Fisher Scientific, Waltham, MA, USA). Every third brain section was rinsed in phosphate-buffered saline (PBS) for 3 × 15 min. All incubations were performed using the free-floating technique at room temperature (except for the incubation with the primary antibody at 4°C) and followed by a 3 × 15 min washing step in PBS. The sections were first treated with 0.3% H_2O_2 in PBS for 30 min to block endogenous peroxidase activity. After rinsing the sections, nonspecific binding was blocked by 2% normal goat serum (NGS, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for another 30 min. Sections were washed again and incubated in rabbit polyclonal anti-cFos (1:20,000, Catalog No. ABE457, Merck Millipore, Darmstadt, Germany) as primary antibody (2 h at room temperature followed by overnight at 4°C). Sections were rinsed again and incubated with biotinylated secondary goat anti-rabbit IgG (1:1000, Catalog No. 111-065-144, Jackson ImmunoResearch) for 2 h. After rinsing, this was followed by the incubation with the avidin-biotin-peroxidase complex (ABC, 1:500, Vector Laboratories,

Burlingame, CA, USA) in 0.3% Triton-PBS for 1 h. Staining was visualized with diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, Darmstadt, Germany) and nickel ammonium sulfate (Fisher Scientific, Waltham, MA, USA). The color development was frequently checked with a light microscope and stopped after about 10 min. After staining, sections were mounted, air-dried, completely dehydrated through a gradient of ethanol, cleared in xylene and cover-slipped with Entellan™ new (Merck Millipore).

In a separate experiment, specificity of the cFos antibody was assessed by pre-absorption with synthetic SGFNADYEASSSRC (amino acids 4–17 of rat c-Fos, JPT Peptide Technologies GmbH, Berlin, Germany). The peptide (5 μg/ml) was incubated with the anti-c-Fos antibody diluted at 1:20,000 (Merck Millipore, antigen:antibody ratio of 100:1) for 2 h at room temperature followed by 22 h at 4°C. The solution was centrifuged for 15 min at 13,000 × g and the supernatant used for immunostaining as described above.

Immunoreactivity of brain sections was examined using a light microscope (Axiophot, Zeiss, Jena, Germany) and images were acquired using a connected camera (AxioCam HRc, Zeiss). The density of Fos positive cells in each brain section was determined semi-quantitatively using a 10x objective and described as −, no; +, low (~1–10 cells); ++, medium (~10–20 cells); and +++, high (>20 Fos positive cells in a 100 μm × 100 μm area of an ocular grid with a 10x objective) density of expression. Coordinates of the brain nuclei were identified according to the rat brain atlas (Paxinos and Watson, 2007). The investigator was blinded to the experimental group. The average density of Fos immunoreactive cells derived from the total number of sections analyzed for each nucleus was determined for each animal and used to calculate the mean density of expression per group.

Statistical Analysis

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

RESULTS

Activity-Based Anorexia Rats Show the Greatest Reduction in Body Weight

During the first week of the experiment (access to the running wheel in single housing cages for activity and activity-based anorexia group; regular single housing conditions for ad libitum and restricted feeding group) no body weight differences were observed between the four groups (**Figure 1**). After the start of food restriction, rats of the restricted feeding group as well as activity-based anorexia rats showed a body weight decrease, while the ad libitum and activity groups continued to gain body weight (**Figure 1**). At the end of the 14-day food restriction period, rats of the restricted feeding group showed a body weight decrease of −13% vs. day eight ($p < 0.001$) and vs. AC (−22%, $p < 0.001$) and AL (−26%, $p < 0.001$) that gained body weight (+10% and +13%, respectively; $p < 0.001$; **Figure 1**). Activity-based anorexia rats showed an additional body weight loss of −9% compared to rats of the restricted feeding group ($p < 0.001$; **Figure 1**) reaching an average body weight loss of −22% during the 14-day observation period. Three-way ANOVA showed a significant influence of time [$F_{(13, 504)} = 5.6$, $p < 0.001$], activity [$F_{(1, 504)} = 436.1$, $p < 0.001$] and feeding regimen [$F_{(1, 504)} = 9806.3$, $p < 0.001$] as well as an interaction of these three factors [$F_{(13, 504)} = 2.0$, $p = 0.02$].

It is to note that three out of 14 rats subjected to activity-based anorexia failed to lose body weight (or even gained body weight during the experimental period, data not shown) and were therefore excluded from further analyses (final $n = 11$).

Activity-Based Anorexia Rats Show a Similar Reduction in Food Intake As Observed in the Restricted Feeding Group

The food intake observed in the two ad libitum fed groups (ad libitum and activity group) did not differ from each other and was fairly stable over the 21-day observation period (**Figure 2**). While during the first 3 days of the habituation period—although during this time also fed ad libitum—the food intake was lower in the activity as well as activity-based anorexia group compared to the ad libitum and the restricted feeding groups ($p < 0.001$) giving rise to more time spent for physical activity and less for food intake, food intake was similar on days 6 and 7 (before food restriction) in all four groups (**Figure 2**). After food restriction to 1.5 h per day in the restricted feeding and activity-based anorexia group, food intake significantly dropped by −88% in these groups on the first day compared to the ad libitum group ($p < 0.001$) and slowly increased afterwards to reach the same level as observed in the ad libitum fed groups on the last day of the observation period (**Figure 2**). Overall, rats of the restricted feeding and activity-based anorexia group ate less (−38 and −41%, respectively) compared to the ad libitum group during the 14-day food restriction period (**Figure 2**). Three-way ANOVA indicated a significant impact of time [$F_{(20, 776)} = 24.5$, $p < 0.001$], activity [$F_{(1, 776)} = 68.3$, $p < 0.001$] and feeding regimen [$F_{(1, 776)} = 626.5$, $p < 0.001$] as well as an interaction of these three factors [$F_{(20, 776)} = 1.8$, $p = 0.02$]. Food intake during 1.5 h did not differ between the restricted feeding and activity-based anorexia group, while 24-h food intake did not differ between the ad libitum and activity group ($p > 0.05$; **Figure 3**).

Activity-Based Anorexia Rats Show a Similar Physical Activity As Observed in the Activity Group

Physical activity assessed using a running wheel slightly increased during the first week from ~1500 to ~2000 wheel rotations/day (**Figure 4**). During the food restriction period, physical activity more prominently increased reaching ~3500 wheel rotations/day in both, the activity and activity-based anorexia groups (**Figure 4**), corresponding to ~1300 m/day. No daily differences were observed either in the 1.5-h (data not

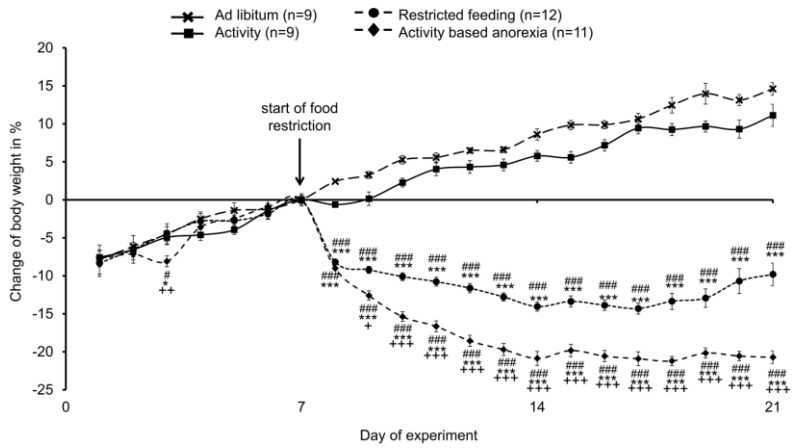

FIGURE 1 | Activity-based anorexia rats show the greatest body weight loss compared to all other groups. Animals had access to a running wheel for 24 h/day (activity and activity-based anorexia group) or were housed without wheel access under otherwise similar conditions (ad libitum and restricted feeding group). On day eight food intake was restricted to 1.5 h/day in the restricted feeding and activity-based anorexia group, while the activity and ad libitum group retained access to food for 24 h/day. Body weight changes are expressed in % changes from the day of food restriction. Data are expressed as mean ± SEM. *p < 0.05 and ***p < 0.001 vs. ad libitum group; # p < 0.05 and # # # p < 0.001 vs. activity group; +p < 0.05, ++p<0.01 and +++p < 0.001 vs. restricted feeding group.

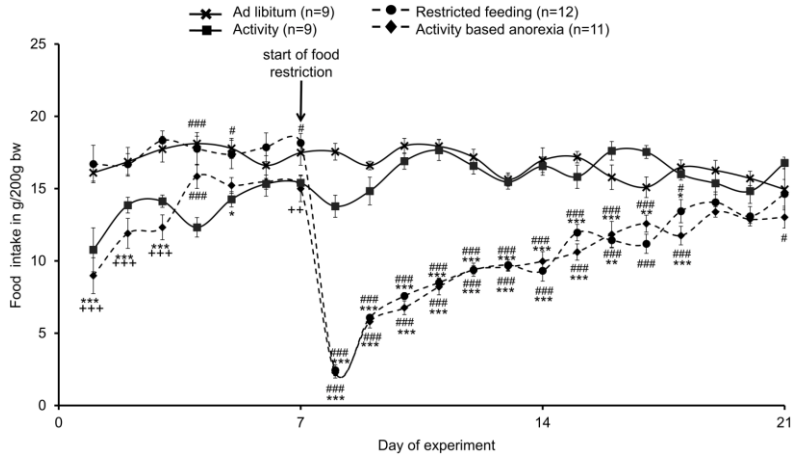

FIGURE 2 | Activity-based anorexia and restricted feeding group show a similar reduction in food intake over the whole observation period. Animals had access to a running wheel for 24 h/day (activity and activity-based anorexia group) or were housed without wheel access under otherwise similar conditions (ad libitum and restricted feeding group). On day eight food intake was restricted to 1.5 h/day in the restricted feeding and activity-based anorexia group, while the activity and ad libitum group retained access to food for 24 h/day. Food intake is calculated as g/200 g body weight; all data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. ad libitum group; # p < 0.05 and # # # p < 0.001 vs. activity group; ++p < 0.01 and + + +p < 0.001 vs. restricted feeding group.

shown) or 24-h wheel rotations between the activity and activity-based anorexia group ($p > 0.05$; **Figure 4**).

The daily energy expenditure including calculated resting energy expenditure and energy expenditure while running was ~35 kcal/200 g body weight at the beginning of the observation period (data not shown). This value slightly increased during the food restriction period in both the activity and activity-based anorexia group to ~38 kcal/200 g body weight (data not

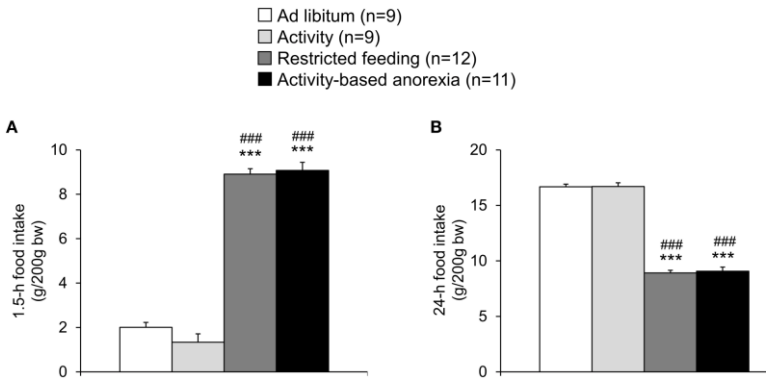

FIGURE 3 | Activity-based anorexia and restricted feeding group show a similar reduction in 1.5-h and 24-h food intake. Animals had access to a running wheel for 24 h/day (activity and activity-based anorexia group) or were housed without wheel access under otherwise similar conditions (ad libitum and restricted feeding group). On day eight food intake was restricted to 1.5 h/day in the restricted feeding and activity-based anorexia group, while the activity and ad libitum group retained access to food for 24 h/day. Food intake was monitored continuously using an automated food intake monitoring device and 1.5-h (A) as well as 24-h (B) food intake analyzed over a period of 4 days starting 4 days after food restriction (when a relatively stable food intake was observed). Data are expressed as mean ± SEM. ***$p < 0.001$ vs. ad libitum group and $^{\#\#\#}p < 0.001$ vs. activity group.

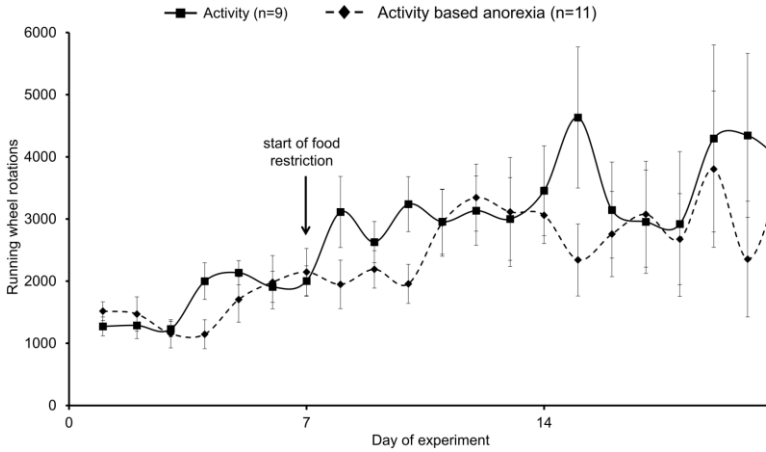

FIGURE 4 | Activity-based anorexia and activity group fed ad libitum show similar levels of physical activity. Animals had access to a running wheel for 24 h/day. On day eight food intake was restricted to 1.5 h/day in the activity-based anorexia group, while the activity group retained access to food for 24 h/day. Physical activity was monitored by measuring the wheel rotations per day. Data are expressed as mean ± SEM. $p > 0.05$.

shown). Two-way ANOVA indicated a signif cant impact of time [$F_{(20, 377)} = 3.6$, $p < 0.001$] and feeding regimen [$F_{(1, 377)} = 4.3$, $p = 0.04$].

Caloric def cit was calculated by subtracting energy expenditure from calculated caloric intake. All four groups showed a caloric surplus during the f rst 7 days of the experimental period (ranging from 9.6 to 21.4 kcal/day on day 7, data not shown). While this surplus remained visible in the ad libitum fed groups over the remaining 14-day observation period (14.2 ± 4.6 kcal in the ad libitum and 17.1 ± 2.3 kcal in the activity group), in the restricted feeding groups a caloric def cit was observed from the start of the food restriction (greatest levels on day 8: restricted feeding: −30.5 ± 1.4 kcal, activity-based anorexia: −29.6 ± 0.9 kcal) with a progressive decrease of caloric def cit reaching a surplus again on day 21 (restricted feeding: 10.4 ± 3.6 kcal, activity-based anorexia: 2.0 ± 0.9 kcal, data not

shown). Three-way ANOVA indicated a signif cant impact of time [$F_{(20, 771)} = 22.3$, $p < 0.001$], activity [$F_{(1, 771)} = 125.8$, $p < 0.001$] and feeding regimen [$F_{(1, 771)} = 666.7$, $p < 0.001$] as well as an interaction of these three factors [($F_{(20, 771)} = 2.0$, $p < 0.01$].

Activity-Based Anorexia Rats Show a Similar Food Intake Microstructure Compared to the Restricted Feeding Group

After analysis of overall daily 24-h (in the ad libitum fed groups) and 1.5-h (in the restricted feeding groups) food intake, the underlying food intake microstructure was assessed using an automated food intake monitoring device. When analyzing the 1.5-h food intake microstructure no dif erence was observed between both restricted feeding groups (restricted feeding and activity-based anorexia, $p > 0.05$; **Figures 5A–G**). Similarly, the two ad libitum fed groups did not show a dif erence except for the bout size which was smaller in the activity compared to the ad libitum group ($p < 0.01$; **Figure 5A**). The two restricted feeding groups showed signif cantly higher levels for several parameters of the food intake microstructure such as meal size ($p < 0.001$; **Figure 5B**), bout frequency ($p < 0.001$; **Figure 5C**), meal duration ($p < 0.001$; **Figure 5E**), time spent in meals ($p < 0.001$; **Figure 5F**) and eating rate ($p < 0.001$; **Figure 5G**), while for meal frequency the dif erence was only observed when compared to the activity group ($p < 0.05$; **Figure 5D**).

Also when analyzing the 24-h food intake microstructure, no dif erence was observed between the two ad libitum fed groups (ad libitum and activity) or the two restricted feeding groups (restricted feeding and activity-based anorexia, $p > 0.05$; **Figures 6A–G**). While both restricted feeding groups showed higher levels in bout size ($p < 0.001$; **Figure 6A**), meal size ($p < 0.001$; **Figure 6B**), meal duration ($p < 0.001$; **Figure 6E**) and eating rate ($p < 0.001$; **Figure 6G**), values were lower for—as expected based on the feeding schedule—bout frequency ($p < 0.001$; **Figure 6C**), meal frequency ($p < 0.001$; **Figure 6D**) and time spent in meals ($p < 0.001$; **Figure 6F**).

Activity-Based Anorexia Robustly Activates Several Brain Nuclei in Different Areas of the Brain

To investigate neuronal activation of brain areas under conditions of activity-based anorexia we performed immunohistochemistry for the activity marker Fos. After pre-absorption of the Fos antibody with a synthetic Fos fragment no immunostaining was observed (data not shown) indicating the specif city of the antibody.

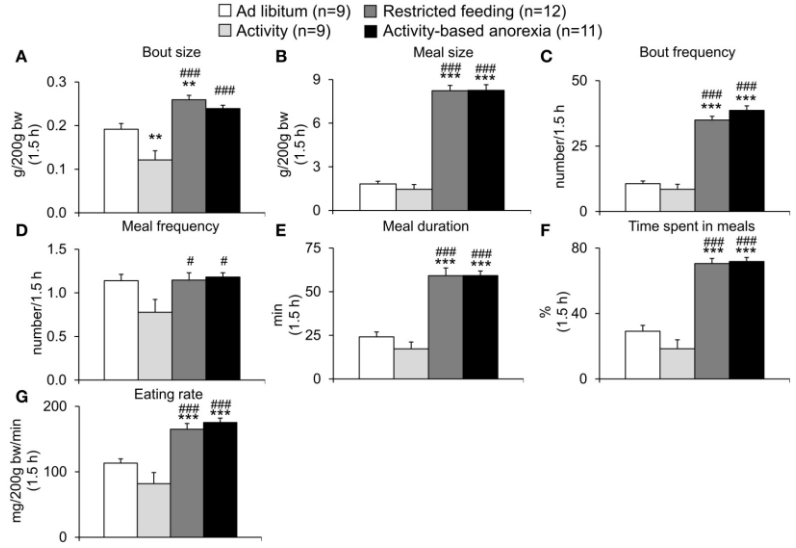

FIGURE 5 | Activity-based anorexia and restricted feeding group show similar changes in the 1.5-h food intake microstructure. Animals had access to a running wheel for 24 h/day (activity and activity-based anorexia group) or were housed without wheel access under otherwise similar conditions (ad libitum and restricted feeding group). On day eight food intake was restricted to 1.5 h/day (from 9:00 to 10:30 a.m.) in the restricted feeding and activity-based anorexia group, while the activity and ad libitum group retained access to food for 24 h/day. Food intake was monitored continuously using an automated food intake monitoring device and parameters of the food intake microstructure during the 1.5-h restricted feeding period, namely bout size (A), meal size (B), bout frequency (C), meal frequency (D), meal duration (E), time spent in meals (F), and eating rate (G) analyzed over a period of 4 days starting 4 days after food restriction (when a relatively stable food intake was observed). Data are expressed as mean ± SEM. **p < 0.01 and ***p < 0.001 vs. ad libitum group; # p < 0.05 and # # # p < 0.001 vs. activity group.

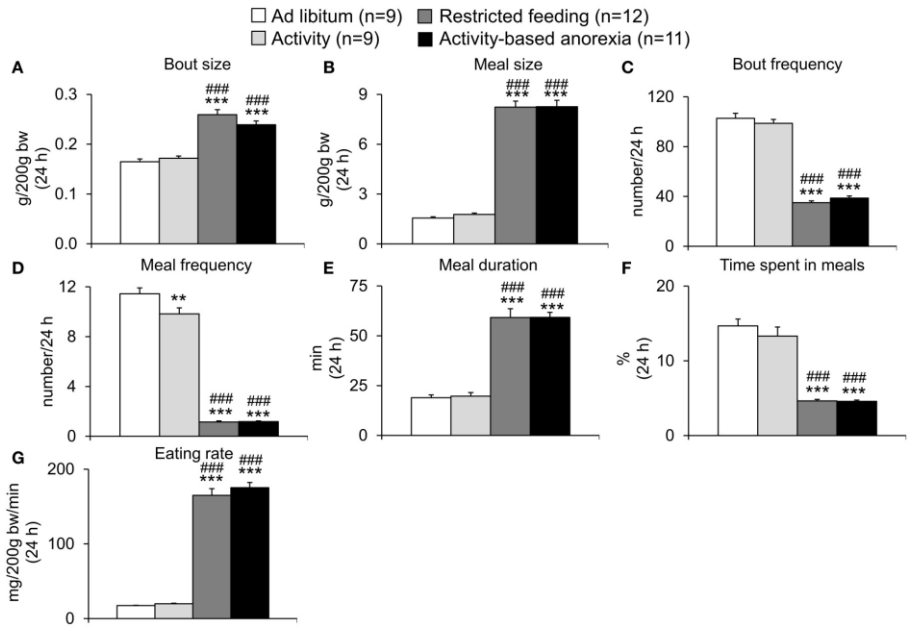

FIGURE 6 | Activity-based anorexia and restricted feeding group show similar changes in the 24-h food intake microstructure. Animals had access to a running wheel for 24 h/day (activity and activity-based anorexia group) or were housed without wheel access under otherwise similar conditions (ad libitum and restricted feeding group). On day eight food intake was restricted to 1.5 h/day (from 9:00 to 10:30 a.m.) in the restricted feeding and activity-based anorexia group, while the activity and ad libitum group retained access to food for 24 h/day. Food intake was monitored continuously using an automated food intake monitoring device and parameters of the 24-h food intake microstructure, namely bout size (A), meal size (B), bout frequency (C), meal frequency (D), meal duration (E), time spent in meals (F), and eating rate (G) analyzed over a period of 4 days starting 4 days after food restriction (when a relatively stable food intake was observed). Data are expressed as mean ± SEM. **$p < 0.01$ and ***$p < 0.001$ vs. ad libitum group and $^{\#\#\#}$ $p < 0.001$ vs. activity group.

Overall, activity-based anorexia rats showed higher Fos activation levels compared to ad libitum fed rats (**Table 1**). In the forebrain, higher activity was observed in the piriform cortex, cingulate cortex, somatomotor cortex, lateral septal nucleus, caudate putamen and hippocampus (**Figures 7A,B**) of activity-based anorexia rats compared to the ad libitum group, while no activation was observed in the amygdala (**Table 1**). In the thalamus higher Fos activation was observed in the paraventricular thalamic nucleus of activity-based anorexia rats compared to the ad libitum group, while in the lateral habenula similar numbers of Fos positive cells were detected using semiquantitative assessment (**Table 1**). Evaluation of hypothalamic nuclei showed more Fos signals in the suprachiasmatic nucleus, supraoptic nucleus (**Figures 7C,D**), anterior hypothalamic area, both magno- and parvocellular parts of the paraventricular nucleus (**Figures 7E,F**), lateral hypothalamic area (**Figures 7G,H**), dorsomedial hypothalamic nucleus (**Figures 7I,J**) and the medial part of the arcuate nucleus (**Figures 7K,L**) of activity-based anorexia rats compared to the ad libitum group, while similar levels were observed in the ventromedial hypothalamic nucleus (**Table 1**). In the midbrain similar levels were detected in the Edinger-Westphal nucleus (**Figures 8A,B**), while higher activation was observed in the dorsal raphe nuclei (**Figures 8C,D**) and locus coeruleus (**Figures 8E,F**) of activity-based anorexia compared to the ad libitum fed rats (**Table 1**). Lastly, also in the medulla a higher activation was observed in activity-based anorexia rats compared to the ad libitum group, namely in the raphe pallidus nucleus (**Figures 9A,B**), area postrema, rostral part of the nucleus of the solitary tract (**Figures 9C,D**) and the dorsal motor nucleus of the vagus nerve (**Table 1**).

DISCUSSION

In the present study we first established the activity-based anorexia model combining voluntary physical activity in a running wheel and a time-restricted feeding protocol and showed a body weight loss of −22% following 2 weeks of restricted feeding for 1.5 h per day and 24-h access to a running wheel. This body weight loss was greater (−9%) than that observed in the restricted feeding group, while rats of the ad libitum and activity group gained body weight. These data indicate the importance

TABLE 1 | Localization of Fos positive neurons in brains of rats fed ad libitum or under conditions of activity-based anorexia.

Area	Brain structure	Ad libitum	Activity based anorexia
Forebrain	Piriform cortex	++	+++
	Cingulate cortex	+	++
	Somatomotor cortex	+	+++
	Lateral septal nucleus	+ – ++	+++
	Caudate putamen	++	++ – +++
	Amygdala (central, medial and basolateral)	–	–
	Hippocampus	+ – ++	+++
Thalamus	Paraventricular thalamic nucleus, anterior part	+	++
	Lateral habenula	+ – ++	+ – ++
Hypothalamus	Suprachiasmatic nucleus	++	++ – +++
	Supraoptic nucleus	–	+++
	Anterior hypothalamic area	+	++
	Paraventricular nucleus	+	++
	Lateral hypothalamic area	++	+++
	Ventromedial hypothalamic nucleus	++	++
	Dorsomedial hypothalamic nucleus	–	++
	Arcuate nucleus, medial	+	+++
Midbrain	Edinger-Westphal nucleus	++	++
	Dorsal raphe nuclei	+	++
	Locus coeruleus	–	+
Medulla	Raphe pallidus nucleus	+	++
	Area postrema	–	+ – ++
	Nucleus of the solitary tract, rostral	–	+ – ++
	Dorsal motor nucleus of the vagus nerve	–	– – +

Data are expressed as mean derived from three rats/group. It is to note that rats in the activity-based anorexia group received a fixed amount of 1.5 g during the feeding period in order to avoid signals derived from overfilling of the stomach and/or nausea. Fos expression is expressed semi-quantitatively as +, low (~1–10 cells); ++, medium (~10–20 cells); and +++, high (>20 Fos positive cells in a 100 μm × 100 μm area of an ocular grid with a 10x objective) density of expression; –, no cells.

of food restriction for the present model. To gain further insight into the underlying changes, we next analyzed the food intake microstructure which greatly differed from the microstructure observed in the ad libitum fed groups (ad libitum and activity group) but was very similar to the one observed in the restricted feeding group. The increase in 1.5-h food intake observed in the two groups kept on the restricted feeding schedule was based on a larger meal size and an increase in eating rate. During these 1.5 h, rats spent 71% of the time in meals (eating and interacting with the food hopper). It is important to note that despite this great drive to eat, rats still exercised during this 1.5-h period.

Interestingly, daily activity was similar between the activity and activity-based anorexia group; however, daily activity increased over time in both groups. These data are in line with previous data from mice where daily activity also increased over time; however, in mice daily activity decreased during the last days of the 2.5-week observation period (Jésus et al., 2014). Whether this represents a species difference (rats vs. mice), sex difference (female vs. male) or is related to the slower weight loss in rats (−20% reached at day 15 vs. 11 in mice) warrants further investigation. At the same time we did not observe a decrease in food intake (which rather plateaued) which is different from the human situation where food intake decreases while activity increases with the progression of the disease (Davis et al., 1994). The relatively short observation period of 3 weeks likely contributes to/explains this difference. Taken together, the combination of both food restriction and activity is key in order to exert the pronounced weight loss observed in the activity-based anorexia group.

It is to note that in the present study three out of 14 rats did not develop activity-based anorexia and were therefore excluded from further analyses. This finding is in line with previous studies reporting that 20–30% of rats are not interested in running (Mondon et al., 1985) and do not develop activity-based anorexia (Carrera et al., 2014). These data well match the dropout rate described here (3 out of 14 = 21%). Interestingly, this finding also parallels human data where up to 80% of anorexic subjects display hyperactivity, whereas 20% do not (Davis et al., 1997). Whether a difference in leptin levels, hypothesized to play a role in the semi-starvation-induced hyperactivity in rats before (Exner et al., 2000), contributes to these differences will have to be further investigated. On the other hand, all rats of the restricted feeding group (12/12) show a reduction in body weight indicating that this effect cannot be eluded. However, the fact that rats of the activity-based anorexia group show a greater body weight loss than the restricted feeding group highlights the importance of the physical activity.

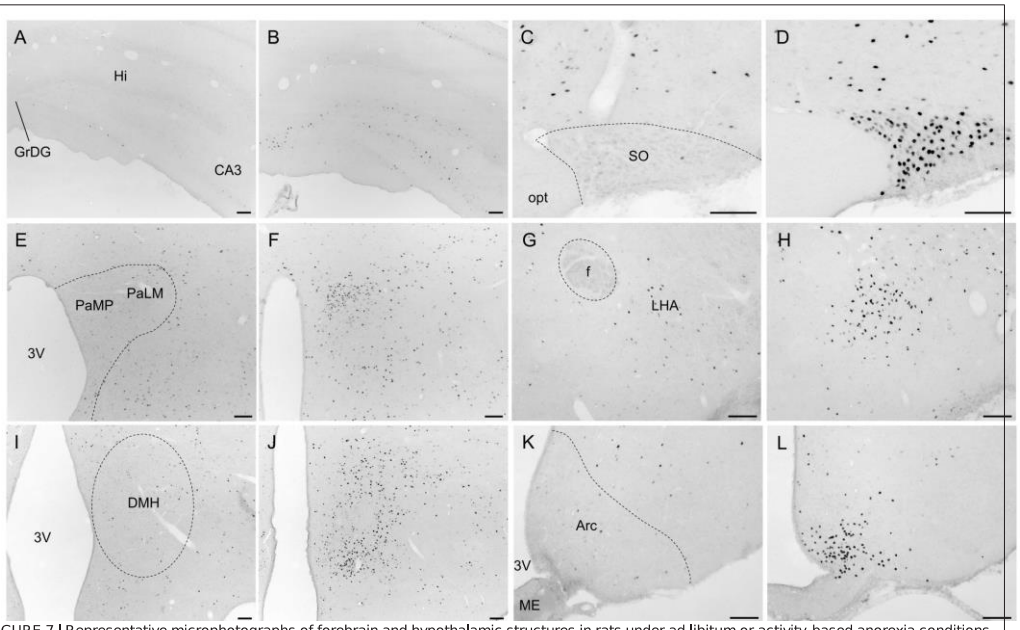

FIGURE 7 | Representative microphotographs of forebrain and hypothalamic structures in rats under ad libitum or activity-based anorexia conditions. Following the 21-day observation period rats received food for 1.5 h (activity-based anorexia) or were fed ad libitum and were transcardially perfused directly after this 1.5-h feeding period. It is to note that rats in the activity-based anorexia group received a fixed amount of 1.5 g during this feeding period in order to avoid signals derived from overfilling of the stomach and/or nausea. Brains were processed for Fos immunohistochemistry. Signals were observed in the hippocampus (B), supraoptic nucleus (D), magno- und parvocellular parts of the paraventricular nucleus (F), lateral hypothalamic area (H), dorsomedial hypothalamic nucleus (J) and medial part of the arcuate nucleus (L) of activity-based anorexia rats, while in the respective nuclei of the ad libitum group no (C,I) or few (A,E,G,K) signals were detected. The scale bars indicate 100 μm. Abbreviations: 3V, third ventricle; Arc, arcuate nucleus; CA3, field CA3 of the hippocampus; DMH, dorsomedial hypothalamic nucleus; f, fornix; GrDG, granular layer of the dentate gyrus; Hi, hippocampus; LHA, lateral hypothalamic area; ME, median eminence; opt, optic tract; PaLM, lateral magnocellular part of the paraventricular nucleus of the hypothalamus; PaMP, medial parvocellular part of the paraventricular nucleus of the hypothalamus; SO, supraoptic nucleus.

Despite the fact that the data mentioned above give rise to the use of the activity-based anorexia model as a suited tool to study pathophysiological alterations of AN, several limitations should be kept in mind. Although restriction of food and increased physical activity, two mean features of AN (Treasure et al., 2015) are used in this model, several other aspects such as genetic susceptibility (Clarke et al., 2012) or psychosocial and interpersonal factors (Zipfel et al., 2015) are not respected. Moreover, rats do not voluntarily reduce their body weight in contrast to human anorexic subjects. Whenever the rats' access to food is increased again, they start to regain body weight (Dixon et al., 2003; Ratnovsky and Neuman, 2011). Furthermore, the changes induced here are rather acute or subacute, while human AN is a chronic disease. Interestingly, after the initial sharp decline of food intake rats of the activity-based anorexia and restricted feeding group show a gradual increase of food intake reaching similar levels of daily food intake as observed in the ad libitum fed groups. Whether different dietary patterns as observed in human anorexia (Huse and Lucas, 1984; Elran-Barak et al., 2014) or a change of dietary patterns over time occurs in these rats as well will have to be further determined, preferably in a study with a longer monitoring time. Taken together, cautious interpretation of the data obtained in this model is necessary.

Several other anorexia models have been developed encompassing genetically engineered mouse models that share similarities with changes observed in AN; however, none of these reflect the multiple hormonal changes observed in AN (Méquinion et al., 2015). Lastly, other models use access to low caloric food or expose rats to various kinds of stressors (Méquinion et al., 2015). However, it is important to note that so far activity-based anorexia is considered the best animal model (Gutierrez, 2013) as it recapitulates two main features, physical activity and reduced food intake, of human AN.

To further characterize the activity-based anorexia rats we also investigated the activation of brain nuclei using the activity marker Fos (Sagar et al., 1988) and performed a whole brain mapping for activity-based anorexia and ad libitum fed rats. Neuronal activation was observed in brain areas involved in

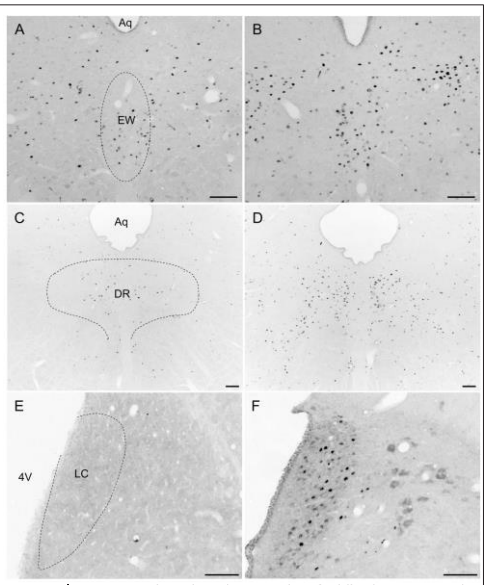

FIGURE 8 | Representative microphotographs of midbrain structures in rats under ad libitum or activity-based anorexia conditions. Following the 21-day observation period rats received food for 1.5 h (activity-based anorexia) or were fed ad libitum and were transcardially perfused directly after this 1.5-h feeding period. It is to note that rats in the activity-based anorexia group received a fixed amount of 1.5 g during this feeding period in order to avoid signals derived from overfilling of the stomach and/or nausea. Brains were processed for Fos immunohistochemistry. Signals were observed in the Edinger-Westphal nucleus (B), dorsal raphe nuclei (D) and locus coeruleus (F) of the activity-based anorexia group, while in the respective nuclei of the ad libitum group no (E), or few (C) signals were detected. It is to note that signals of similar density were observed in the Edinger-Westphal nucleus (A) of the ad libitum group. The scale bars indicate 100 µm. Abbreviations: 4V, fourth ventricle; Aq, aqueduct; DR, dorsal raphe nuclei; EW, Edinger-Westphal nucleus; LC, locus coeruleus.

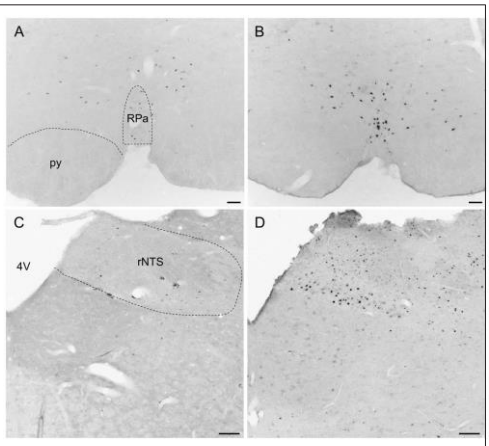

FIGURE 9 | Representative microphotographs of medulla structures in rats under ad libitum or activity-based anorexia conditions. Following the 21-day observation period rats received food for 1.5 h (activity-based anorexia) or were fed ad libitum and were transcardially perfused directly after this 1.5-h feeding period. It is to note that rats in the activity-based anorexia group received a fixed amount of 1.5 g during this feeding period to avoid signals derived from overfilling of the stomach and/or nausea. Signals were observed in the raphe pallidus nucleus (B) and the rostral part of the nucleus of the solitary tract (D) of the activity-based anorexia group, while in the respective nuclei of the ad libitum group no (C) or few (A) Fos positive neurons were detected. The scale bars indicate 100 µm. Abbreviations: 4V, fourth ventricle; py, pyramidal tract; rNTS, rostral part of the nucleus of the solitary tract; RPa, raphe pallidus nucleus.

the regulation of several functions such as motor activity, stress response, food intake and thermogenesis.

Analyzing brain areas involved in olfaction and the processing of olfactory stimuli (Roullet et al., 2005) an increased activation of neurons was observed in the piriform cortex, while in the lateral habenula similar Fos expression was observed in activity-based anorexia and ad libitum fed rats. This activation is likely associated with the increased interaction with food (as reflected in the increased number of bouts in activity-based anorexia rats compared to the ad libitum group) as well as the stimulated food intake during the 1.5-h feeding period. In line with this assumption, key areas of food intake regulation were activated as well, namely the lateral septal nucleus (Mitra et al., 2015), lateral hypothalamic area (Bernardis and Bellinger, 1993), the dorsomedial hypothalamic nucleus and the medial part of the Arc, both expressing the potent orexigenic transmitter neuropeptide Y (Wang et al., 2002; Bi et al., 2012) and lastly also the nucleus of the solitary tract (Stengel and Taché, 2011). Further corroborating the involvement of these nuclei in the orexigenic drive under conditions of activity-based anorexia, a previous study reported a robust upregulation of orexigenic agouti-related peptide and neuropeptide Y, whereas the anorexigenic transmitters pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) were reduced in the Arc of activity-based anorexia rats compared to sedentary food-restricted controls (de Rijke et al., 2005). Moreover, in the lateral hypothalamic area melanin-concentrating hormone mRNA expression was increased in activity-based anorexia rats (de Rijke et al., 2005). Associated with the orexigenic response, also brain nuclei involved in the regulation of gastrointestinal motility were activated, namely the lateral hypothalamic area (Gong et al., 2013), nucleus of the solitary tract and the dorsal motor nucleus of the vagus nerve (Stengel and Taché, 2011). This pronounced activation likely underlies the robust orexigenic response of activity-based anorexia rats observed during the 1.5-h feeding period. It is to note that—although food intake was restricted to 1.5 g in the last feeding period before brain processing for Fos immunohistochemistry to avoid unspecific gastric distention and nauseating signals—a moderate activation of the area postrema, known to be involved in the mediation of nausea (Horn, 2014), has been observed in the activity-based anorexia but not in the ad libitum fed group. Therefore, nauseating signals—at least to a certain extent—might play a

role in this model as well and may modulate/limit food intake displayed during the restricted feeding period.

Besides the restriction of food intake, the stimulation of activity contributes to the weight loss observed in activity-based anorexia rats. Respective nuclei activated under these conditions and therefore likely implicated in the stimulation of activity encompass the somatomotor cortex (Elias et al., 2008) and caudate putamen (David et al., 2005). Interestingly, especially the dorsomedial hypothalamic nucleus has been implicated in the mediation of food-anticipatory activity under fixed-feeding conditions (Verhagen et al., 2011) as also observed in the present study. This activation likely also involves the suprachiasmatic nucleus working in a modulatory manner as part of an intrahypothalamic system (Acosta-Galvan et al., 2011). Moreover, the dorsomedial hypothalamic nucleus was implicated in the food-entrainable-related preprandial rise of body temperature, an effect that vanished after lesion of the nucleus (Gooley et al., 2006). This thermogenic response might also contribute to the observed decrease in body weight. However, it is important to note that activity-based anorexia was associated with a hypothermic response before (Hillebrand et al., 2005a) and an increase in ambient temperature was reported to reduce physical activity (Gutierrez et al., 2008). Future studies should further investigate these—likely very dynamic—changes of body temperature in activity-based anorexia rats.

Also stress mediated via the hypothalamic-pituitary-adrenal gland axis might play a role in the reduction of food intake and stimulation of physical activity. In the present study we observed a robust activation of lateral parvocellular neurons of the hypothalamic paraventricular nucleus of activity-based anorexia compared to ad libitum fed rats. This region is known for its predominant expression of corticotropin-releasing factor (CRF). Moreover, CRF mRNA expression was also reported to rise in the dorsomedial hypothalamic nucleus in rats with access to a running wheel (Kawaguchi et al., 2005). Interestingly, intracerebroventricular injection of the CRF antagonist, alpha-helical CRF attenuated the wheel-induced reduction of food intake and body weight (Kawaguchi et al., 2005) giving rise to a role of stimulated CRF signaling in activity-based anorexia. Lastly, this likely contributes to the increased circulating levels of corticosterone in rats (Burden et al., 1993) and cortisol in human anorexic subjects (Casper et al., 1979).

Lastly, also psychological parameters such as anxiety (Swinbourne and Touyz, 2007) and depressiveness (Deboska et al., 2011) are often altered under conditions of AN. In the present study we observed an increased activation of the dorsal raphe nuclei and the raphe pallidus nucleus under conditions of activity-based anorexia, serotonergic nuclei that might play a role in the pathogenesis of depression (Michelsen et al., 2008). Interestingly, intraperitoneal injections of the serotonin agonist fenfluramine accelerated weight loss under conditions of activity-based anorexia compared to pair-fed controls giving rise to a mechanism other than reduced food intake (Atchley and Eckel, 2005). While the amygdala was not activated in the present study, the noradrenergic locus coeruleus showed a moderate activation in activity-based anorexia rats possibly leading to increased arousal (Aston-Jones and Waterhouse, 2016). Ascending projections might be involved in the observed activation of the paraventricular thalamic nucleus, the cingulate cortex and the hippocampus, brain structures involved in the processing of emotions and memory (Rolls, 2015). Interestingly, also the supraoptic nucleus as well as some magnocellular neurons of the paraventricular nucleus of the hypothalamus, two brain nuclei prominently expressing oxytocin, showed a robust activation in activity-based anorexia rats. It is important to note that oxytocin has been—besides its well-defined role during pregnancy—implicated in social memory, aggression and anxiety (Caldwell et al., 2016). Whether there is a direct link between anxiety or depressiveness and physical activity as suggested in humans (Holtkamp et al., 2004) warrants further investigation.

In summary, the activity-based anorexia model combines voluntary physical activity in a running wheel and time-restricted feeding to greatly reduce body weight. Interestingly, the food intake microstructure observed in activity-based anorexia rats did not differ from the one observed in the restricted feeding group arguing against a specific feeding phenotype. Also physical activity did not differ from the respective control group. Activity-based anorexia rats displayed an activation of distinct brain nuclei involved in the mediation of food intake, physical activity, thermoregulation as well as depression/anxiety and stress. Although these animal data have to be interpreted with caution, current data point toward the usefulness of the model to better understand pathophysiological alterations also occurring in AN.

AUTHOR CONTRIBUTIONS

SS performed the experiments and drafted the manuscript. PP performed the experiments and analyzed the data. MGS and PK performed the experiments and reviewed the manuscript. TH wrote and reviewed the manuscript. MR gave critical input throughout the study and reviewed the manuscript. AS planned the experiments, analyzed the data and wrote the manuscript.

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REFERENCES

Acosta-Galvan, G., Yi, C. X., Van Der Vliet, J., Jhamandas, J. H., Panula, P., Angeles-Castellanos, M., et al. (2011). Interaction between hypothalamic dorsomedial nucleus and the suprachiasmatic nucleus determines intensity of food anticipatory behavior. Proc. Natl. Acad. Sci. U.S.A. 108, 5813–5818. doi: 10.1073/pnas.1015551108

American Psychiatric Association (2013). Diagnostic and Statistical Manual of Mental Disorders, 5th Edn (DSM-5). Washington, DC.

Aoki, C., Sabaliauskas, N., Chowdhury, T., Min, J. Y., Colacino, A. R., Laurino, K., et al. (2012). Adolescent female rats exhibiting activity-based anorexia express elevated levels of GABA(A) receptor alpha4 and delta subunits at the plasma membrane of hippocampal CA1 spines. Synapse 66, 391–407. doi: 10.1002/syn.21528

Arcelus, J., Mitchell, A. J., Wales, J., and Nielsen, S. (2011). Mortality rates in patients with anorexia nervosa and other eating disorders. A meta-analysis of 36 studies. Arch. Gen. Psychiatry 68, 724–731. doi: 10.1001/archgenpsychiatry.2011.74

Aston-Jones, G., and Waterhouse, B. (2016). Locus coeruleus: from global projection system to adaptive regulation of behavior. Brain Res. 1645, 75–78. doi: 10.1016/j.brainres.2016.03.001

Atchley, D. P., and Eckel, L. A. (2005). Fenfluramine treatment in female rats accelerates the weight loss associated with activity-based anorexia. Pharmacol. Biochem. Behav. 80, 273–279. doi: 10.1016/j.pbb.2004.11.006

Bernardis, L. L., and Bellinger, L. L. (1993). The lateral hypothalamic area revisited: neuroanatomy, body weight regulation, neuroendocrinology and metabolism. Neurosci. Biobehav. Rev. 17, 141–193. doi: 10.1016/S0149-7634(05)80149-6

Bi, S., Kim, Y. J., and Zheng, F. (2012). Dorsomedial hypothalamic NPY and energy balance control. Neuropeptides 46, 309–314. doi: 10.1016/j.npep.2012.09.002

Burden, V. R., White, B. D., Dean, R. G., and Martin, R. J. (1993). Activity of the hypothalamic-pituitary-adrenal axis is elevated in rats with activity-based anorexia. J. Nutr. 123, 1217–1225.

Caldwell, H. K., Aulino, E. A., Freeman, A. R., Miller, T. V., and Witchey, S. K. (2016). Oxytocin and behavior: lessons from knockout mice. Dev. Neurobiol. doi: 10.1002/dneu.22431. [Epub ahead of print].

Carrera, O., Fraga, Á., Pellón, R., and Gutiérrez, E. (2014). Rodent model of activity-based anorexia. Curr. Protoc. Neurosci. 67, 9.47.41–11. doi: 10.1002/0471142301.ns0947s67

Casper, R. C., Chatterton, R. T. Jr., and Davis, J. M. (1979). Alterations in serum cortisol and its binding characteristics in anorexia nervosa. J. Clin. Endocrinol. Metab. 49, 406–411. doi: 10.1210/jcem-49-3-406

Casper, R. C., Sullivan, E. L., and Tecott, L. (2008). Relevance of animal models to human eating disorders and obesity. Psychopharmacology (Berl) 199, 313–329. doi: 10.1007/s00213-008-1102-2

Casteels, C., Gérard, N., Van Kuyck, K., Pottel, L., Nuttin, B., Bormans, G., et al. (2014). Small animal PET imaging of the type 1 cannabinoid receptor in a rodent model for anorexia nervosa. Eur. J. Nucl. Med. Mol. Imaging 41, 308–321. doi: 10.1007/s00259-013-2522-8

Chowdhury, T. G., Ríos, M. B., Chan, T. E., Cassataro, D. S., Barbarich-Marsteller, N. C., and Aoki, C. (2014). Activity-based anorexia during adolescence disrupts normal development of the CA1 pyramidal cells in the ventral hippocampus of female rats. Hippocampus 24, 1421–1429. doi: 10.1002/hipo.22320

Clarke, T. K., Weiss, A. R., and Berrettini, W. H. (2012). The genetics of anorexia nervosa. Clin. Pharmacol. Ther. 91, 181–188. doi: 10.1038/clpt.2011.253

David, H. N., Ansseau, M., and Abraini, J. H. (2005). Dopamine-glutamate reciprocal modulation of release and motor responses in the rat caudate-putamen and nucleus accumbens of "intact" animals. Brain Res. Brain Res. Rev. 50, 336–360. doi: 10.1016/j.brainresrev.2005.09.002

Davis, C., Katzman, D. K., Kaptein, S., Kirsh, C., Brewer, H., Kalmbach, K., et al. (1997). The prevalence of high-level exercise in the eating disorders: etiological implications. Compr. Psychiatry 38, 321–326. doi: 10.1016/S0010-440X(97)90927-5

Davis, C., Kennedy, S. H., Ravelski, E., and Dionne, M. (1994). The role of physical activity in the development and maintenance of eating disorders. Psychol. Med. 24, 957–967. doi: 10.1017/S0033291700029044

Deboska, E., Janas, A., Banczyk, W., and Janas-Kozik, M. (2011). Depression or depressiveness in patients diagnosed with anorexia nervosa and bulimia nervosa - pilot research. Psychiatr. Danub. 23, S87–S90.

de Rijke, C. E., Hillebrand, J. J., Verhagen, L. A., Roeling, T. A., and Adan, R. A. (2005). Hypothalamic neuropeptide expression following chronic food restriction in sedentary and wheel-running rats. J. Mol. Endocrinol. 35, 381–390. doi: 10.1677/jme.1.01808

Dixon, D. P., Ackert, A. M., and Eckel, L. A. (2003). Development of, and recovery from, activity-based anorexia in female rats. Physiol. Behav. 80, 273–279. doi: 10.1016/j.physbeh.2003.08.008

Doerries, L. E., Stanley, E. Z., and Aravich, P. F. (1991). Activity-based anorexia: relationship to gender and activity-stress ulcers. Physiol. Behav. 50, 945–949. doi: 10.1016/0031-9384(91)90419-O

Elias, C. F., Sita, L. V., Zambon, B. K., Oliveira, E. R., Vasconcelos, L. A., and Bittencourt, J. C. (2008). Melanin-concentrating hormone projections to areas involved in somatomotor responses. J. Chem. Neuroanat. 35, 188–201. doi: 10.1016/j.jchemneu.2007.10.002

Elran-Barak, R., Accurso, E. C., Goldschmidt, A. B., Sztainer, M., Byrne, C., and Le Grange, D. (2014). Eating patterns in youth with restricting and binge eating/purging type anorexia nervosa. Int. J. Eat. Disord. 47, 878–883. doi: 10.1002/eat.22289

Exner, C., Hebebrand, J., Remschmidt, H., Wewetzer, C., Ziegler, A., Herpertz, S., et al. (2000). Leptin suppresses semi-starvation induced hyperactivity in rats: implications for anorexia nervosa. Mol. Psychiatry 5, 476–481. doi: 10.1038/sj.mp.4000771

Ghadirian, A. M., Engelsmann, F., Leichner, P., and Marshall, M. (1993). Prevalence of psychosomatic and other medical illnesses in anorexic and bulimic patients. Behav. Neurol. 6, 123–127. doi: 10.1155/1993/356914

Gong, Y., Xu, L., Wang, H., Guo, F., Sun, X., and Gao, S. (2013). Involvements of the lateral hypothalamic area in gastric motility and its regulation by the lateral septum. Gen. Comp. Endocrinol. 194, 275–285. doi: 10.1016/j.ygcen.2013.09.022

Gooley, J. J., Schomer, A., and Saper, C. B. (2006). The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms. Nat. Neurosci. 9, 398–407. doi: 10.1038/nn1651

Gutierrez, E. (2013). A rat in the labyrinth of anorexia nervosa: contributions of the activity-based anorexia rodent model to the understanding of anorexia nervosa. Int. J. Eat. Disord. 46, 289–301. doi: 10.1002/eat.22095

Gutierrez, E., Cerrato, M., Carrera, O., and Vazquez, R. (2008). Heat reversal of activity-based anorexia: implications for the treatment of anorexia nervosa. Int. J. Eat. Disord. 41, 594–601. doi: 10.1002/eat.20535

Herzog, W., Schellberg, D., and Deter, H. C. (1997). First recovery in anorexia nervosa patients in the long-term course: a discrete-time survival analysis. J. Consult. Clin. Psychol. 65, 169–177. doi: 10.1037/0022-006X.65.1.169

Hillebrand, J. J., de Rijke, C. E., Brakkee, J. H., Kas, M. J., and Adan, R. A. (2005a). Voluntary access to a warm plate reduces hyperactivity in activity-based anorexia. Physiol. Behav. 85, 151–157. doi: 10.1016/j.physbeh.2005.03.017

Hillebrand, J. J., Koeners, M. P., de Rijke, C. E., Kas, M. J., and Adan, R. A. (2005b). Leptin treatment in activity-based anorexia. Biol. Psychiatry 58, 165–171. doi: 10.1016/j.biopsych.2005.03.011

Holtkamp, K., Hebebrand, J., and Herpertz-Dahlmann, B. (2004). The contribution of anxiety and food restriction on physical activity levels in acute anorexia nervosa. Int. J. Eat. Disord. 36, 163–171. doi: 10.1002/eat.20035

Horn, C. C. (2014). The medical implications of gastrointestinal vagal afferent pathways in nausea and vomiting. Curr. Pharm. Des. 20, 2703–2712. doi: 10.2174/13816128113199990568

Huber, J., Salatsch, C., Ingenerf, K., Schmid, C., Maatouk, I., Weisbrod, M., et al. (2015). Characteristics of disorder-related autobiographical memory in acute anorexia nervosa patients. Eur. Eat. Disord. Rev. 23, 379–389. doi: 10.1002/erv.2379

Hudson, J. I., Hiripi, E., Pope, H. G. Jr., and Kessler, R. C. (2007). The prevalence and correlates of eating disorders in the National Comorbidity Survey Replication. Biol. Psychiatry 61, 348–358. doi: 10.1016/j.biopsych.2006.03.040

Huse, D. M., and Lucas, A. R. (1984). Dietary patterns in anorexia nervosa. Am. J. Clin. Nutr. 40, 251–254.

Jésus, P., Ouelaa, W., Francois, M., Riachy, L., Guérin, C., Aziz, M., et al. (2014). Alteration of intestinal barrier function during activity-based anorexia in mice. Clin. Nutr. 33, 1046–1053. doi: 10.1016/j.clnu.2013.11.006

Kawaguchi, M., Scott, K. A., Moran, T. H., and Bi, S. (2005). Dorsomedial hypothalamic corticotropin-releasing factor mediation of exercise-induced anorexia. Am. J. Physiol. Regul. Integr. Comp. Physiol. 288, R1800–R1805. doi: 10.1152/ajpregu.00805.2004

Kinzig, K. P., and Hargrave, S. L. (2010). Adolescent activity-based anorexia increases anxiety-like behavior in adulthood. Physiol. Behav. 101, 269–276. doi: 10.1016/j.physbeh.2010.05.010

Kjaersdam Telléus, G., Jepsen, J. R., Bentz, M., Christiansen, E., Jensen, S. O., Fagerlund, B., et al. (2015). Cognitive profile of children and adolescents with anorexia nervosa. Eur. Eat. Disord. Rev. 23, 34–42. doi: 10.1002/erv.2337

Kline, C. L. (1979). Anorexia nervosa: death from complications of ruptured gastric ulcer. Can. J. Psychiatry 24, 153–156.

Legrand, R., Lucas, N., Breton, J., Azhar, S., do Rego, J. C., Déchelotte, P., et al. (2016). Ghrelin treatment prevents development of activity based anorexia in mice. Eur. Neuropsychopharmacol. 26, 948–958. doi: 10.1016/j.euroneuro.2016.03.010

Luyten, L., Welkenhuysen, M., Van Kuyck, K., Fieuws, S., Das, J., Sciot, R., et al. (2009). The effects of electrical stimulation or an electrolytic lesion in the mediodorsal thalamus of the rat on survival, body weight, food intake and running activity in the activity-based anorexia model. Brain Res. Bull. 79, 116–122. doi: 10.1016/j.brainresbull.2009.01.001

Méquinion, M., Chauveau, C., and Viltart, O. (2015). The use of animal models to decipher physiological and neurobiological alterations of anorexia nervosa patients. Front. Endocrinol. (Lausanne) 6:68. doi: 10.3389/fendo.2015.00068

Michelsen, K. A., Prickaerts, J., and Steinbusch, H. W. (2008). The dorsal raphe nucleus and serotonin: implications for neuroplasticity linked to major depression and Alzheimer's disease. Prog. Brain Res. 172, 233–264. doi: 10.1016/S0079-6123(08)00912-6

Mitra, A., Lenglos, C., and Timofeeva, E. (2015). Inhibition in the lateral septum increases sucrose intake and decreases anorectic effects of stress. Eur. J. Neurosci. 41, 420–433. doi: 10.1111/ejn.12798

Mondon, C. E., Dolkas, C. B., Sims, C., and Reaven, G. M. (1985). Spontaneous running activity in male rats: effect of age. J. Appl. Physiol. (1985) 58, 1553–1557.

Nagl, M., Jacobi, C., Paul, M., Beesdo-Baum, K., Höfer, M., Lieb, R., et al. (2016). Prevalence, incidence, and natural course of anorexia and bulimia nervosa among adolescents and young adults. Eur. Child. Adolesc. Psychiatry 25, 903–918. doi: 10.1007/s00787-015-0808-z

Paulukat, L., Frintrop, L., Liesbrock, J., Heussen, N., Johann, S., Exner, C., et al. (2016). Memory impairment is associated with the loss of regular oestrous cycle and plasma oestradiol levels in an activity-based anorexia animal model. World J. Biol. Psychiatry 17, 274–284. doi: 10.3109/15622975.2016.1173725

Paxinos, G., and Watson, C. (2007). The Rat Brain in Stereotaxic Coordinates. San Diego, CA: Academic Press.

Preti, A., Girolamo, G. d., Vilagut, G., Alonso, J., Graaf, R. d., Bruffaerts, R., et al. (2009). The epidemiology of eating disorders in six European countries: results of the ESEMeD-WMH project. J. Psychiatr. Res. 43, 1125–1132. doi: 10.1016/j.jpsychires.2009.04.003

Ratnovsky, Y., and Neuman, P. (2011). The effect of pre-exposure and recovery type on activity-based anorexia in rats. Appetite 56, 567–576. doi: 10.1016/j.appet.2011.01.027

Rolls, E. T. (2015). Limbic systems for emotion and for memory, but no single limbic system. Cortex 62, 119–157. doi: 10.1016/j.cortex.2013.12.005

Roullet, F., Liénard, F., Datiche, F., and Cattarelli, M. (2005). Fos protein expression in olfactory-related brain areas after learning and after reactivation of a slowly acquired olfactory discrimination task in the rat. Learn. Mem. 12, 307–317. doi: 10.1101/lm.89605

Routtenberg, A., and Kuznesof, A. W. (1967). Self-starvation of rats living in activity wheels on a restricted feeding schedule. J. Comp. Physiol. Psychol. 64, 414–421. doi: 10.1037/h0025205

Sagar, S. M., Sharp, F. R., and Curran, T. (1988). Expression of c-fos protein in brain: metabolic mapping at the cellular level. Science 240, 1328–1331. doi: 10.1126/science.3131879

Shepherd, R. E., and Gollnick, P. D. (1976). Oxygen uptake of rats at different work intensities. Pflugers Arch. 362, 219–222. doi: 10.1007/BF00581173

Steinhausen, H. C. (2002). The outcome of anorexia nervosa in the 20th century. Am. J. Psychiatry 159, 1284–1293. doi: 10.1176/appi.ajp.159.8.1284

Steinhausen, H. C., and Jensen, C. M. (2015). Time trends in lifetime incidence rates of first-time diagnosed anorexia nervosa and bulimia nervosa across 16 years in a Danish nationwide psychiatric registry study. Int. J. Eat. Disord. 48, 845–850. doi: 10.1002/eat.22402

Stengel, A., Goebel, M., Wang, L., Rivier, J., Kobelt, P., Monnikes, H., et al. (2009). Central nesfatin-1 reduces dark-phase food intake and gastric emptying in rats: differential role of corticotropin-releasing factor2 receptor. Endocrinology 150, 4911–4919. doi: 10.1210/en.2009-0578

Stengel, A., and Taché, Y. (2011). "The physiological relationships between the brainstem, vagal stimulation and feeding," in The International Handbook of Behavior, Diet and Nutrition, Chapter 54, ed V. R. Preedy (London: Springer).

Swinbourne, J. M., and Touyz, S. W. (2007). The co-morbidity of eating disorders and anxiety disorders: a review. Eur. Eat. Disord. Rev. 15, 253–274. doi: 10.1002/erv.784

Teufel, P., Wang, L., Prinz, P., Goebel-Stengel, M., Scharner, S., Kobelt, P., et al. (2015). Treatment with the ghrelin-O-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats. J. Physiol. Pharmacol. 66, 493–503.

Treasure, J., Zipfel, S., Micali, N., Wade, T., Stice, E., Claudino, A., et al. (2015). Anorexia nervosa. Nat. Rev. Dis. Primers 1, 15074. doi: 10.1038/nrdp.2015.74

Verhagen, L. A., Luijendijk, M. C., de Groot, J. W., Van Dommelen, L. P., Klimstra, A. G., Adan, R. A., et al. (2011). Anticipation of meals during restricted feeding increases activity in the hypothalamus in rats. Eur. J. Neurosci. 34, 1485–1491. doi: 10.1111/j.1460-9568.2011.07880.x

Verhagen, L. A., Luijendijk, M. C., Korte-Bouws, G. A., Korte, S. M., and Adan, R. A. (2009). Dopamine and serotonin release in the nucleus accumbens during starvation-induced hyperactivity. Eur. Neuropsychopharmacol. 19, 309–316. doi: 10.1016/j.euroneuro.2008.12.008

Wang, L., Goebel-Stengel, M., Stengel, A., Wu, S. V., Ohning, G., and Taché, Y. (2011). Comparison of CRF-immunoreactive neurons distribution in mouse and rat brains and selective induction of Fos in rat hypothalamic CRF neurons by abdominal surgery. Brain Res. 1415, 34–46. doi: 10.1016/j.brainres.2011.07.024

Wang, L., Saint-Pierre, D. H., and Taché, Y. (2002). Peripheral ghrelin selectively increases Fos expression in neuropeptide Y - synthesizing neurons in mouse hypothalamic arcuate nucleus. Neurosci. Lett. 325, 47–51. doi: 10.1016/S0304-3940(02)00241-0

Wu, H., Van Kuyck, K., Tambuyzer, T., Luyten, L., Aerts, J. M., and Nuttin, B. (2014). Rethinking food anticipatory activity in the activity-based anorexia rat model. Sci. Rep. 4:3929. doi: 10.1038/srep03929

Zipfel, S., Giel, K. E., Bulik, C. M., Hay, P., and Schmidt, U. (2015). Anorexia nervosa: aetiology, assessment, and treatment. Lancet Psychiatry 2, 1099–1111. doi: 10.1016/S2215-0366(15)00356-9

Zipfel, S., Wild, B., Groß, G., Friederich, H. C., Teufel, M., Schellberg, D., et al. (2014). Focal psychodynamic therapy, cognitive behaviour therapy, and optimised treatment as usual in outpatients with anorexia nervosa (ANTOP study): randomised controlled trial. Lancet 383, 127–137. doi: 10.1016/S0140-6736(13)61746-8

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Publication 3:
Scharner S, Prinz P, Goebel-Stengel M, Lommel R, Kobelt P, Hofmann T, Rose M, Stengel A. Activity-based anorexia activates nesfatin-1 immunoreactive neurons in distinct brain nuclei of female rats. Brain Res. 2017;1677:33-46 https://doi.org/10.1016/j.brainres.2017.09.024 (No open access)

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10. Curriculum vitae

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11. List of publications

Original papers

1. Teuffel P, Wang L, Prinz P, Goebel-Stengel M, **Scharner S**, Kobelt P, Hofmann T, Rose M, Klapp BF, Reeve JR Jr, Stengel A. Treatment with the ghrelin-O-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats. J Physiol Pharmacol. 2015; 66:493-503.
2. Teuffel P, Goebel-Stengel M, Hofmann T, Prinz P, **Scharner S**, Körner JL, Grötzinger C, Rose M, Klapp BF, Stengel A. A RAPID method for blood processing to increase the yield of plasma peptide levels in human blood. J Vis Exp. 2016; 28;(110).
3. **Scharner S**, Prinz P, Goebel-Stengel M, Kobelt P, Hofmann T, Rose M, Stengel A. Activity-based anorexia reduces body weight without inducing a separate food intake microstructure or activity phenotype in female rats – mediation via an activation of distinct brain nuclei. Front Neurosci. 2016; 10:475.
4. Prinz P, Kobelt P, **Scharner S**, Goebel-Stengel M, Harnack D, Faust K, Winter Y, Rose M, Stengel A. Deep brain stimulation alters light phase food intake microstructure in rats. J Physiol Pharmacol. 2017; 68:345-354.
5. Schalla M, 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.
6. Prinz P*, **Scharner S***, Friedrich T, Schalla M, Goebel-Stengel M, Rose M, Stengel A. Central and peripheral expression sites of phoenixin-14 immunoreactivity in rats. Biochem Biophys Res Commun. 2017; 31808-9.
7. **Scharner S**, Prinz P, Goebel-Stengel M, Lommel R, Kobelt P, Hofmann T, Rose M, Stengel A. Activity-based anorexia induces activation of nesfatin-1 immunoreactive neurons in distinct brain nuclei of female rats. Brain Res. 2017; 1677:33-46.

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Abstracts

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

2. **Scharner S**, Prinz P, Kobelt P, Rose M, Stengel A. Activity-based anorexia as an animal model for anorexia nervosa. Annual Meeting of the German Society for Neurogastroenterology & Motility, February 2016, Freising, Germany.
3. Prinz P, **Scharner S,** Goebel-Stengel M, Kobelt P, Rose M, Stengel A. "Activity-based anorexia" – ein Tiermodell für Anorexia Nervosa. Z Gastroenterol. 2016; 54 – KV338.
4. **Scharner S**, Prinz P, Goebel-Stengel M, Kobelt P, Hofmann T, Rose M, Stengel A. Brain activation under conditions of activity-based anorexia – an immunohistochemical study in rats. Annual Meeting of the German Society for Neurogastroenterology & Motility, March 2017, Berlin, Germany.
5. Prinz P, Kobelt P, **Scharner S,** Goebel-Stengel M, Harnack D, Faust K, Winter Y, Rose A, Stengel A. Deep brain stimulation increases body weight gain without affecting food intake in rats. Annual Meeting of the German Society for Neurogastroenterology & Motility, March 2017, Berlin, Germany.
6. Prinz P, Kobelt P, **Scharner S**, Goebel-Stengel M, Harnack D, Faust K, Winter Y, Rose M, Stengel A. Deep brain stimulation of the nucleus accumbens shell augments body weight without affecting food intake in rats. J Neurogastroenterol Motil. 2017;29:3-140.
7. Prinz P, **Scharner S**, Goebel-Stengel M, Kobelt P, Rose M, Stengel A. Activity- based anorexia rats show an increased activation of nesfatin- 1 immunoreactive neurons in distinct brain nuclei. J Neurogastroenterol Motil. 2017;29:3-140.
8. Goebel-Stengel M, **Scharner S,** Prinz P, Kobelt P, Rose M, Stengel A. Ratten mit Aktivitäts-basierter Anorexie zeigen eine erhöhte Aktivierung Nesfatin-1-haltiger Neurone in verschiedenen Gehirnkernen. Z Gastroenterol. 2017; 55: e57-e299.
9. Goebel-Stengel M, Prinz P, Kobelt P, **Scharner S,** Harnack D, Faust K, Winter Y, Rose M, Stengel A. Tiefe Hirnstimulation der Schalenregion des Nucleus accumbens erhöht das Körpergewicht bei Ratten ohne die Nahrungsaufnahme zu beeinflussen. Z Gastroenterol. 2017; 55: e57-e299.

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