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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.

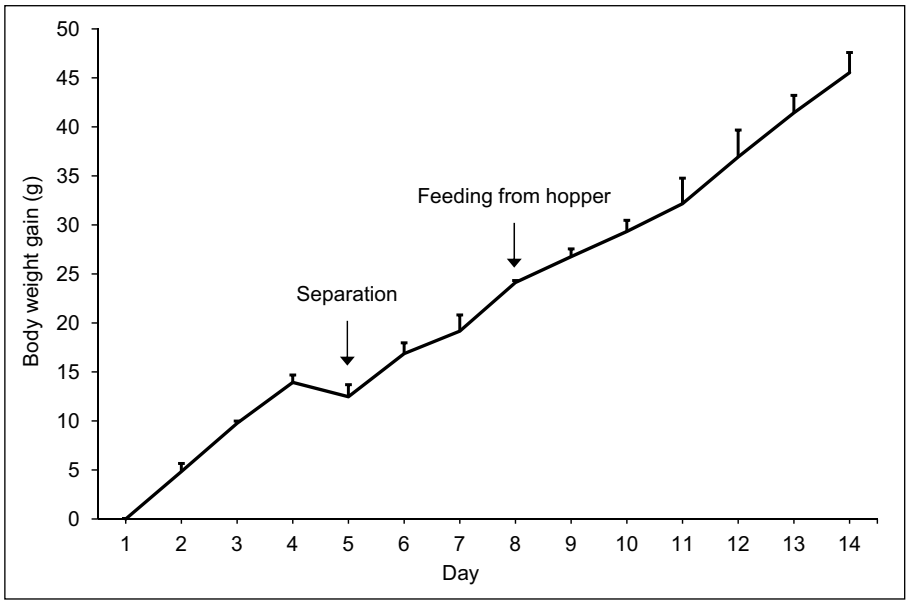

Fig. 1. Body weight gain in rats before and after separation. Rats were housed in groups of three and then on day five separated in single housing cages with eye and odor contact. Food was provided from the top of the cage and on day eight from the hopper of the automated feeding monitoring system. Body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

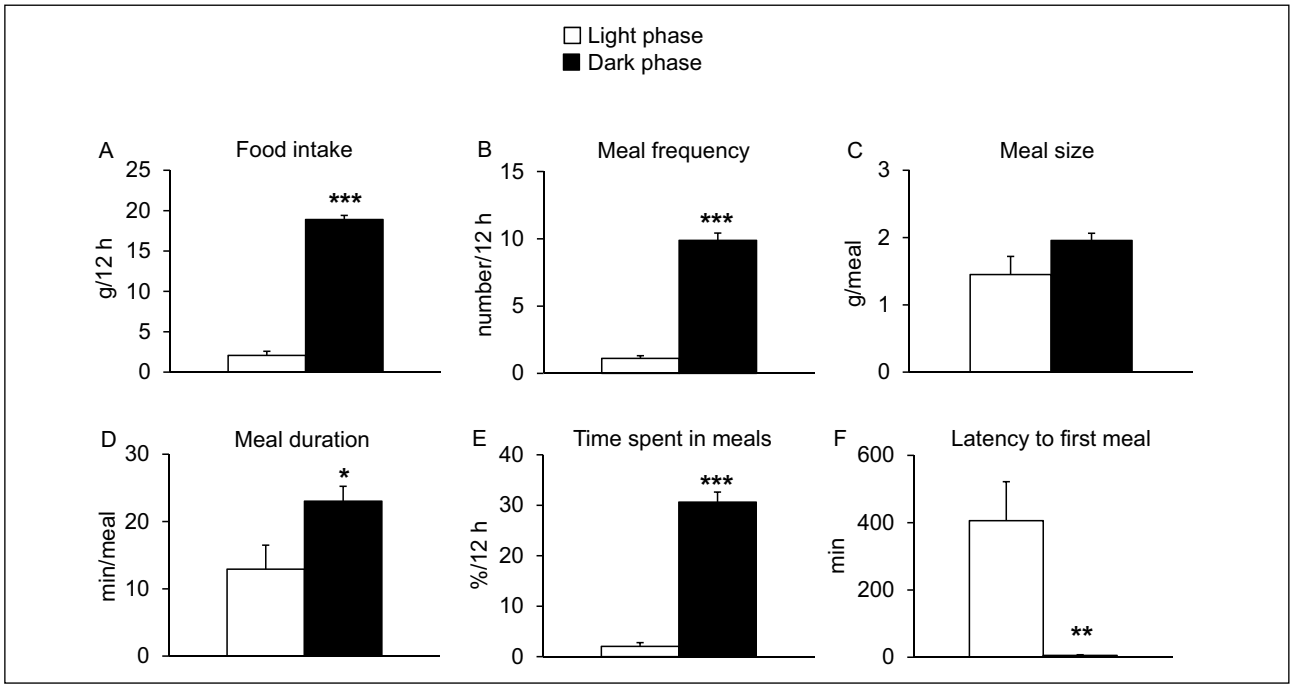

Fig. 2. Food intake microstructure during the light and dark photoperiod. Food intake (A) and the underlying food intake microstructure encompassing meal frequency (B), meal size (C), meal duration (D), time spent in meals (E) and the latency to the first meal (F) were assessed over a period of 24 h 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

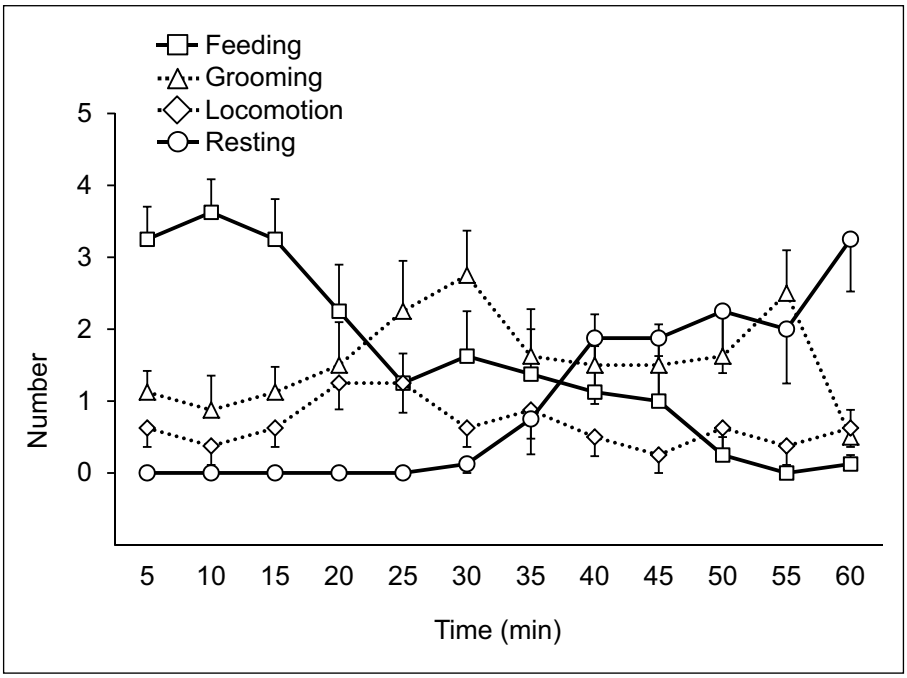

Fig. 3. The behavioral satiety sequence observed in rats housed in cages of the automated feeding monitoring system. Rats were single-housed in regular cages connected to the automated food intake monitoring system. While food intake was measured automatically, the behavior consisting of feeding, grooming, 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.

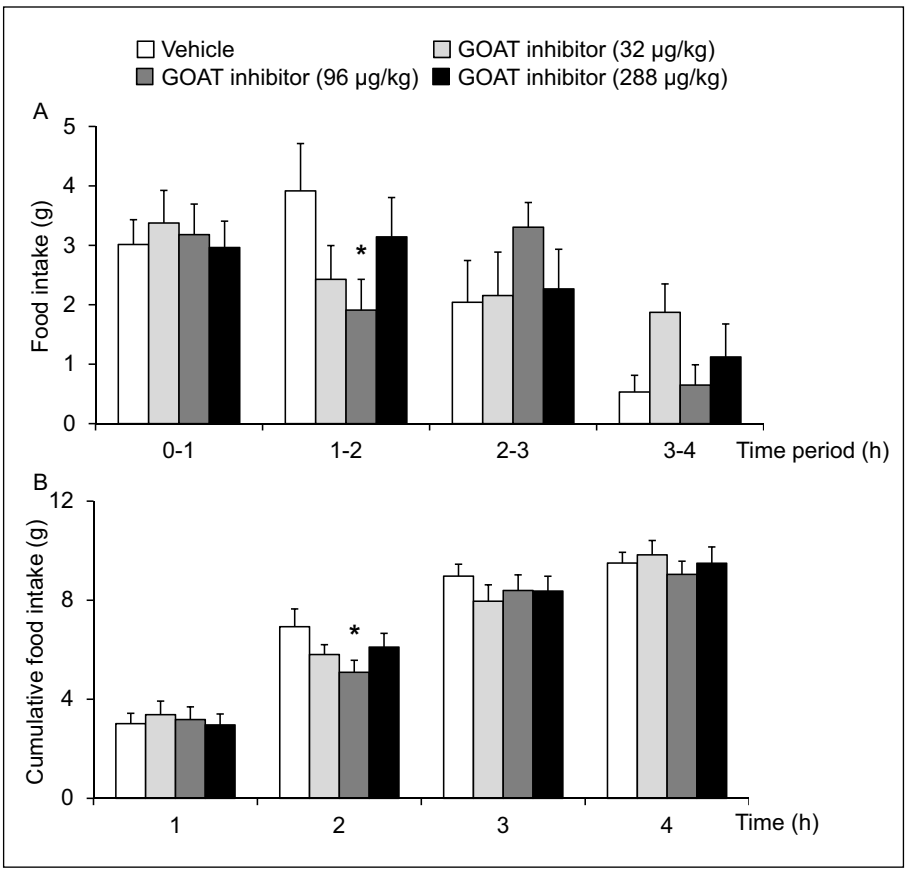

Fig. 4. Dark phase food intake in rats intraperitoneally injected with the GOAT inhibitor. *Ad libitum* fed rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor, GO-CoA-Tat (32, 96 or 288 µg/kg in 300 µl saline) directly at the beginning of the dark phase 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.

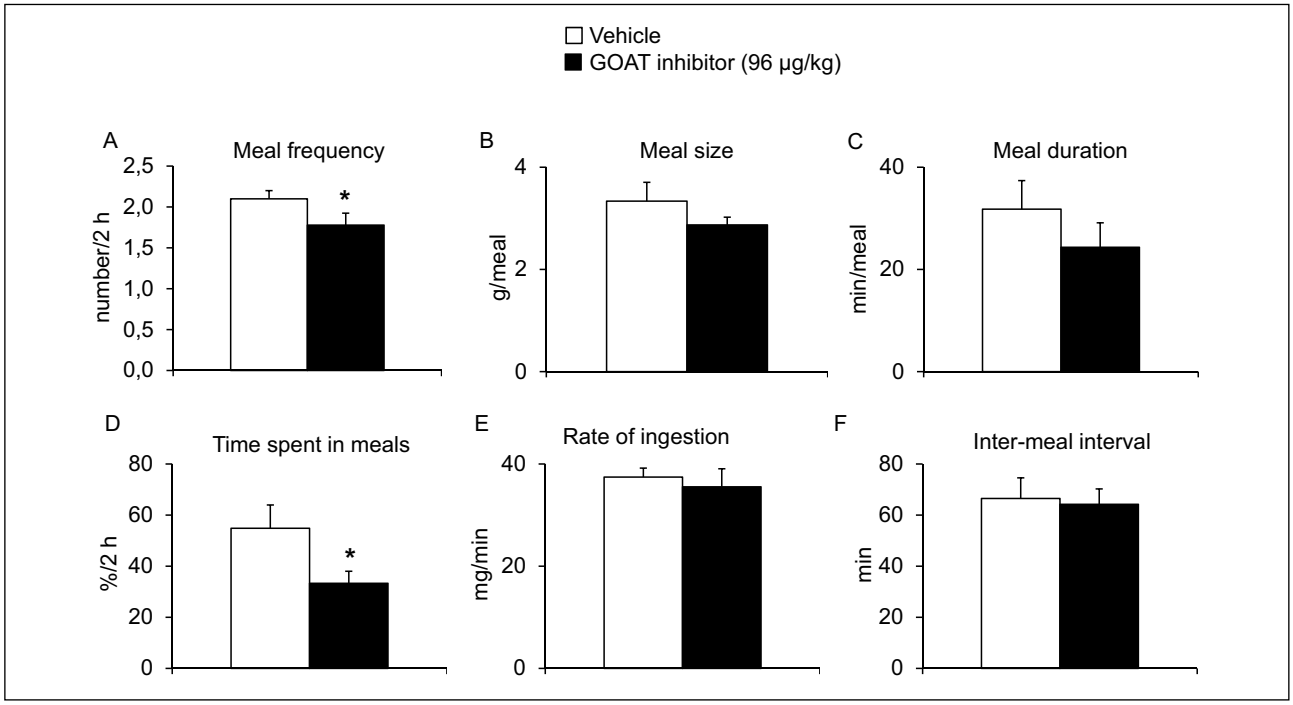

Fig. 5. Food intake microstructure in rats intraperitoneally injected with the GOAT inhibitor. *Ad libitum* fed rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor, GO-CoA-Tat (96 µg/kg in 300 µl saline) directly at the beginning of the dark phase and 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.

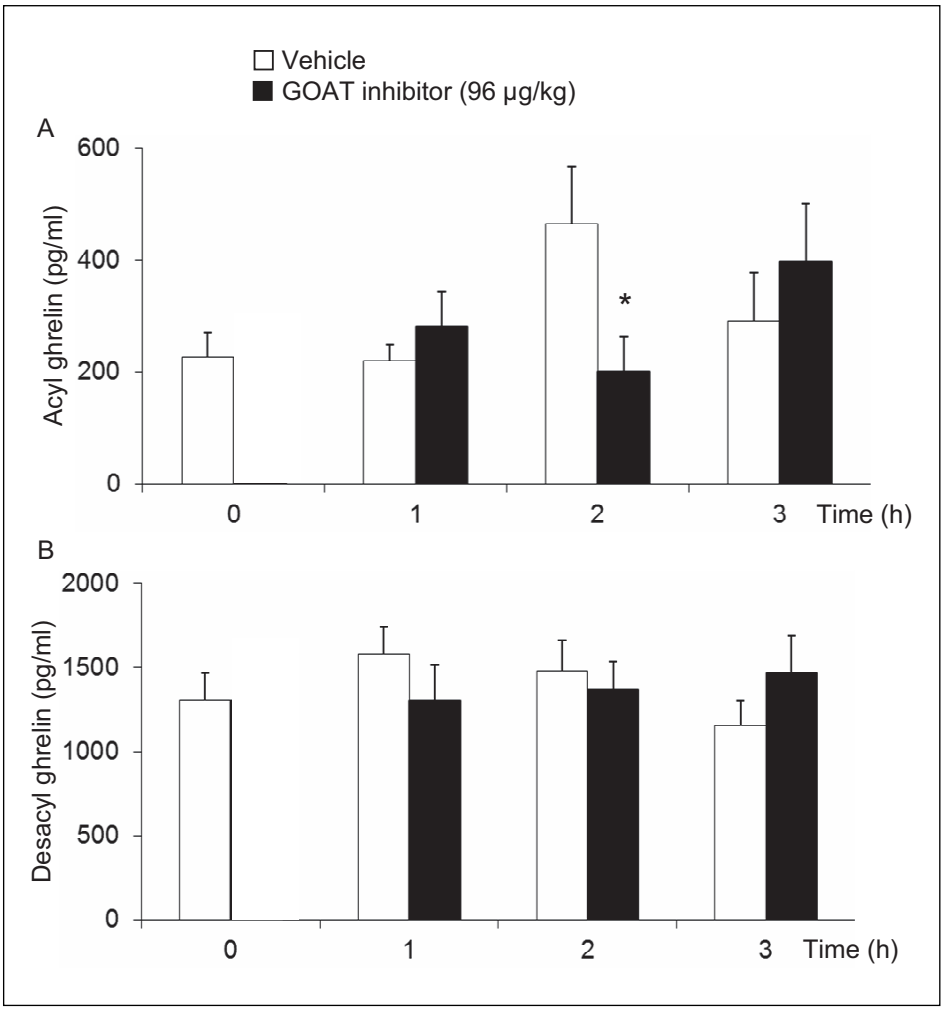

Fig. 6. Circulating acyl and desacyl ghrelin levels in rats intraperitoneally injected with the GOAT inhibitor. *Ad libitum* fed rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor, GO-CoA-Tat (96 µg/kg in 300 µl saline) directly at the beginning of the 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

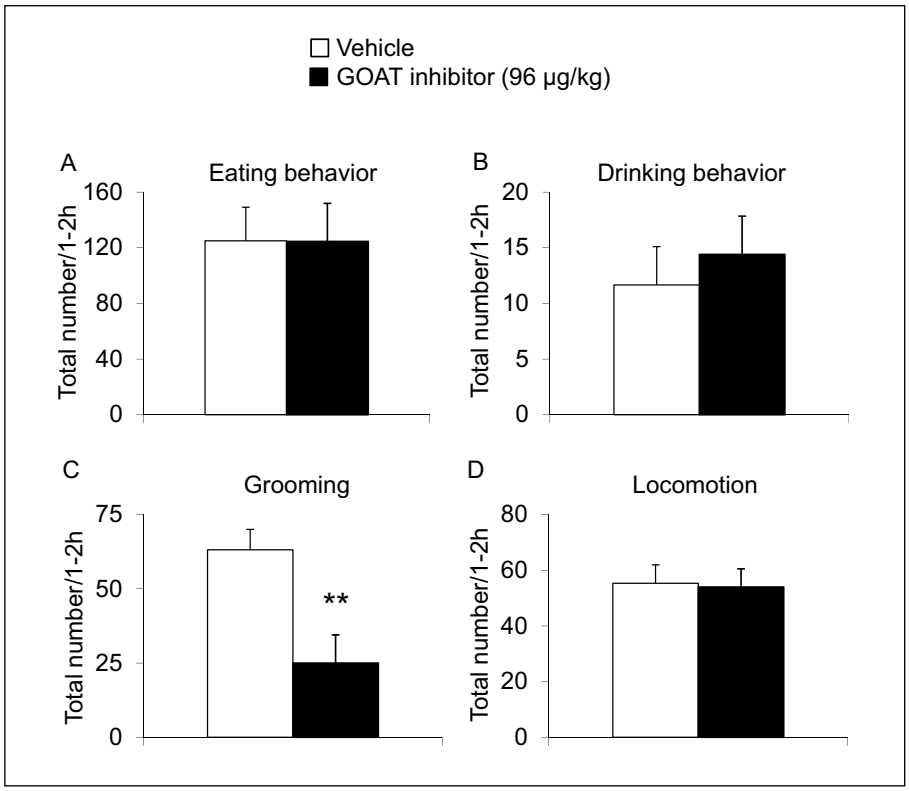

Fig. 7. Behavior in rats intraperitoneally injected with the GOAT inhibitor. *Ad libitum* fed rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor, GO-CoA-Tat (96 µg/kg in 300 µl saline) directly at the beginning of the dark phase. Single housed rats with 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.

reduction of food intake, indicated that eating behavior (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 the system has been used in rats before (20-22) and 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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