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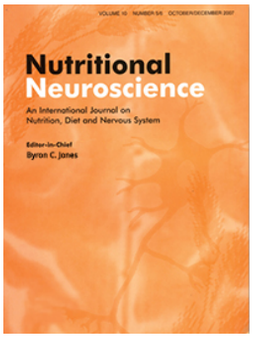

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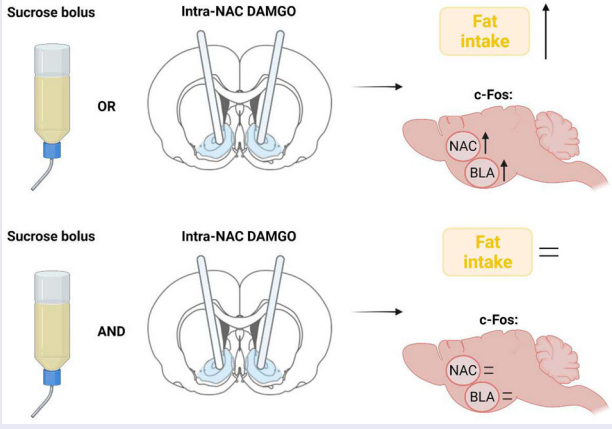
ABSTRACT

Objectives: We have previously shown that the combined consumption of fat and a sucrose solution induces overeating, and there is evidence indicating that sucrose drinking directly stimulates fat intake. One neurochemical pathway by which sucrose may enhance fat intake is through the release of endogenous opioids in the nucleus accumbens (NAC).

Methods: To test this hypothesis, we provided rats with a free-choice high-fat diet for two weeks. During the second week, rats had access to an additional bottle of water or a 30% sucrose solution for five minutes per day. After these two weeks, we infused vehicle or the μ-opioid receptor agonist [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) into the NAC 30 min after their daily access to the additional bottle of water or the sucrose solution.

Results: Sucrose drinking had two effects, (1) it stimulated fat intake in the absence of DAMGO infusion, (2) it diminished sensitivity to DAMGO, as it prevented the rapid increase in fat intake typically seen upon DAMGO infusion in the nucleus accumbens. In a second experiment, we confirmed that these results are not due to the ingested calories of the sucrose solution. Lastly, we investigated which brain areas are involved in the observed effects on fat intake by assessing c-Fos-expression in brain areas previously linked to DAMGO's effects on food intake. Both intra-NAC DAMGO infusion and sucrose consumption in the absence of DAMGO infusion had no effect on c-Fos-expression in orexin neurons and the central amygdala but increased c-Fos-expression in the NAC as well as the basolateral amygdala.

Discussion: In conclusion, we confirm that sucrose drinking stimulates fat intake, likely through the release of endogenous opioids.



KEYWORDS
Nucleus accumbens; DAMGO; μ-opioid receptor; Sugar; High-fat diet; Basolateral amygdala; Central amygdala; Orexin neurons; c-Fos

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Introduction

A Western diet, typically containing large amounts of saturated fat and added sugar, is thought to be one of the main contributors to the current obesity epidemic [1]. While attempts were made to unravel whether specifically saturated fat or sugar consumption negatively affects health, an increasing amount of evidence suggests that it might be the combination of fat and sugar that is responsible for a synergistic and negative effect on energy metabolism [2–5]. For example, we found in healthy men that a hypercaloric high-fat high-sucrose (HFHS) diet altered the brain's serotonergic system in a way that a hypercaloric high-fat (HF) diet does not [2]. Furthermore, subjects were willing to pay more for a high-fat high-sucrose reward than for a high-fat or a high-sucrose reward, and this was associated with greater activation of the brain's reward system [3]. In rodent studies, a choice diet that consisted of a dish of fat, a sucrose solution, and regular chow induced persistent hyperphagia, whereas having only a dish of fat or a sucrose solution in addition to a regular chow diet did not have this effect [4,5].

As it appears that the combined intake of fat and sugar have synergistic effects on feeding behavior, the question arose whether fat and sugar also stimulate each other's consumption. When rats receive a free-choice HF diet, consumption of the fat component of the choice diet decreases over time, whereas rats that receive a free-choice HFHS diet continue to consume the fat component [4]. On the contrary, fat intake actually lowers sucrose consumption: rats that receive a sucrose solution in addition to a chow diet consume more sucrose than rats who have a chow diet, a dish of fat, and a sucrose solution available. Therefore, we hypothesize that sucrose stimulates the consumption of fat and not vice versa. In line with this hypothesis, when mice were intermittently offered a sucrose solution, they consumed significantly more of an HF diet in the hours after sucrose consumption [6]. The exact mechanisms by which sucrose consumption stimulates fat intake remain to be unraveled.

We have previously hypothesized that sucrose stimulates fat intake through the release of endogenous opioids [7]. Infusions of opioids – both endogenous and synthetic – in several different brain areas are known to increase fat intake (for excellent review [8]), but the effects of opioid stimulation in the nucleus accumbens (NAC) have been studied most thoroughly. Specifically, activation of the μ-opioid receptor in the NAC has been found to potently stimulate fat intake [9–13]. Interestingly, in a porcupine model, sucrose drinking causes the release of opioids in the NAC [14]. Furthermore, repeated exposure to sucrose drinking alters μ-opioid receptor expression in the NAC [14–16]. These results underline the possibility that sucrose drinking increases fat intake through changes in NAC opioid transmission.

In this study, we hypothesized that sucrose drinking would cause an opioid release in the NAC. To test this hypothesis, we fed rats a free-choice HF (fcHF) diet for two weeks and gave the rats access to a sucrose solution for five minutes per day during the second week. After these two weeks, rats were infused with either vehicle or DAMGO in the NAC 30 min after drinking a sucrose bolus to test whether sucrose drinking stimulated fat intake and altered the response to the DAMGO infusion. If sucrose drinking increases opioid release, we expect: (1) an increase in fat intake after sucrose drinking and (2) a change in sensitivity for the μ-opioid receptor agonist [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO).

Material and methods

Animals

All experiments were performed in male Wistar rats (Charles River Breeding Laboratories, Sulzfeld, Germany) weighing 240–280 g at arrival in the animal facility of The Netherlands Institute for Neuroscience (Amsterdam, The Netherlands). Rats were housed in a temperature – (21 ± 2°C), humidity – (60 ± 5%) and light-controlled (12/12 h = light/dark; lights on 07:00–19:00) rooms with background noise (radio) during the entire experiment. During acclimatization, rats were group-housed and had free access to a container with nuggets of a control diet (chow; Teklad global diet 2918; 24% protein, 58% carbohydrate, and 18% fat, 3.1 kcal/g, Envigo, Horst, The Netherlands) and a bottle of tap water. The animal ethics committee of the Netherlands Institute for Neuroscience approved all experiments according to Dutch legal, ethical guidelines.

Surgery

Rats were anesthetized with an intraperitoneal injection of a mixture of 80 mg/kg Ketamine (Eurovet Animal Health, Bladel, The Netherlands), 8 mg/kg Rompun® (xylazine, Bayer Health Care, Mijdrecht, The Netherlands), and 0.1 mg/kg Atropine (Pharmachemie B.V., Haarlem, The Netherlands). Rats were fixed in a stereotact (Kopf®, David Kopf instruments, Tujunga,

California) and two 26-gauge stainless steel guide cannula (C315G-SPC 8 mm, Plastics One, Bilaney Consultants GmbH, Düsseldorf, Germany) aimed bilaterally at the NAC were implanted with a 10° angle at anteroposterior (AP): +1.4 mm, mediolateral (ML): ±2.8 mm, dorsoventral (DV): −7.1 mm. Cannulas were fixed on the skull with dental cement and anchor screws. From surgery onwards, rats were individually housed. Rats received Carprofen (5 mg/kg, subcutaneous) during surgery and the first post-surgery day. During the recovery period of 14 days, food and water intake and body weight were measured daily.

Experiments

Three experiments were performed: in experiment one, we measured food intake after water or sucrose drinking in combination with DAMGO infusion in the NAC, in Experiment 2, we determined whether DAMGO affected food intake when drinking a daily bolus of a triglyceride solution, and in experiment three, we assessed c-Fos-expression in brain areas previously linked to DAMGO's effects on food intake, after water and sucrose drinking in combination with DAMGO infusion. In all experiments, after the recovery period, rats received a free-choice HFD (consisting of a container of standard chow pellets, a dish of beef tallow (Ossenwit/Blanc de boeuf, Belgium) as well as a bottle of tap water) for two weeks.

Experiment 1

In Experiment 1, during the second week, rats were split into two groups: fcHF W and fcHF S. The fcHF W group daily received a second bottle of tap water for five minutes (between 16:00 and 16:05), whereas the fcHF S group received a bottle containing a 30% sucrose solution during the same timeframe. This time window was chosen as we previously found that rats increase their fat intake towards the end of the light period, potentially triggered by sucrose drinking that occurs during the light period [17]. Furthermore, μ-opioid receptor availability is significantly higher towards the end of the light phase, compared to the beginning [18]. Food, water intake, and body weight were measured daily (at 16:00). On the eighth consecutive day of exposure to the extra bottle, food was removed, rats received their daily bottle of water or 30% sucrose solution at 16:00–16:05, and 30 min later received a bilateral intra-NAC infusion of either vehicle (0.3 μl 0.9% PBS; Fresenius Kabi GmbH, Zeist, The Netherlands) or DAMGO (0.1 μg in 0.3 μl 0.9% PBS; Sigma-Aldrich, St. Louis, USA) (fcHF W vehicle $n = 7$, fcHF W DAMGO $n = 11$, fcHF S vehicle $n = 8$, and fcHF S DAMGO $n = 10$). Injectors were left in the guide cannula for one minute after completion of the infusion to allow for diffusion. Food was returned, and food intake was measured two and five hours after infusion. Data for the two- and five-hour measurements are expressed as a percentage of baseline intake, where baseline intake is the daily intake of fat in kcal, as measured during the two weeks prior to infusion, to account for individual variability in overall fat intake. At the end of the experiment, rats were anesthetized with a CO_2/O_2 mixture (6:4) and killed by decapitation. Brains were rapidly removed, frozen on dry ice and stored at −80°C. Two rats were removed from analysis for not consuming sufficient sucrose solution prior to vehicle infusion.

Experiment 2

In Experiment 2, during the second week, rats received a bottle of a 20% triglyceride solution (Intralipid®, Fresenius Kabi GmbH, Zeist, The Netherlands) for a maximum of 5 min per day (between 16:00 and 16:05) as in experiment one. However, the triglyceride solution was removed as soon as rats drank a similar amount of calories as the rats drinking sucrose in experiment one. On the eighth consecutive day of triglyceride solution consumption, food was removed, rats received their daily bottle of water or triglyceride solution, and subsequently received vehicle or DAMGO infusion in a similar manner as in experiment one (fcHF T vehicle $n = 5$ and fcHF T DAMGO $n = 6$). Food intake was measured two and five hours after infusion. At the end of the experiment, rats were anesthetized with a CO_2/O_2 mixture (6:4) and killed by decapitation. Brains were rapidly removed, frozen on dry ice, and stored at −80°C.

Experiment 3

In Experiment 3, the same paradigm as in experiment one was performed, with the exception that food was not returned after vehicle or DAMGO infusion (fcHF W vehicle $n = 5$, fcHF W DAMGO $n = 6$, fcHF S vehicle $n = 5$, and fcHF S DAMGO $n = 6$) and rats were intraperitoneally injected with pentobarbital (120 mg/kg) and perfused with 0.9%NaCl and 4% paraformaldehyde 60 min after vehicle or DAMGO infusion. One rat was removed from analysis for not consuming sufficient sucrose solution prior to vehicle infusion.

Brain tissue sectioning

Brains from experiments one and two were cut at 35 μm sections and mounted on Superfrost Plus slides (Fisher, Gerhard Menzel GmbH, Germany), fixed with a 4% paraformaldehyde solution (Sigma-Aldrich, St. Louis,

USA), and stained for Nissl staining with thionine (Sigma-Aldrich, St. Louis, USA). Stained sections were examined under the microscope to determine the placement of the cannula (Supplemental Table 1). Animals receiving DAMGO infusion with unilateral or bilateral misplacement were excluded from the analysis. Approximately 10% of all cannulas were misplaced.

Brains from experiment three were left in 4% paraformaldehyde for 24 h and subsequently submerged in a 30% sucrose solution. Next, they were frozen, cut at 30 µm sections, and collected in PBS + 0.04% sodiumazide. Sections were collected in series of 10 sections, 10 series in total for the NAC and 12 series for the lateral hypothalamus/amygdala. For the NAC, every third section was mounted on Superfrost Plus slides fixed with a 4% paraformaldehyde solution and stained for Nissl staining with thionine to assess cannula placement. For c-Fos staining in the NAC, one series of 10 sections was stained. For staining of orexin neurons and amygdala neurons, two series (at least three series apart) of 10 sections were stained as orexin neurons were not present in the most anterior and posterior sections of a series, and we wanted to obtain a minimum of eight orexin-containing sections per rat.

Immunohistochemical staining

For the c-Fos staining in the NAC, free-floating sections were washed in tris–buffered saline (TBS; 50 mM Tris–Cl, 150 mM NaCl; pH 7.6), blocked with 5% normal goat serum (Cat# S26-M, Sigma-Aldrich, USA) and 0.5% Triton X-100 in TBS for 15 min, and incubated with a 1:1000 rabbit anti-c-Fos antibody (Cat#: sc52, Santa Cruz Biotechnology, Inc., USA) in 5% normal goat serum and 0.5% Triton X-100 in TBS for one hour at room temperature followed by overnight incubation at 4°C. The next day, after TBS washes, sections were incubated with a 1:400 biotinylated horse anti-rabbit antibody (Cat#: BA9500, Vector Laboratories, USA) in 5% normal goat serum and 0.5% Triton X-100 in TBS for an hour at room temperature. After TBS washes, sections were incubated with Vectastain Elite ABC-HRP (1:800, Vector Laboratories, PK-6100) in 5% normal goat serum and 0.5% Triton X-100 in TBS for an hour at room temperature. After TBS washes, sections were incubated in TBS containing 0.5 mg/ml DAB, 0.23 mg/ml Nickel Ammonium Sulfate and 0.03% H_2O_2 for 20 min at room temperature, after which the reaction was stopped by transfer of sections into distilled water. After TBS washes, sections were mounted on Superfrost Plus slides, dried overnight, dehydrated through a series of alcohol, soaked in xylene, covered with Entellan and a coverslip.

For the combined c-Fos and orexin staining, free-floating sections were washed in tris–buffered saline (TBS; 50 mM Tris–Cl, 150 mM NaCl; pH 7,6) and incubated with 3% H_2O_2 and 10% methanol in TBS for 10 min. After TBS washes, sections were blocked with 10% normal donkey serum (Cat#566460, Sigma-Aldrich, USA) and 0.3% Tween-20 in TBS for one hour at RT, and incubated with a 1:1000 rabbit anti-c-Fos in 5% normal donkey serum and 0.3% Tween-20 in TBS for one hour at room temperature followed by overnight incubation at 4°C. After TBS washes, sections were incubated with a 1:400 biotinylated horse anti-rabbit antibody in TBS supermix (0.15 M NaCl, 0.05 M Tris, 0.25%w/v gelatin, 0.5%v/v Triton X-100, pH 7.6) for two hours at room temperature. After TBS washes, sections were incubated with 1:800 Vectastain Elite ABC-HRP in supermix for one hour at room temperature. After TBS washes, sections were incubated in TBS containing 0.5 mg/ml DAB, 0.46 mg/ml Nickel Ammonium Sulfate and 0.03% H_2O_2 for 20 min at room temperature, after which the reaction was stopped by transfer of sections into distilled water. After TBS washes, sections were incubated with a 1:1000 goat anti-orexin antibody (Cat#: sc8070, Santa Cruz Biotechnology, Inc., USA) in supermix for one hour at room temperature followed by overnight incubation at 4°C. After TBS washes, sections were incubated with a 1:400 biotinylated horse anti-goat antibody (Cat#: BA1100, Vector Laboratories, USA) in supermix for two hours at room temperature. After TBS washes, sections were incubated with 1:800 Vectastain Elite ABC in supermix for an hour at room temperature. After TBS washes, sections were incubated in TBS containing 0.5 mg/ml DAB and 0.03% H_2O_2 for four minutes at room temperature, after which the reaction was stopped by the transfer of sections into distilled water. After TBS washes, sections were mounted on Superfrost Plus slides, dried overnight, dehydrated through a series of alcohol, soaked in xylene, covered with Entellan and a coverslip. These sections were used both for assessing c-Fos+orexin-positive cells, as well as c-Fos-positive cells in the amygdala.

c-Fos counting

Microscopic images were acquired using the Carl Zeiss Axio Scan, using the Carl Zeiss ZEN software version 3.3 (Carl Zeiss AG, Oberkochen, Germany). c-Fos-positive cells in NAC sections, c-Fos-positive cells in amygdala sections, and c-Fos+orexin-positive neurons were counted using Fiji software [19]. For the NAC, images were acquired using a 10× magnification and sections where scars of the cannula tract were visible were

excluded, as they generated an abnormal high background signal. The average number of c-Fos-positive cells in all sections between Bregma +3.00 mm and Bregma +0.72 mm was calculated. For the central amygdala (CeA), the average of c-Fos-positive cells in sections between Bregma −1.56 mm and Bregma −3.24 mm was calculated. For the basolateral amygdala (BLA), images were acquired using a 10× magnification, and the average of c-Fos-positive cells in sections between Bregma −1.56 mm and Bregma −4.08 mm was calculated. For c-Fos+orexin-positive cells, images were acquired using a 20× magnification. Every section containing orexin-positive neurons was counted, and the percentage of c-Fos+orexin-positive cells per section was calculated. Subsequently, the average of all percentages was calculated.

Statistics

Data are shown as mean ± SEM. In addition, individual points are shown in the graphs. Graphpad Prism 9.0. (GraphPad Software, USA) was used to perform statistical analyzes. To assess the effects of water/sucrose drinking and vehicle/DAMGO infusion on food intake or c-Fos-expression, a two-way ANOVA was performed. If significant effects were found with the two-way ANOVA, a one-way ANOVA using an uncorrected Fisher's LSD was used for *post hoc* testing. To assess the effects of triglyceride solution consumption on food intake, a student's *t*-test was performed. Statistical outliers were determined using a Grubb's test for statistical outliers (GraphPad Software, USA) and removed if they were statistically an outlier. Only *p*-values below .05 were accepted as significant.

Results

Both intra-NAC DAMGO infusion and sucrose drinking increase fat intake

Rats received an fcHF diet for two weeks, and in the second week, they had daily access for five minutes to an additional bottle of water or 30% sucrose solution. Daily access to a sucrose solution did not affect daily chow or fat intake, nor did it alter final body weight (Tables 1 and 2). After these two weeks, rats received an intra-NAC infusion of vehicle or DAMGO 30 min after access to the additional water bottle or sucrose solution. In the group that received an additional water bottle, DAMGO markedly increased fat intake during the first two hours after infusion (*Drink* × *Infusion* $p = .0376$, *post hoc* fcHF W vehicle vs. fcHF W DAMGO, $p < .05$) (Figure 1(A) and Table 1). Interestingly, DAMGO no longer induced this increase in fat intake in rats that consumed a sucrose bolus 30 min prior to DAMGO infusion (Figure 1(A)). Sucrose drinking itself (in the absence of DAMGO infusion) also stimulated fat intake, but more evenly throughout the five hours (24% of baseline intake during 0–2 h, 28% of baseline intake during 2–5 h) (Figures 1(A,B)). Specifically, in the 2–5 h window, this differed from the other tested groups: the fcHF S vehicle group

Table 1. Statistical outcomes of two-way ANOVA tests. Bold values indicate *p*-values <0.05.

		Main effect of drink		Main effect of infusion		Interaction effect *Drink* × *infusion*	
	Experiment	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value	*F*-value
0–2 h fat intake	**1**	**.0466**	$F(1, 32) = 4.284$	**.0246**	$F(1, 32) = 5.564$	**.0376**	$F(1, 32) = 4.706$
2–5 h fat intake	**1**	**.0301**	$F(1, 32) = 5.150$	**.0063**	$F(1, 32) = 8.567$	.1844	$F(1, 32) = 1.840$
0–5 h fat intake	1	.5589	$F(1, 32) = 0.3488$	.6311	$F(1, 32) = 0.2351$	**.0115**	$F(1, 32) = 7.194$
0–2 h chow intake	1	.3512	$F(1, 32) = 0.8952$	.9679	$F(1, 32) = 0.001643$	.4503	$F(1, 32) = 0.5842$
2–5 h chow intake	1	.8346	$F(1, 32) = 0.0443$	.1460	$F(1, 32) = 2.220$	.2514	$F(1, 32) = 1.364$
0–5 h chow intake	1	.6967	$F(1, 32) = 0.1547$	.2166	$F(1, 32) = 1.589$	.6134	$F(1, 32) = 0.2603$
Final body weight	1	.6444	$F(1, 56) = 0.2154$	.4294	$F(1, 56) = 0.6336$	.4586	$F(1, 56) = 0.5569$
Baseline chow intake	1	.3760	$F(1, 56) = 0.7965$	.6769	$F(1, 56) = 0.1755$	.1713	$F(1, 56) = 1.921$
Baseline fat intake	1	.7651	$F(1, 56) = 0.0901$	0,8040	$F(1, 56) = 0.06215$	.6975	$F(1, 56) = 0.1526$
c-Fos-positive cells nucleus accumbens	3	.5181	$F(1, 18) = 0.4345$	.2361	$F(1, 18) = 1.502$	**.0239**	$F(1, 18) = 6.084$
c-Fos+orexin-positive neurons	3	.4631	$F(1, 18) = 0.5621$	**.0313**	$F(1, 18) = 5.451$	.1021	$F(1, 18) = 2.968$
c-Fos-positive cells central amygdala	3	.2201	$F(1, 18) = 1.614$	.7836	$F(1, 18) = 0.07769$	.2689	$F(1, 18) = 1.302$
c-Fos-positive cells basolateral amygdala	3	.8924	$F(1, 17) = 0.0189$	.8721	$F(1, 17) = 0.02673$	**.0449**	$F(1, 17) = 4.685$

Table 2. Body weight and food intake during experiment 1. For statistical outcomes, see Table 1.

	fcHF W vehicle	fcHF W DAMGO	fcHF S vehicle	fcHF S DAMGO
Final body weight (g)	389.95 ± 4.95	389.68 ± 4.74	396.62 ± 6.54	388.12 ± 5.42
Baseline chow intake (kcal/day)	44.64 ± 0.96	46.11 ± 1.71	45.39 ± 1.38	42.65 ± 1.49
Baseline fat intake (kcal/day)	51.85 ± 4.09	48.91 ± 4.93	51.43 ± 5.11	52.08 ± 3.45
Daily sucrose intake (kcal/day)	—	—	4.66 ± 0.38	4.59 ± 0.37

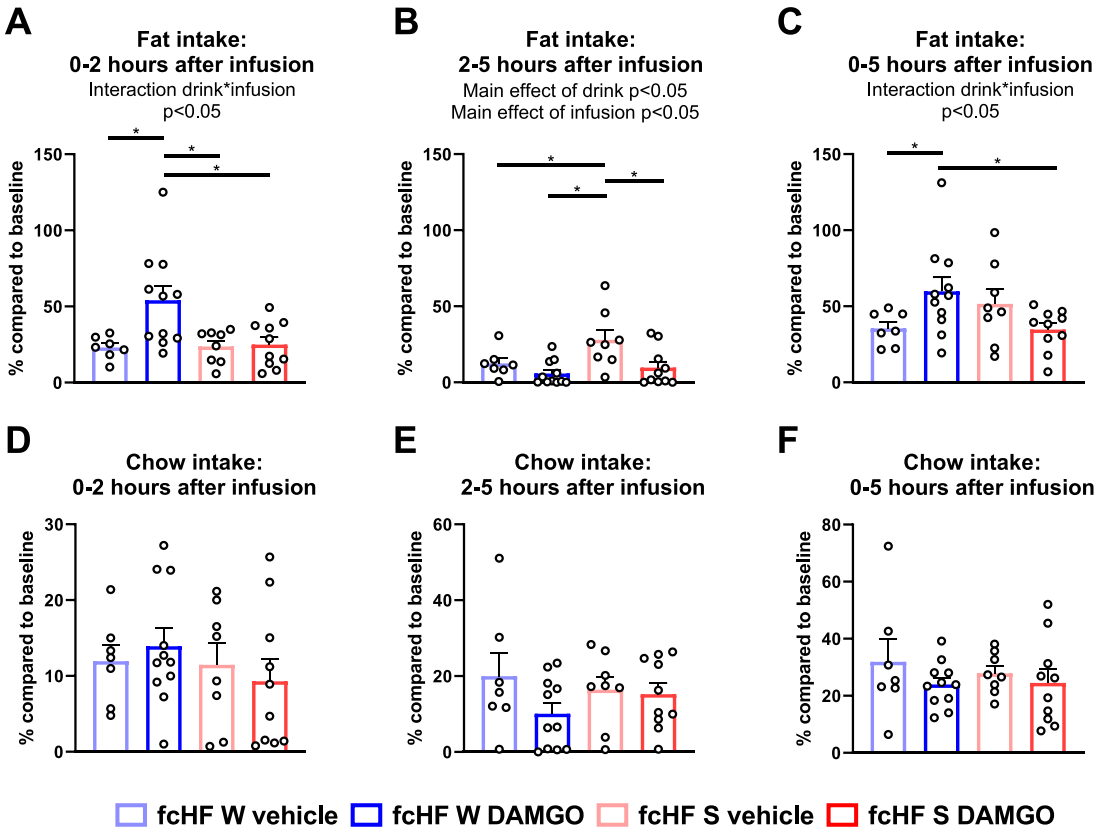

Figure 1. DAMGO infusion and sucrose drinking (in the absence of DAMGO infusion) both increase fat intake but during different time frames. (A–C) percentage of baseline fat intake consumed during the 0–2 h (A), 2–5 h (B) or 0–5 h (C) after vehicle or DAMGO infusion. D–F: percentage of baseline fat intake consumed during the 0–2 h (D), 2–5 h (E) or 0–5 h (F) after vehicle or DAMGO infusion. fcHF W = free-choice high-fat diet plus daily 5 min access to an additional bottle of water, fcHF S = free-choice high-fat diet plus daily 5 min access to 30% sucrose solution. fcHF W vehicle $n = 7$, fcHF W DAMGO $n = 11$, fcHF S vehicle $n = 8$, fcHF S DAMGO $n = 10$. Data are shown as mean ± SEM. Main or interaction effects tested with a two-way ANOVA, *$p < .05$ as tested with a *post hoc* Fisher's LSD test.

continued to consume fat, whereas the other three groups showed minimal fat intake (*Main effect of drink*, $p = .0301$, *Main effect of infusion*, $p = .0063$. *Post hoc* testing fcHF S vehicle vs fcHF W vehicle, fcHF W DAMGO or fcHF S DAMGO, $p < .05$) (Figure 1(B) and Table 1). Overall, 5 h after infusion, an interaction effect of *Drink* and *Infusion* was found, where the fcHF W DAMGO group consumed significantly more fat than the fcHF W vehicle and the fcHF S DAMGO group, but not more than the fcHF S vehicle group (Figure 1(C) and Table 1). Neither DAMGO infusion nor sucrose drinking affected chow intake (Figure 1(D–F)). In conclusion, both sucrose drinking and intra-NAC DAMGO infusion increased fat intake.

Drinking of a triglyceride solution does not prevent DAMGO's stimulatory effects on fat intake

As we observed that rats no longer increased fat intake in the first two hours after DAMGO infusion if they consumed a sucrose bolus prior to the infusion, we wondered whether this was due to a satiating effect of the calories which are present in the sucrose solution. In Experiment 2, we, therefore, used the same paradigm as in experiment one, but now using a 20% triglyceride solution instead of a sucrose solution to test whether drinking a similar amount of calories from a triglyceride solution also diminishes the response to intra-NAC DAMGO infusion. Unlike the sucrose bolus, a bolus of triglyceride solution did not prevent the increased fat intake induced by an intra-NAC infusion of DAMGO infusion (Tables 2 and 3), (Figure 2(A–C)).

Table 3. Body weight and food intake during experiment 2.

	fcHF T vehicle	fcHF T DAMGO	*p*-value
Final body weight (g)	412.4 ± 5.75	400.86 ± 8.53	.3308
Baseline chow intake (kcal/day)	44.33 ± 178	43.99 ± 1.60	.8911
Baseline fat intake (kcal/day)	63.36 ± 4.76	68.99 ± 7.91	.5962
Daily triglyceride solution intake (kcal/day)	4.42 ± 0.75	5.41 ± 0.73	.3769

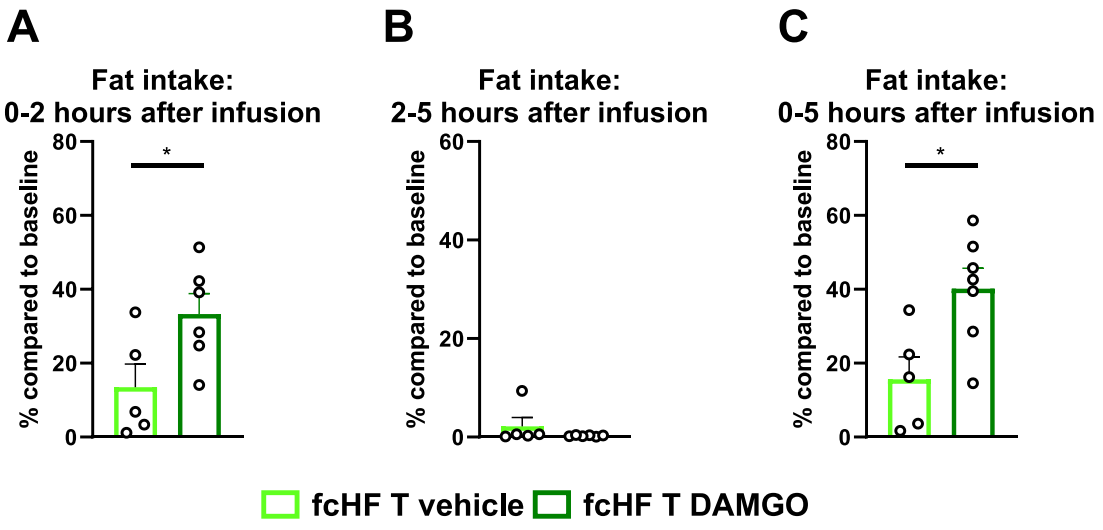

Figure 2. Drinking of a triglyceride solution does not affect the intra-NAC DAMGO-induced increase in fat intake. A–C: percentage of baseline fat intake consumed during the 0–2 h (A), 2–5 h (B) or 0–5 h (C) after vehicle or DAMGO infusion. fcHF T = free-choice high-fat diet plus daily 5 min access to a triglyceride solution. fcHF T vehicle $n = 5$, fcHF T DAMGO $n = 6$. Data are shown as mean ± SEM. *$p < .05$ as tested with a student's *t*-test.

Both intra-NAC DAMGO infusion and sucrose drinking by itself increase the number of c-Fos-positive cells in the NAC and BLA

To unravel which brain areas are involved in the effects of DAMGO and sucrose on fat intake, in experiment three, we again repeated the paradigm of experiment one, but instead of measuring food intake, rats were sacrificed after vehicle or DAMGO infusion and the number of c-Fos cells was counted in different brain areas. We first investigated the direct effects of DAMGO infusion on the number of c-Fos-positive cells in the NAC. We found an interaction effect of *Drink* and *Infusion* on the number of c-Fos-positive cells in the NAC (Figures 3(A) and 4, Table 1) (*Drink* × *Infusion*, $p = 0239$). *Post hoc* testing revealed that both in the fcHF W DAMGO and the fcHF S vehicle group, an increased number of c-Fos-positive cells was found compared to the fcHF W vehicle group. Next, we tested c-Fos-expression in orexin neurons and in the amygdala because previous studies indicated that orexin neurons [11], as well as CeA and BLA neurons, are involved in the effects of intra-NAC infusion of DAMGO on fat intake [20]. In our study, we found that the DAMGO infusion increased the percentage of c-Fos+orexin-positive cells, independent of sucrose drinking (*Main effect of infusion*, $p = .0313$) (Figure 3 (B)). No effect of *Drink* or *infusion* on the number of c-Fos-positive neurons in the CeA was found (Figure 3(C)). However, again an interaction effect of *Drink* and *Infusion* was found for the number of c-Fos-positive cells in the BLA (Figure 3(D) and Figure 5 and Table 1).

Discussion

In this study, we assessed the stimulatory effect of sucrose consumption on fat intake and the possible involvement of NAC μ-opioid receptors. In line with our hypothesis, we found an altered sensitivity for μ-opioid receptor stimulation after sucrose drinking, as intra-NAC DAMGO infusion increased fat intake in the first two hours after infusion, but consumption of a sucrose bolus prior to the DAMGO infusion completely diminished this effect. Interestingly, drinking a sucrose bolus by itself also increased fat intake but more evenly spread out throughout the hours after sucrose drinking. In the end, this resulted in a similar amount of fat intake five hours after intra-NAC infusion in rats that received DAMGO and rats that drank a sucrose bolus. We verified that the inhibitory effect of sucrose drinking on DAMGO-induced fat intake was not due to a satiating effect of the sucrose bolus, as DAMGO was still capable of increasing fat intake in rats consuming a similar amount of calories from a 20% triglyceride solution prior to the DAMGO infusion. Lastly, we showed that both intra-NAC DAMGO infusion, as well as sucrose drinking by itself, induced changes in c-Fos-expression in the NAC and BLA that could underlie the observed increase in fat intake.

While the effects of intra-NAC infusion of DAMGO on fat intake have been well described [9–13], we now show for the first time that a prior bolus of sucrose drinking diminishes this DAMGO-induced increase in fat intake. Sucrose has known analgesic effects [21], which are thought to be due to the release of

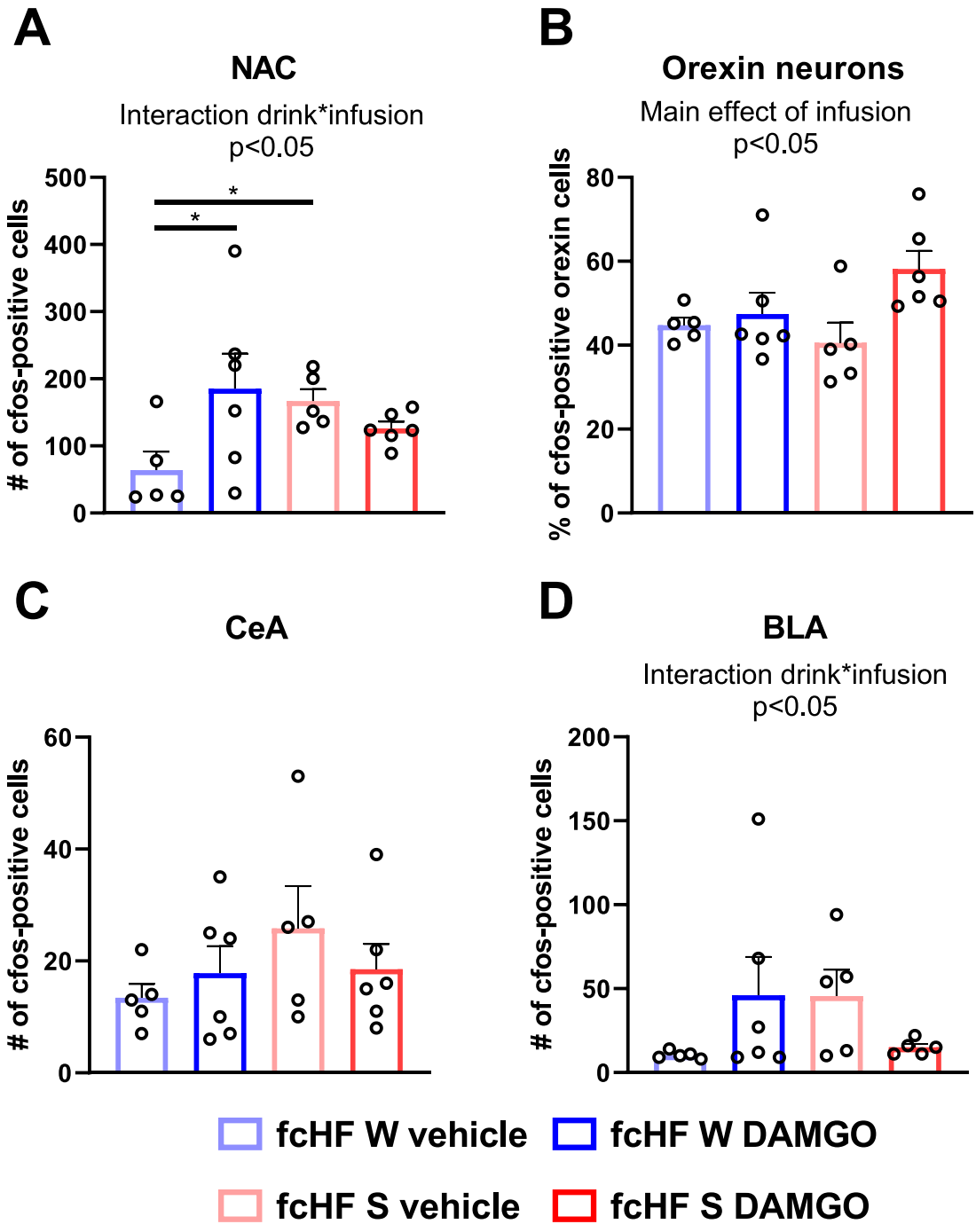

Figure 3. DAMGO infusion and sucrose drinking (in the absence of DAMGO infusion) both increase c-Fos-expression in the NAC and BLA. (A) Number of c-Fos-positive cells in the NAC. (B) Percentage of orexin-positive cells that were also c-Fos-positive. (C) Number of c-Fos-positive cells in the CeA. (D) Number of c-Fos-positive cells in the BLA. NAC = nucleus accumbens, CeA = central amygdala, BLA = basolateral amygdala, fcHF W = free-choice high-fat diet plus daily 5 min access to additional bottle of water, fcHF S = free-choice high-fat diet plus daily 5 min access to 30% sucrose solution. fcHF W vehicle $n = 5$, fcHF W DAMGO $n = 6$, fcHF S vehicle $n = 5$, fcHF S DAMGO $n = 6$. Data are shown as mean ± SEM. Main or interaction effects measured with a two-way ANOVA, * $p < .05$ as tested with a *post hoc* Fisher's LSD test.

endogenous opioids [22]. Indeed, sucrose drinking has been found to trigger endogenous opioid release in multiple brain areas, including the dorsal striatum [23] and the NAC [15]. Remarkably, the binding of the endogenous opioids β-endorphin and met-enkephalin can cause the internalization of the μ-opioid receptor [24]. We, therefore, hypothesize that due to the release of endogenous opioids in the NAC upon sucrose drinking and subsequent internalization of the μ-opioid receptor, fewer μ-opioid receptors are available once DAMGO is

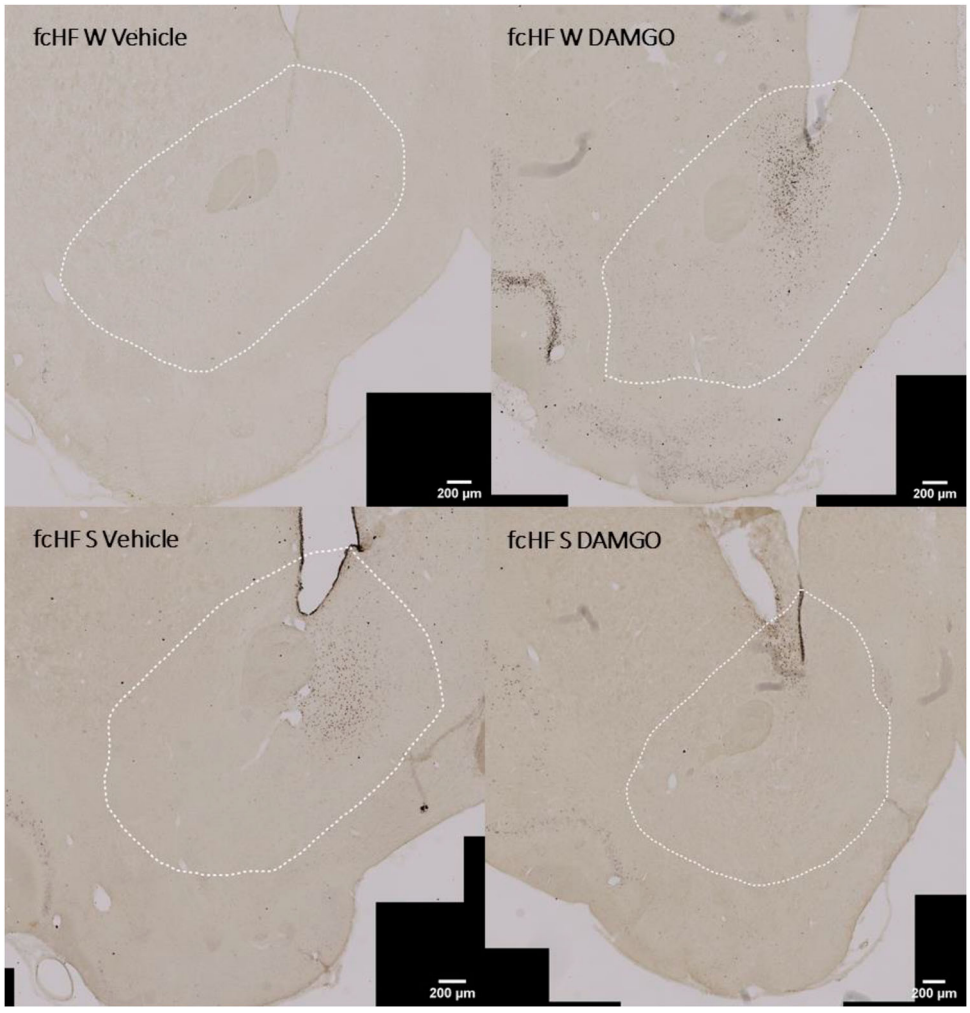

Figure 4. The number of c-Fos-positive cells in the nucleus accumbens is increased in the fcHF W DAMGO and the fcHF S vehicle group compared to the fcHF W vehicle group. Example pictures of each treatment group. The white dotted line indicates the area that defines nucleus accumbens. fcHF *W* = free-choice high-fat diet plus daily 5 min access to an additional bottle of water, fcHF S = free-choice high-fat diet plus daily 5 min access to 30% sucrose solution.

infused. This reduced availability prevents the increased fat intake that is typically observed upon intra-NAC DAMGO infusion.

As it is known that stimulation of the NAC μ-opioid receptors, both by endogenous as well as synthetic opioids, can increase food intake [9–13, 25], one would expect that if sucrose drinking indeed causes the release of endogenous opioids in the NAC, this would trigger food intake as well. Indeed, we found increased consumption of fat throughout the five hours after vehicle infusion. Specifically, in the second half of those five hours, the other experimental groups showed minimal fat intake, indicating that sucrose drinking prolongs the period of fat consumption at the beginning of the dark period, compared to control groups. Thus, while intra-NAC DAMGO infusion causes a sharp increase in fat intake in the first two hours, sucrose drinking causes a steady increase in fat intake, resulting in comparable amounts of fat consumed after five hours. Strikingly, when DAMGO was infused after sucrose drinking, it did not increase fat intake during the first two hours (as seen in the fcHF W DAMGO group), but it also lowered fat intake in the two-five hour window after infusion compared to the fcHF S vehicle group. One possible explanation for this is that DAMGO is known to enhance the internalization of μ-opioid receptors [26], thereby possibly altering the effects that the sucrose drinking-induced release of endogenous opioids has on fat intake.

To further unravel the neural network involved in the altered fat intake that we observe after DAMGO infusion and sucrose intake, we measured c-Fos in multiple brain areas. c-Fos is an immediate-early gene and is typically used as a proxy for neuronal activity [27]. However, activation of the μ-opioid receptor also has a direct effect on the transcription of the c-Fos gene,

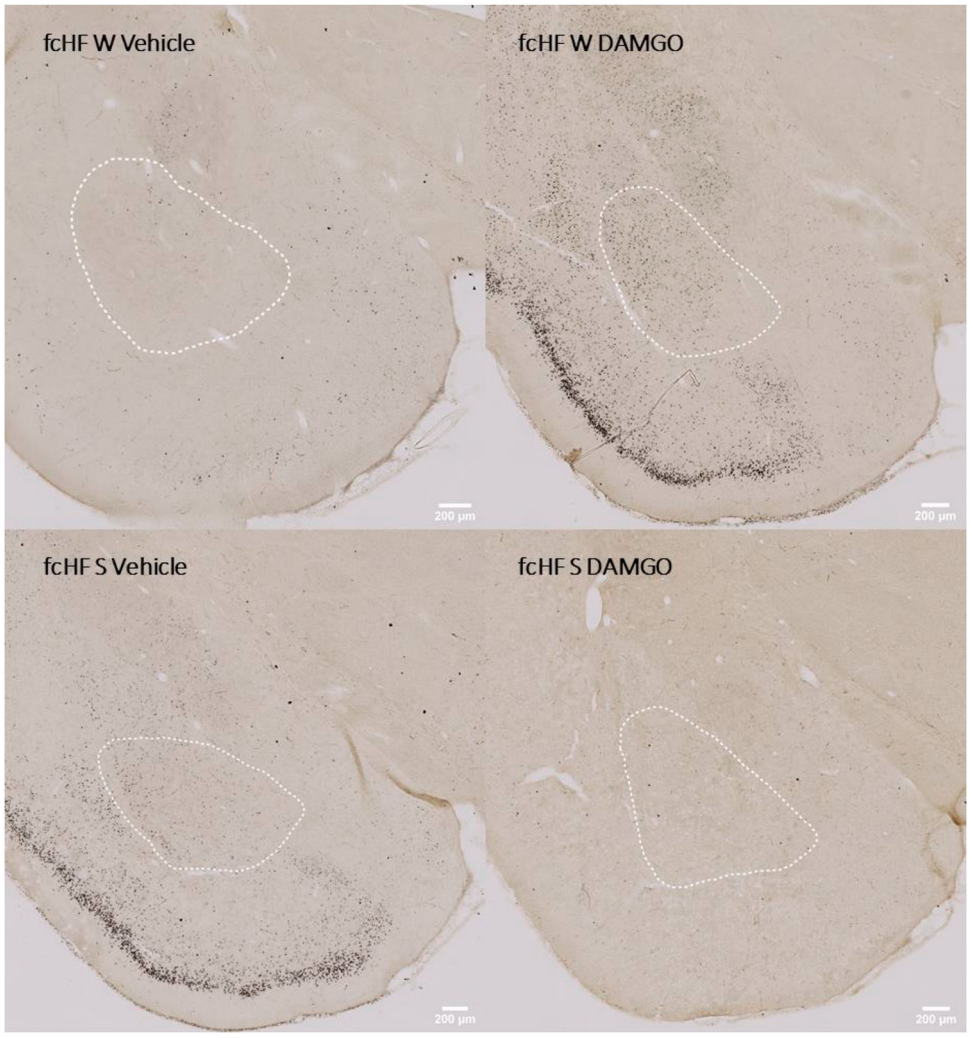

Figure 5. The number of c-Fos-positive cells in the basolateral amygdala is increased in the fcHF W DAMGO and the fcHF S vehicle group. Example pictures of each treatment group. The white dotted line indicates the area that defines the basolateral amygdala. fcHF W = free-choice high-fat diet plus daily 5 min access to an additional bottle of water, fcHF S = free-choice high-fat diet plus daily 5 min access to 30% sucrose solution.

causing an increase in c-Fos-expression [28]. Especially considering the known inhibitory effect opioids have on neuronal activity [29,30], an increased number of c-Fos-positive cells in the NAC is more likely a reflection of μ-opioid receptor activation. The increased number of c-Fos-positive cells in the fcHF W DAMGO and fcHF S vehicle group, therefore, supports the hypothesis that DAMGO and sucrose drinking (through the release of endogenous opioids) both cause μ-opioid receptor activation in the NAC. Interestingly, comparable to the fat intake results, DAMGO seems to have opposite effects in the sucrose drinking group, as there was a smaller and insignificant increase in c-Fos-positive cells compared to the fcHF W vehicle group. How endogenous opioids released after sucrose drinking and DAMGO interact in the NAC will have to be determined in future experiments.

We found that overall, DAMGO increased the percentage of c-Fos+orexin-positive cells and that this increase was mainly driven by the group that was drinking sucrose. In the water-drinking groups, the difference between vehicle and DAMGO treated animals was much smaller than previously reported [11,31]. This discrepancy might be explained by differences in the timing of the experiment [32] (end of the light period versus the beginning of the light period) or the motivational state (rats in our experiments had continuous access to the fcHF diet, compared to two hours a day [11,31]), which both have been shown to affect orexin neuronal activity [32–34]. Furthermore, the observed c-Fos+orexin-positive cell pattern does not reflect the changes we found in fat intake. Orexin neurons project to a variety of brain areas [35], but only orexin signaling in the ventral tegmental area, and not the arcuate

nucleus or paraventricular hypothalamus, has been shown to be crucial for the effects of intra-NAC DAMGO on fat intake [11]. This indicates that orexin signaling is not always linked to increased fat intake, depending on where the orexin is released. Therefore, we speculate that the orexin neurons that are activated in the fcHF W vehicle or fcHF S DAMGO group (in which we did not observe an increase in fat intake) might be primarily orexin neurons that project to other brain areas than the ventral tegmental area.

Unlike the c-Fos-expression in orexin neurons, c-Fos-expression in the BLA (and not the CeA) neurons was affected by sucrose drinking and intra-NAC DAMGO infusion. Both the BLA and the CeA have been investigated for their involvement in intra-NAC DAMGO-induced increases in food intake. In line with our findings, opioid transmission in the BLA was found necessary for the effects that intra-NAC DAMGO infusion has on fat intake, whereas blocking opioid transmission in the CeA had no effects on DAMGO-induced food intake [12,13]. We, therefore, speculate that sucrose drinking or intra-NAC DAMGO infusion will trigger the release of endogenous opioids in the BLA, causing an increase in c-Fos-expression. While it may seem counterintuitive that opioids, which are inhibitory by nature [29,30], would induce BLA neuronal activity, opioid receptor activation in the BLA inhibits GABAergic interneurons, thereby disinhibiting the majority of BLA neurons [36,37]. BLA neuronal activity is linked to palatability-driven feeding [38], can override satiety, and promote feeding in sated-rats [39]. Thus, the increased BLA neuronal activity is likely part of the neural network that underlies the enhanced fat intake seen after intra-NAC DAMGO infusion or sucrose consumption.

In this study, we confirm that sucrose drinking increases fat intake, possibly through the release of endogenous opioids in the NAC. Together with previous reports on the interacting effects of sucrose and fat intake [4–6], these findings might explain how a Western diet that contains large amounts of sucrose, sweetened beverages can induce overeating of hypercaloric fat-containing foods. The possible involvement of endogenous opioids identifies a neurobiological mechanism that may underlie this eating behavior. Interestingly, decreased NAC μ-opioid receptor binding has been found in obese individuals [40]. Potentially, the decreased μ-opioid receptor availability reflects a continuous stimulation of the receptor due to overeating of palatable foods. Further understanding of how sucrose-containing foods affect the opioid release and contribute to the development of obesity will help to identify and potentially target the neurochemical pathways involved in overeating.

To conclude, we find that sucrose drinking alters the sensitivity to NAC μ-opioid receptor stimulation and stimulates fat intake. Sucrose drinking mimics intra-NAC DAMGO infusion by similarly increasing c-Fos-expression in the NAC and by activating BLA neurons, likely a result of μ-opioid receptor activation. These findings help to better understand how increased consumption of sucrose-sweetened beverages can cause overeating and have detrimental effects on the development of obesity.

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The datasets generated and analyzed during the current study are available from the corresponding author on request.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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