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Peripherally injected cholecystokinin-induced neuronal activation is modified by dietary composition in mice

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ABSTRACT

The aim of this study was to investigate the effect of long-term nutrient intake on the central response to the anorexigenic gut hormone CCK. C57BL/6 mice were fed one of three diets for 6 weeks: standard high carbohydrate (HC), high fat (HF), or high protein (HP). Assessment of brain response to cholecystokinin (CCK) by manganese-enhanced MRI (MEMRI) showed a reduction in neuronal activity both in an appetite-related area (ventromedial nucleus of the hypothalamus) and areas associated with reward (nucleus accumbens and striatum) regardless of diet. When comparing diet effects, while the HF diet did not induce any change in activity, reductions in MEMRI-associated signal were found in the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) when comparing the HP to the HC diet. In addition, a significant interaction was found between CCK administration and the HF diet, shown by an increased activation in the PVN, which suggests a decrease the inhibiting action of CCK. Our results put forward that the long-term intake of an HP diet leads to a reduction in basal hypothalamic activation while a high-fat diet leads to desensitization to CCK-induced effects in the hypothalamus.

Introduction

The central nervous system (CNS) is able to sense changes in nutrient availability and to respond by a feedback control on energy balance (Schwartz, 2009). During digestion a broad range of satiety signals generated within the gut wall participate to promote meal termination (i.e., satiation) either by activating vagal afferents or by direct effects on the brain (Schwartz, 2006; Moran et al., 2001; Raybould et al., 2006; Raybould, 2007). Vagal afferents activate the nucleus of the solitary tract (NTS) neurons, which project extensively to other key regions involved in controlling food intake, including the hypothalamus, the amygdala, and the nucleus accumbens (Gibbs et al., 1973; Moran, 2000).

Circulating gut peptides released in response to nutritional stimuli constitute a key means by which the gastrointestinal (GI) tract communicates with central nervous system (CNS) centers of appetite (Schwartz et al., 2000). Cholecystokinin (CCK) is an anorexigenic neuropeptide normally secreted by enteroendocrine cells in the upper gastrointestinal tract in response to mechanical and chemical stimuli including fat in the duodenum. The release of CCK contributes to the inhibition of feeding during the meal (Kissileff et al., 1981; Stanley et al., 2005) and it has been shown that central administration of CCK (intracerebroventricularly or in the lateral hypothalamus) suppresses feeding behavior in numerous species (Schick et al., 1994).

Several pieces of evidence have suggested that diet can alter neural networks controlling food intake. For instance, in murine models, long-term exposure to a high-fat diet has been shown to decrease the efficiency of neural pathways for controlling meal size. This causes an increase in food intake and as a consequence elevated body weight (Savastano and Covasa, 2005; Paulino et al., 2008). Desensitization of the gut–brain axis to anorexic signals such as CCK is likely to be at the core of this phenomenon. Although such plastic processes may be of importance because they could cause early body weight drifts and obesity, the precise molecular and cellular bases for these adaptation processes remain unclear.

Increasingly, functional neuroimaging techniques have been employed to study aspects of metabolic physiology in both rodents (Stark et al., 2006) and humans (Liu et al., 2000; Tataranni and DelParigi, 2003; Batterham et al., 2006). In manganese-enhanced magnetic resonance imaging (MEMRI), manganese is used as a biological MRI detectable contrast agent. The ability of paramagnetic manganese (Mn^{2+}) ions to enter cells via voltage-gated calcium channels allows it to be used as an activity-dependent contrast agent especially for functional mapping of animal brain in vivo (Silva et al., 2004; Lin and Koretsky, 1997). Previous work indicates that MEMRI enables detection of a significant difference in activity-dependent Mn^{2+} uptake into neurons in response to gut hormone administration

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Table 1
Diet compositions.

Diet	High carbohydrate (g/kg)	High fat (g/kg)	High protein (g/kg)
Milk proteins	140	170	530
Cornstarch	622.4	436	287
Sucrose	100.3	71.1	45.7
Soybean oil	40	225	40
AIN 93M mineral mix	35	35	35
AIN 93M vitamin mix	10	10	10
α-Cellulose	50	50	50
Choline	2.3	2.3	2.3
Metabolizable energy (kJ/g)	14.6	18.8	14.6

(Kuo et al., 2005, 2006; Chaudhri et al., 2009). In addition, MEMRI overcomes some of the limitations associated with utilizing the expression of the proto-oncogene c-Fos as an indirect marker of neuronal activation (Harris, 1998; Hoffman and Lyo, 2002). We therefore used this technique to investigate changes in neuronal activity in the brain in response to peripheral administration of exogenous CCK.

More precisely, we looked at the effect of dietary macronutrient composition on the central response to CCK. Our hypothesis is that the localization and intensity of CCK-induced activity in the brain may be dependent on the macronutrient composition of the dietary regimen.

Materials and methods

Dietary treatment

Fifty-one male C57BL/6 mice (Harlan Laboratories, France) initially 8–10 weeks old were housed in a temperature- and humidity-controlled room under a 12:12 light/dark cycle (lights on at 0700 h). After 1 week of adaptation to environmental conditions where the mice were fed standard chow pellets, they were randomly assigned to 3 dietary groups: standard high carbohydrate (HC), high fat (HF), or high protein (HP; INRA, Jouy en Josas; Table 1). The diets were given ad libitum for 6 weeks. All experiments were performed under an approved protocol according to the guidelines of the French National Animal Care Committee.

Analysis of feeding sequence

After the dietary treatment, mice ($n=5$ from each dietary group) were placed in cages fitted with custom-built electronic equipment that measures food and water ingestion by the continuous weighing of food troughs and water bottles (sensitivity 0.01 g), both of which are fixed to the sides of the cage. The cage equipment was controlled and measurements recorded in 5-s time bins by a computer running a program designed in the laboratory. Cumulative food intake was recorded during 23 h (9:00 to 8:00 h) on each of 4 consecutive days. Only the last two 23-h recordings were kept for analyses. Our previous work using this device has shown that these two time intervals are the most stable after the initial period of adaptation. For each animal, the microstructure of feeding was analyzed according to the following criteria: an eating bout was defined as a feeding event of more than 0.01 g and lasting more than 10 s (criteria described by Clifton et al., 2000). Analysis of the feeding sequence lead to the extraction of the following parameters for a 23-h recording session and for their day/night distribution: number of bouts, size (g), length (min), ingestion speed (g/min, calculated as the ratio meal size/meal length), and inter-bout interval (min). Because the mean mice body weights were similar in the three groups, the data were not adjusted accordingly.

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Animal handling

After 6 weeks of the dietary treatment, 36 mice ($n=12$ per dietary group) were split again, this time into saline and CCK injection groups for MEMRI. Mice had their chow removed 20 h prior to scanning but were allowed ad libitum access to water. All scans were performed in the early light phase. Mice were anesthetized with isoflurane via a facemask (3% for induction, reduced to 1% for maintenance). The tail vein was cannulated and an i.p. catheter sited. The rectal temperature of each mouse was monitored electronically (SA Instruments) and maintained at 37.0 ± 0.5 °C by a waterbed heating system.

MRI parameters

The protocol used for MEMRI scanning was adapted from previously described methods (Kuo et al., 2006; Chaudhri et al., 2009). A 7-T Bruker Pharmascan MRI system was used. The mouse head was centrally located inside a Bruker-supplied quadrature mouse coil with an internal diameter of 31 mm. After standard calibration and piloting, shimming was performed on a $4\times4\times4$ mm voxel using FASTMAP (Gruetter, 1993; Tkác et al., 2004). A time course of 150 volumes was acquired using a T_1-weighted RARE sequence with the following parameters: TR = 1300 ms, TEeff = 5 ms (6 echoes), matrix = 192×96, field of view = 38.4×19.2 mm, 27 contiguous 0.3-mm-thick transverse slices, 0.33 mm apart, and 2 averages (acquisition time per volume 46 s, total 1 h, 55 min). IV infusion of $MnCl_2$ was commenced after six initial baseline acquisitions. Each mouse received 5 µl/g of 100 mM $MnCl_2$ at a rate of 0.2 ml/h (approximate duration of CCK/saline infusion was 40 min). Simultaneously with this, each mouse received an i.p. injection of 3 nmol/kg CCK or an equivalent volume of 0.9% saline. A further 144 acquisitions were then obtained. The resolution was $0.2\times0.2\times0.33$ mm. The CCK dose was selected on the basis of the results of previous experiments showing brain activation and decrease in food intake following i.p. CCK injections (Fan et al., 2004; Bechtold and Luckman, 2006).

Statistical analysis

Body weights (g) were analyzed separately using a one-way repeated measures ANOVA with time being the repeated measure and

Table 2
Comparative table of food intake parameters for mice fed either the HC, HF, or HP diet ($n=5$ animals per group).

	HC diet	HF diet	HP diet	*P*-value (ANOVA)
Body weight at $t=0$ (g)	16.27 ± 0.07	16.47 ± 0.10	16.52 ± 0.07	0.7413
Body weight at $t=6$ weeks (g)	27.08 ± 0.31	27.74 ± 0.49	26.82 ± 0.52	0.3597
Total energy intake (kJ)	45.8 ± 3.30	43.1 ± 1.25	48.4 ± 4.12	0.1181
Total bout number	38.2 ± 3^{ab}	33.8 ± 4.21^{b}	48 ± 3.58^{a}	0.0465*
Average bout size (kJ)	1.2 ± 0.1^{ab}	1.4 ± 0.06^{a}	1.01 ± 0.04^{b}	0.007*
Average bout duration (min)	2.4 ± 0.10	2.2 ± 0.10	2.2 ± 0.07	0.5195
Average instant ingestion speed (kJ/min)	0.6 ± 0.02	0.7 ± 0.06^{a}	0.51 ± 0.03^{b}	0.0374*
Average inter-bout interval (min)	30.3 ± 3.52	40 ± 5.06	25.9 ± 1.99	0.0555
Delay to consume the first bout (min)	152.2 ± 26.6^{a}	72 ± 26.6^{ab}	58.6 ± 16.18^{b}	0.0332*

Data expressed as mean ± SEM, *$p<0.05$.

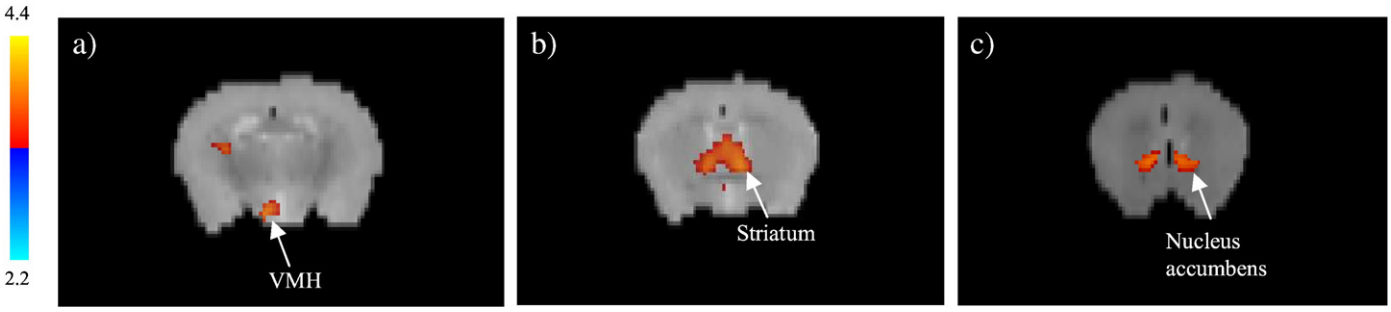

Fig. 1. Statistical maps of differential manganese-related signal for saline vs. CCK. Mice injected with CCK demonstrated significantly reduced MEMRI signal intensity in the ventromedial nucleus of the (a) hypothalamus, (b) striatum, and (c) nucleus accumbens. Results are presented as color-coded *z*-scores thresholded using clusters to a significance level of $p<0.05$. Brain regions were identified by overlaying the statistical map to a high-resolution MRI mouse brain atlas (Dorr et al., 2008).

diet (HC versus HF versus HP) as the main effect. The dietary intake parameters including number of bouts, inter-bout intervals, delay to consume the first bout, and ingestion speed were analyzed using one-way ANOVA with diet (HC versus HF versus HP) as the main effect. Descriptive data are presented as means ± SEM. For all the analyses, the procedure used in SAS was PROC MIXED that is specific for models with fixed and random variables. Significant main effect differences were tested using Tukey–Kramer's *post-hoc* test for multiple comparisons. All data were analyzed with the statistical package SAS (Version 8.02) and a $p<0.05$ was considered significant.

MEMRI time courses were motion corrected with SPM5 (http://www.fil.ion.ucl.ac.uk/spm): images from the same mouse were adjusted in order to be in the same plane. The images from all mice were then coregistered to a mouse brain template (Dorr et al., 2008) using AFNI (Cox and Hyde, 1997) before GLM analysis in the FSL tool FEAT (Smith et al., 2004). Here, to determine Mn uptake, single subject data were fitted to a model of signal change induced by $MnCl_2$ infusion. This model generates two different uptake curves representing manganese uptake outside the blood–brain barrier (BBB) and inside the BBB. The model was derived from a tensor ICA analysis (using the FSL tool MELODIC; Beckmann et al., 2006) of independently acquired MEMRI data. Data from inside the BBB are then taken into consideration for statistical analysis. Group analysis of the Mn uptake maps used a 2 × 3 ANOVA model with factors of diet (at three levels—HC, HF and HP) and injection (at two levels—saline and CCK). The statistical equation used is given as follows:

$$SI = \text{mean} + CCK + HF + HF{:}CCK + HP + HP{:}CCK$$

SI is the signal intensity of the voxel, the mean represents the effect of the HC diet and a saline injection, CCK is the effect of the cholecystokinin injection, HF and HP represent the effect of each corresponding diet, and the terms HF:CCK and HP:CCK are interaction terms. The term HC:saline was chosen as the overall mean because the HC diet is perceived as a standard balanced diet that would be representative of normal conditions (based on American Institute of Nutrition recommendations, AIN-93 modified diet, Reeves et al., 1993). This same diet has already been used in several dietary intervention studies where it was considered as the control group (Nefti et al., 2009; Savastano and Covasa, 2005; Darcel et al., 2005). Results are presented as color-coded *z*-score maps thresholded using clusters that were corrected to a significance level of $p<0.05$ using random field theory (http://imaging.mrc-cbu.cam.ac.uk/imaging/PrinciplesRandomFields).

Results

Feeding sequence

There were no significant differences in body weight or in overall energy intake between the different dietary groups (Table 2). Mice fed the HP diet had a larger number of eating bouts than those fed the HF diet (48 ± 3.5 vs. 33.8 ± 4.2, $P = 0.04$), but these were of smaller size (1.01 ± 0.04 kJ vs. 1.41 ± 0.06 kJ, $P = 0.001$). HP-fed mice had a shorter delay to consume their first bout (58.6 ± 16.2 min compared to 72 ± 26.6 min for the HF and 152.2 ± 26.6 min for the HC, $P = 0.02$) as well as a lower ingestion rate (0.51 ± 0.03 kJ/min) compared to the HF diet (0.68 ± 0.06 kJ/min, $P = 0.04$).

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CCK versus saline

Assessment of the response to CCK showed a significant reduction in MEMRI-associated signal in the ventromedial nucleus (VMN) of the

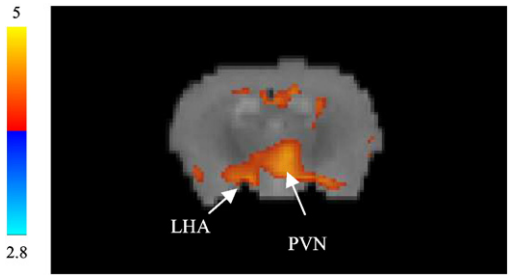

Fig. 2. Statistical map of differential MEMRI-associated signal for HC vs. HP. The HP group had lower basal activation in the paraventricular nucleus (PVN) of the hypothalamus and the lateral hypothalamic area (LHA) compared to the HC group. Results are presented as color-coded *z*-scores thresholded using clusters to a significance level of $p<0.05$. Brain regions were identified by overlaying the statistical map to a high-resolution MRI mouse brain atlas (Dorr et al., 2008).

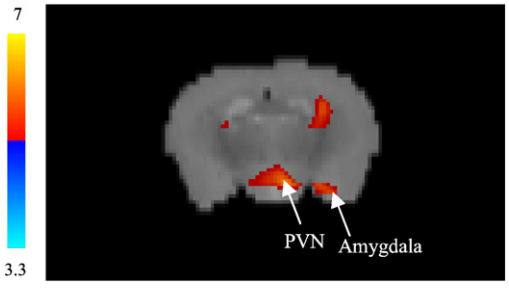

Fig. 3. Statistical map of the interaction between CCK and high-fat diet groups of mice in the MEMRI experiment. Mice fed a high-fat diet and injected with CCK demonstrated higher manganese-related signal intensities notably in the PVN and the basomedial and medial amygdaloid nuclei. Results are presented as color-coded *z*-scores thresholded using clusters to a significance level of $p<0.05$. Brain regions were identified by overlaying the statistical map to a high-resolution MRI mouse brain atlas (Dorr et al., 2008).

hypothalamus (Fig. 1a), striatum (Fig. 1b), and nucleus accumbens (Fig. 1c).

Comparison between diets

When comparing the effects of the different diets, while the HF diet did not induce any significant difference in the activity patterns when compared to the other two diets, some fairly large reduction in MEMRI-associated signal was found when comparing the HP to the HC diet. The HP group had lower activation in a large area of the ventral forebrain including the hypothalamus, with particularly significant foci of activations in the paraventricular nucleus (PVN) of the hypothalamus and the lateral hypothalamic area (LHA) compared to the HC group (Fig. 2).

Interactions between CCK and diets

When looking at interactions between CCK and the different diets, there was a significant interaction between the HF diet and CCK (Fig. 3). Mice injected with CCK and fed the HF diet showed higher MEMRI signal in certain brain areas, indicative of higher neuronal activity. Notably, significant peaks of activation were found in the PVN and amygdala. No significant interaction was found between CCK and HP diet.

Discussion

The objective of this study was to investigate by MEMRI the influence of the adaptation to different diet on the central response to the anorexigenic gut hormone CCK. The results showed a reduction in neuronal activity in the ventromedial nucleus of the hypothalamus, nucleus accumbens, and striatum in response to CCK administration. In addition, while body weight and energy intake were similar across the different dietary groups, the analysis of the patterns of food intake revealed some differences. Mice fed the HP diet had a larger number of eating bouts but of smaller size compared to mice fed the HF diet. Since we did not observe any reduction of total energy intake in the HP group, we think that the lower eating bout size compared to the HF group reflects the known satiating properties of proteins (reviewed by Noakes, 2008) but that it is then compensated by more numerous bouts. Mice fed the HF diet had larger inter-bout intervals than the HC animals during the day only. HP-fed mice had a shorter delay to consume their first meal as well as a lower ingestion speed. These results reveal an effect of long-term diets on altering eating patterns. When comparing diet effects, while the HF diet did not induce any change in activity, reduction in MEMRI-associated signal was found in the PVN and LHA when comparing the HP to the HC diet. Moreover, a significant interaction was found between CCK administration and the HF diet as shown by an increased activation in the PVN.

Assessment of the response to CCK by MEMRI showed a significant reduction in neuronal activity in appetite and reward-related areas. First, mice injected with CCK demonstrated significantly reduced Mn uptake in the ventromedial hypothalamus (VMH). This reduction in MEMRI-associated signal agrees with findings that CCK suppresses hunger by downregulating hormones involved in initiation of feeding in the hypothalamus (reviewed by Chandra and Liddle, 2007). In addition, the VMH is known to be strongly involved in the control of food intake and its destruction results in hyperphagia whereas its electrical stimulation inhibits ongoing feeding behavior (Shimizu et al., 1987; Takaki et al., 1992; King, 2006). Hence the VMH is thought to play a crucial role in the regulation of food intake because it is the target of many mediators of satiety including CCK (Shiraishi, 1990). Notably, CCK microinjection in the VMH of rats was found to suppress food intake during the first hour post-injection (Blevins et al., 2000).

This study did not show any difference in activation of the dorsomedial hypothalamic nucleus following the peripheral CCK injection as it was demonstrated in a study where CCK-induced neuronal activation was assessed through Fos immunohistochemistry (Kobelt et al., 2006). This discrepancy could be explained by differences in the fasting state of the animals, which may affect the response to CCK. In addition, both c-Fos labeling and MEMRI are indirect methods to measure neuronal activation. The fact that the results do not exactly converge therefore emphasizes the need to combine different experimental methods to study brain responses since each technique has its own characteristic features especially in terms of spatial and temporal resolutions (Sagar et al., 1988; Shibasaki, 2008). CCK-injected mice also demonstrated significantly reduced Mn uptake in the nucleus accumbens and striatum of their brains, which are known to be parts of the "reward area" of the brain. Although they have traditionally been studied for their role in addiction, these brain regions play an equal role in processing many rewards such as food. Indeed, the nucleus accumbens and its associated circuitry constitute a system that subserves motivated behaviors, such as feeding, drinking, sexual behavior, and incentive learning. Reward pathways and homeostatic pathways interact in the control of food intake (Robbins and Everitt, 1996; Kelley, 1999; Kelley et al., 2002). The question that remains to be answered is whether the satiety-related signals generated by these pathways are influenced by diet macronutrient composition.

When comparing the effects of the different diets, significant reduction in MEMRI-associated signal was found when comparing the HP to the HC diet. Indeed, the HP group had lower activation in the paraventricular nucleus (PVN) of the hypothalamus and the lateral hypothalamic area compared to the HC group. This result reflects differences in basal activity between HC and HP dietary conditions. The PVN is known to be an integrating brain area on which converge many neural pathways that influence energy homeostasis. It is rich in terminals containing numerous appetite-modifying neurotransmitters and is particularly sensitive to these neurotransmitters' effects on feeding and energy expenditure (Berthoud, 2002; Valassi et al., 2008). In addition, both the PVN and LHA are known to contain glucose-sensing neurons that respond to circulating glucose levels (reviewed by Williams et al., 2001). Since it has been shown that low-carbohydrate diets induce a decrease of fasting plasma glucose levels (reviewed by Adam-Perrot et al., 2006), this may explain the lower

Table 3
Z-scores and coordinates of peak voxels in our areas of interest.

Parameter studied	Area of activation	*Z*-score of the peak voxel	Peak voxel coordinate*		
			x	*y*	*z*
Saline versus CCK	Nucleus accumbens	2.70	0.61	3.30	0.16
	Striatum	3.53	0.61	2.25	0.16
	VMH	3.52	−0.41	0.90	−1.82
HC versus HP	LHA	3.52	−1.43	1.05	−1.16
	PVN	4.34	0.41	1.05	−0.50
The CCK:HF interaction	Amygdala	4.35	2.05	0.90	−1.82
	PVN	5.71	0.41	0.90	−1.49

All tabulated *z*-scores correspond to $P<0.05$ (cluster-level corrected for multiple comparisons). *The coordinates system is based on the MRI mouse brain atlas that we used (Dorr et al., 2008). The system has its origin (0, 0, 0) close to the midline at a point in the caudal thalamus. The *x*-axis corresponds left–right (sagittal), *y* is the antero-posterior or rostro-caudal axis (coronal/axial), and *z* is the superior–inferior or dorso-ventral axis (transverse).

activation in these regions consequently to the lower carbohydrate content of the HP diet.

The significant interaction between CCK administration and the high-fat diet in the PVN and amygdala suggests that mice habituated to a high-fat diet respond less to satiety signaling through CCK. When considering that CCK suppresses Mn uptake by inhibition of orexigenic peptides (reviewed by Chandra and Liddle, 2007), the increased MEMRI signal resulting from the interaction between CCK and the high-fat diet indicated a decrease in the inhibiting action of CCK. The amygdala is a brain region traditionally studied for its role in behaviors such as fear and anxiety but it also influences ingestive behaviors. In fact, it was found to be involved in both the homeostatic regulation of energy intake and the selection of macronutrients (King et al., 1998). In addition, a recent study by Boghossian et al. (2009) showed that high-fat diets induce a rapid loss of the insulin anorectic response in the amygdala of rats. The higher activation of the amygdala in the case of high-fat fed animals that were injected with CCK might therefore reflect modifications in the homeostatic regulation of energy intake through CCK. It should be noted that MEMRI can give us information about differences in neuronal activity but it cannot indicate whether the neurons concerned are activating or inhibitory. Therefore we cannot tell if the interaction with fat is caused by the inhibition being reduced or being balanced by increased stimulation. In order to look at possible HC:CCK interactions, we conducted the statistical analysis with the HP diet as the mean value. No significant HF:CCK nor HC:CCK was found (PVN peak z-score = 2.24, amygdala peak z-score = 2.01; Table 3). The reason that the HF:CCK interaction disappears in a high-protein background is, we believe, that it gets partitioned between HF:CCK and HC:CCK. This indicates that the observed significant interaction between HF and CCK in a high-carbohydrate background might be due to specific effects of the high-fat diet or to the attenuation of effects of the HC diet. Our results are in line with a recent publication by Nefti et al. (2009) who demonstrated that a high-fat diet altered the short-term response to CCK-8 and intragastric macronutrient loads while decreasing vagal activation by CCK-8 and modifying the receptor expression of vagal neurons compared to a normal fat diet. However, our findings may not only be attributed to the fat content of the HF diet but also to its superior energy density. Since our dietary data indicate that there were no differences in overall energy intake or body weight between the different groups, we were able to rule out increased caloric intake as a potential confounding factor.

The present study therefore indicates a significant relation between maintenance under a high-fat diet and the function of brain areas controlling food intake. Moreover, it puts forward that a high-fat diet leads to a decrease in CCK-induced effects in the paraventricular nucleus of the hypothalamus.

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