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Functional brain responses in the maintenance of energy balance

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Functional brain responses in the maintenance of energy balance

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CHAPTER 1

General introduction

The regulation of feeding behavior and energy balance is crucial for maintaining health. Various modern day health problems like obesity, metabolic syndrome, diabetes type 2 and eating disorders are all linked to a combination of disrupted feeding behavior and energy balance. In addition to the well-known endocrinological aspects in this, recently it was recognized that the brain is very important in regulation of our feeding behavior, both on a conscious and sub-conscious level.
In the regulation of feeding behavior, various internal and external factors play a role. Hunger and the basic need for energy for both the body and brain are the main driving factor to start eating. Energy intake is regulated by the homeostatic system. The most important brain area of this system is the hypothalamus, which uses objective signal from the periphery to determine the energy status of the body and combines these with signals of hunger and satiety. However, besides caloric need, the pleasure and reward of eating and drinking may also lead to the intake of more energy than strictly necessary. Unfortunately, highly palatable food is often high in carbohydrates, high in fats and usually strongly flavored. All of these characteristics can be very stimulating and elicit strong rewarding responses from the brain. Other factors such as memory and learned habits, social circumstances, personal preference and inhibitory control can also influence hedonic feeding. The hedonic regulation is mainly determined by the limbic and executive control systems of the brain, which can inhibit or override the homeostatic system. If so, this often leads to energy intake that is not in line with homeostatic need. Altogether, the central regulation of energy balance by the brain is a very complex, but is important in maintaining health. Extending knowledge on how these process are influenced and disrupted is crucial for a better understanding of feeding behavior and effects of diet on the brain, and for the development of markers aiding in the management of obesity, metabolic syndrome and eating disorders.
In this chapter the neuronal brain systems involved in central regulation of energy balance will be explained in more detail. The differences in function and responses of these systems when energy balance is disrupted will also be introduced. Furthermore, the effects that different food characteristics can have on the functional responses of the brain in the maintenance of energy balance will be described. And a short introduction of Magnetic Resonance Imaging techniques to investigate these functions and responses will be given. Finally, the overall aim of this thesis and the aim of the various chapters will be discussed.

Central regulation of energy balance by the homeostatic and hedonic systems
In the last decades the regulatory role of the brain in directing energy balance, glucose homeostasis, and eating behavior, is increasingly being recognized [1-3]. Energy

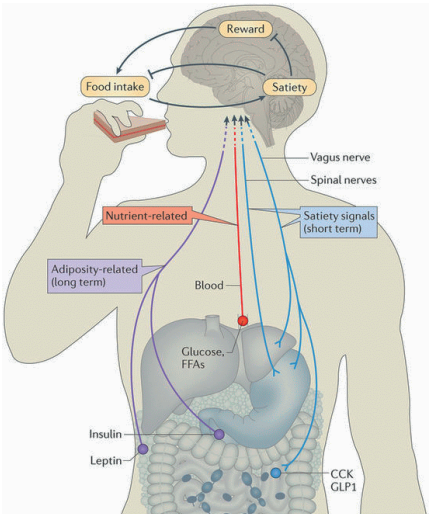


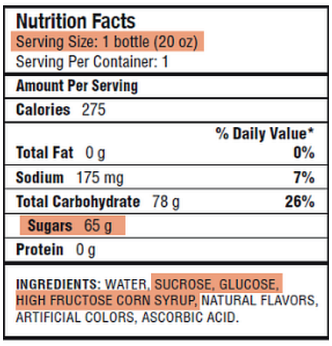
Figure 1. Central regulation of energy balance. The brain coordinates food intake based on various homeostatic (satiety) signals from the periphery and gut combined with reward signals. CCK, cholecystokinin; FFAs, free fatty acids; GLP1, glucagon-like peptide 1. Adapted from Morton, G.J., et al. Nat. Rev. Neurosci, 2014. 15(6)

balance is centrally regulated by the brain through several interacting neuronal systems. The homeostatic system and the hedonic system are mainly involved in maintaining energy balance [2, 4]. The homeostatic system, consisting of the hypothalamus and several nuclei in the brainstem, regulates energy intake by combining satiety signals with metabolic and hormonal cues from the periphery [4, 5]. In this system, the hypothalamus is the most important structure. It regulates energy balance by integrating information from glucose and insulin trajectories with varying levels of hormones and peptides from the gut and stomach [6-10]. Circulating metabolic cues that are related to the energy status of the body, such as ghrelin, leptin and insulin, all influence the homeostatic regulation by initiating signals of hunger and satiety [11, 12]. This information is than integrated with signals from the reward system [13]. A schematic depiction of these interactions between the periphery and homeostatic satiety and reward signalling is shown in figure 1. The reward pathway is responsible for the hedonic response to food. The ventral tegmental area (VTA) and other areas of the limbic system, such as the amygdala and nucleus accumbens, are brain areas that are important in this hedonic response [14]. The VTA is the origin of dopaminergic signaling in the mesolimbic reward system, which is a key substrate for reward prediction and response [15]. The VTA is anatomically and functionally connected to the hypothalamus and integrates homeostatic signals with reward responses [16-18]. Hedonic processes can, completely without awareness, override the homeostatic system and lead to disrupted energy balance, eating behavior and finally to obesity [14, 19].

Understanding of homeostatic and hedonic functional brain responses yields insights into satiety signaling, nutrient sensing, energy seeking and feeding behavior. Moreover, it may aid in the development of neurophysiological markers for (de)regulation of these systems in obesity, eating disorders or type 2 diabetes.

Effects of food characteristics and different nutrients on functional brain responses regulating energy balance

The palatability of food and beverages plays an important role in the consumption of energy. Various food characteristics can influence palatability and therefore influence regulation of energy intake. Several aspects of food and beverages such as taste, smell, visual cues and energy content have been found to elicit or influence functional brain responses [20-22]. The ingestion of different mixtures of macro nutrients, fats, proteins and carbohydrates, which all have specific effects on the brain, can elicit various brain responses [23, 24]. Circulating glucose is the primary source of energy for the brain, and its metabolism is kept under tight regulation to maintain optimal (brain) physiology [25, 26]. Both homeostatic and mesolimbic pathways in the brain have glucose sensing neurons and respond readily to glucose ingestion [25, 27, 28]. Glucose has been shown to elicit a homeostatic satiety response from the hypothalamus almost immediately after ingestion indicated by a decrease in hypothalamic neuronal activity [8, 29-31].
Glucose is a commonly consumed natural sugar and is used a sweetener in various foods and beverages. In addition to glucose, various other mono- and di-saccharides, such as fructose and sucrose, and low or non-caloric sweeteners are increasingly being used to sweeten foods and beverages [32, 33]. The metabolic pathways of these sweeteners is all different. For instance; in contrast to glucose, fructose cannot be used directly as a source of energy but first has to be metabolized by the liver before it can be taken up in the blood and recognized by the brain [7, 34, 35]. The metabolic effects of non-caloric sweeteners are less straightforward. Although, non-caloric sweeteners are expected to decrease caloric intake and might therefore be useable to control obesity [36], epidemiological studies have found that non-caloric sweeteners might have the opposite effect and could actually lead to increased energy intake [9, 37]. The increased ingestion of glucose, fructose or non-caloric or low caloric sweeteners over the last decades, coincides with increased prevalence of obesity [38, 39]. This increases the particular interest of investigating the homeostatic and hedonic aspects of these substances [9, 32, 39, 40].
An important contributor to the overall increased intake of sugars and sweeteners in the modern diet is the increased consumption of sweetened beverages [40]. Generally, sugar sweetened beverages have a very high sugar content and are sweetened with a combination of several sugars (a typical nutrition label for a sugar sweetened beverages



Nutrition Facts
Serving Size: 1 bottle (20 oz)
Serving Per Container: 1

Amount Per Serving	
Calories 275	
	% Daily Value*
Total Fat 0 g	**0%**
Sodium 175 mg	**7%**
Total Carbohydrate 78 g	**26%**
Sugars 65 g	
Protein 0 g	

INGREDIENTS: WATER, SUCROSE, GLUCOSE, HIGH FRUCTOSE CORN SYRUP, NATURAL FLAVORS, ARTIFICIAL COLORS, ASCORBIC ACID.

Figure 2. Typical nutrition label of a sugar sweetened beverage. Sugar sweetened beverages are often very high in sugar and sweetener content, often containing various other sugars or added artificial sweeteners besides glucose such as sucrose and high fructose corn syrup.

is shown in figure 2). In the palatability and rewarding aspects of consumption of sugar sweetened beverages several factors are involved. In addition to the high energy content and sweetness, the refreshment and thirst quenching features are also important [41]. Drinks consumed at low temperatures have been shown to be more thirst quenching than drinks consumed at room temperature [42], indicating that the relatively low consumption temperature, at which sugar sweetened beverages are generally consumed, could have effects on reward and homeostatic brain responses. In addition to sweetness and energy content and consumption temperature, the pleasantness of food is also affected by factors such as flavor and texture. Flavor can have a direct influence on feeding behavior by making food palatable and attractive and thereby eliciting a reward response in the brain [16, 43, 44]. Taste and flavor are an important part of the palatability and pleasantness of food and have been shown to have an effect on the consumption volume and rewarding effects of food and might cause overeating [43, 45, 46]. Energy ingestion combined with a pleasant flavor has been shown to enhance the rewarding properties of energy ingestion. For instance, when glucose is paired with a congruent flavoring, the perception of flavor is enhanced, and results in a greater feeling of satiety [47-49]. Furthermore, sweet taste that is rated as pleasant has been shown to elicit an insulin response when applied to the oral cavity only [50]. Although the evidence for the effect of taste perception on the insulin response is limited, these results suggest that the taste of food, independent of energy content, might influence the central regulation of energy intake and the peripheral energy metabolism.

Taken together, current scientific evidence indicates that various ingredients and other aspects of food and beverages as consumed in our modern day diet could influence and/or alter functional brain responses important for the maintenance of energy balance. Therefore, investigating these effects is important for our understanding of how they might affect regulation of energy consumption and feeding behavior and what role they could play in the disruption of energy balance.

Altered brain function in disrupted energy balance

Knowledge of the regulation of the energy balance by the brain is also important because various modern day health problems and chronic diseases can be led back to a disrupted energy balance, feeding behavior and altered brain function. One of the most important and most prevalent public health concerns is obesity and its subsequent co-morbidity [51]. Obesity is generally marked by a disrupted energy balance and increased storage of energy (fat) often coinciding with disturbed eating behavior [52]. The regulatory role of the brain in obesity is essential since it has unequivocally been shown that in obese persons the function of homeostatic and hedonic brain areas is altered [53-57]. Generally, in obese persons, brain connectivity and activity is increased compared to lean persons [53, 56-58], especially in brain areas that are involved in reward processes [53-55, 57]. Furthermore, brain responses to fasting but also to food intake are different between lean and obese persons [59]. In diabetes type 2, a common co-morbidity of obesity, the hypothalamus demonstrates a decreased homeostatic response after glucose ingestion [29, 30]. This lack of response can be caused by disrupted insulin sensitivity and signaling. Insulin is a hormone that gives negative feedback to hypothalamic nuclei that control energy and glucose homeostasis, and is vital in the regulation of glucose and energy metabolism [60-63]. Hypothalamic functioning is also important on the other end of the spectrum of a disrupted energy balance. For instance, in patients with anorexia nervosa, who demonstrate a severe negative energy balance and disrupted feeding behavior, the hypothalamic-pituitary-adrenal axis is hyperactive [64-66].

Taken together, disrupted brain function plays an important role in several diseases and conditions associated with a disrupted energy balance. Investigating these alterations in brain function and how they could be influenced and/or normalized is therefore important to determine potential targets for the treatment of obesity, metabolic syndrome and eating disorders.

Magnetic resonance imaging techniques to investigate functional brain responses

To investigate functional brain responses in regulating energy balance, functional Magnetic Resonance Imaging (fMRI) can be used to visualize and quantify both functional brain activity and connectivity. Blood oxygen level dependent (BOLD) fMRI is used as a measure of neuronal activity [67]. BOLD fMRI has been used extensively to analyses the immediate effects of nutrient ingestion on specific brain areas and throughout the brain [8, 20, 30, 31]. Beyond measurements of neuronal activity, analysis of functional connectivity can been used to provide further insights into brain areas interacting together to perform specific functions. Functional connectivity analysis

has been successfully used to identify and investigate various functional networks involved in feeding behavior, reward responses and energy balance. Changes in connectivity in these networks have been found after exposure to food cues and nutrient ingestion [54, 55, 68-70].

Aim of this thesis

The primary aim of this thesis was to gain more insights into brain function in the maintenance of energy balance by determining the effects of several aspects of our modern day diet and the effects of a disrupted energy balance on functional brain responses. With the goal to further elucidate both normal and disrupted central regulation of energy consumption and feeding behavior to find neurophysiological markers aiding in the management of obesity, metabolic syndrome and eating disorders.

As glucose is the preferred source of energy of the brain we first investigated the normal functional brain responses to glucose (**Chapter 2**) in healthy normal weight participants. However, in addition to glucose other sugars and sweeteners are often consumed as part of our daily diet. Therefore, to gain a better understanding of the different effects of the ingestion of other caloric and non-caloric sweeteners in addition to glucose, in **Chapter 3** we investigated the effects of several different sugars and sweeteners on the homeostatic and hedonic responses. With the exception of sugar sweetened beverages, sugars and sweeteners are usually consumed in a mixed meal together with other nutrients. Therefore, in **Chapter 4** we investigated the effects of different sugars and sweeteners throughout the brain in the context of other nutrients. To this end, we investigated the effects of the ingestion of sweetened nutrient shakes containing fats and protein and sweetened with either the nutritive natural sugars or low- /non-nutritive sweeteners. In addition to the effects of sweetness and energy content, we investigated the role of consumption temperature (**Chapter 5)** and flavoring **(Chapter 6)** of beverages on hedonic and homeostatic brain responses.

As discussed, various metabolic cues, such as circulating insulin, are known to influence homeostatic regulation of energy balance via the hypothalamus. Deficits in central insulin action in Diabetes type 2 are theorized to play a role in defective hypothalamic responses to glucose. As a proof of concept we investigated in **Chapter 7** whether centrally administrated insulin could potentiate the BOLD response in the hypothalamus after glucose ingestion in healthy normal weight subjects.

Because of the strongly disrupted energy balance and known hypothalamic dysfunction in patients with anorexia nervosa, we investigated whether the hypothalamic response to energy ingestion in these patients was disrupted (**Chapter 8**). Obesity is also be characterized by a disrupted energy balance and alter feeding behavior. The disrupted

energy balance in obesity is also associated with altered brain function, often in the form of increased connectivity and activity, compared to lean persons [53, 56-58]. However it is not known if these altered functions are the cause or a consequence of excess body weight. Therefore, in **Chapter 9** we investigated whether weight loss could reverse and/or normalize the increased brain activity in obese participants.
Overall conclusions, final remarks on the findings of this thesis and future perspectives are discussed in **Chapter 10**.

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CHAPTER 2

Brain activity and connectivity changes in response to glucose ingestion

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ABSTRACT

Objectives

The regulatory role of the brain in directing eating behavior becomes increasingly recognized in addition to the well-known pathways by stomach and gut. Although many areas in the brain have been found to respond to food cues, very little data is available about functional brain responses after actual caloric intake. The aim of this study was to determine normal whole brain functional responses to the ingestion of glucose after an overnight fast in healthy normal weight subjects using several functional MRI analysis approaches.

Methods

Twenty-five healthy, normal weight, adult males underwent functional MRI on two separate visits. In a single-blind randomized study setup, participants received either glucose solution (50gr/300ml of water) or plain water. We studied changes in Blood Oxygen Level Dependent (BOLD) signal, changes in voxel based connectivity by Eigenvector Centrality Mapping (ECM), and changes in functional network connectivity.

Results

Ingestion of the glucose solution led to a small increase in centrality in the thalamus and to decreases in BOLD signal in various brain areas. Additionally, decreases in connectivity in the sensory-motor and dorsal visual stream networks were found. Ingestion of plain water resulted in widespread increases in centrality across the brain, especially in the insula and posterior cingulate cortex and increases in connectivity in the medial and lateral visual cortex network. Increased BOLD intensity was found in a small cluster in the intracalcarine sulcus and cingulate cortex.

Discussion

Our data show that after an overnight fast, ingestion of glucose leads to decreased activity and connectivity in brain areas and networks linked to energy seeking and satiation. In contrast, drinking plain water leads to increased connectivity in the brain, probably associated with continued food seeking and unfulfilled reward.

Trail registration: The present study combines data of two studies which were registered at clinicaltrails.gov under numbers NCT03202342 and NCT03247114.

INTRODUCTION

The regulatory role of the brain in directing glucose homeostasis, energy homeostasis, and thus eating behavior, is increasingly being recognized [1;2]. Glucose is the primary source of energy for the brain, and its metabolism is kept under tight regulation to maintain optimal brain physiology. The brain consumes about 20% of glucose derived energy in the human body [3]. However, energy consumption is not only regulated by homeostatic processes, it also has hedonic aspects in which the brain has an important role [1]. Next to maintaining energy balance, the hedonic features of food and executive control by the brain are important in driving energy seeking behavior, and may be involved in overconsumption of energy. In the regulation of energy intake, many parts of the brain have been found to be involved. Brain areas such as the ventrolateral prefrontal cortex (executive and inhibitory control), hypothalamus (energy homeostasis), insula (taste response) and Ventral Tegmental Area (VTA, reward) have been found to respond to food cues in the form of taste, smell and visual cues [4;5].

Up until now, only a few studies have investigated the direct brain response to actual nutrient ingestion, by studying changes in functional connectivity throughout the brain and specific Blood Oxygen Level Dependent (BOLD) response in the hypothalamus [6-8]. These studies revealed that hypothalamic activity was suppressed within minutes after consumption, suggesting a relation with alterations in energy homeostasis combined with decreased feelings of hunger. In addition to homeostatic effects, hedonic aspects of energy ingestion are also important [9-11]. It is to be expected that, in addition to the hypothalamus, other parts of the brain that are involved in reward, motivation or inhibition and decision making, also show functional responses after glucose ingestion. Understanding of these functional brain responses yields insights into satiety signaling, nutrient sensing, energy seeking and feeding behavior. Moreover, it may aid in the development of neurophysiological markers for (dys-)regulation of these systems in obesity, eating disorders and type 2 diabetes.

Beyond measurements of local BOLD changes, which has been shown to be a measure of neuronal activity [12], analysis of changes in functional connectivity would provide further insights into functional responses after food ingestion. Network analysis is a proven measure to analyze functional brain networks at rest, reflecting basal cerebral functions [13], for instance in context of feeding behavior [14]. A newer method to determine functional brain connectivity is Eigenvector Centrality Mapping (ECM). ECM is an assumption and parameter-free method to determine the level and quality of connectivity on a voxel-wise level [15;16]. Eigenvector centrality has been shown to be modulated by the physiological state of the subject and ECM has successfully been used to investigate voxel-wise connectivity in states of hunger and satiety[16].

The main aim of this study was to determine normal whole brain functional responses to the ingestion of water and glucose by investigating changes in BOLD activity, voxel based Eigenvector centrality and functional network connectivity. We used functional MRI to investigate these effects in normal weight, adult male persons through the ingestion of plain water and glucose dissolved in water after an overnight fast..

METHODS

Subject characteristics

Twenty-five non-smoking, Caucasian men, aged 18 to 25 years were recruited through local advertising for two studies on functional brain responses to nutrient ingestion. Exclusion criteria for both studies were: a history of disturbances in glucose metabolism (e.g. diabetes mellitus), any significant chronic disease, psychiatric disease, BMI below 20 or above 23 kg/m^2, body height below 170 or above 190 cm, recent weight changes (>3kg gain or loss) within the last 3 months, having smoked within the last 6 months, recent blood donation, alcohol consumption of more than 21 standard servings per week, recent use of recreational drugs, and contra-indications to MRI scanning. The present study combines data of two studies which were approved by the local Medical Ethical Committee and registered at clinicaltrails.gov under numbers NCT03202342 and NCT03247114. All volunteers gave written informed consent before participation.

Study design

Study design for both studies was a randomized cross-over observational design, consisting of four or five study occasions respectively. Both studies included glucose and water ingestion, data from these sessions were combined for the current study. For all sessions subjects were asked to refrain from strenuous physical activity and/or alcohol consumption the day before scanning and were admitted to the research site after having fasted overnight (12 hours). To minimize circadian influence, all subjects were examined in the morning between 9:00 and 11:00 AM. The test solution for the study consisted of either 50 grams of glucose (in the form of dextrose powder) dissolved in 300 ml of tap water or 300 ml of plain tap water. This glucose dose is comparable to sugar amounts found in several high energy beverages and was chosen to provide a strong blood glucose and insulin response. Both solutions were consumed at room temperature. The protocol consisted of a 30-minute acclimatization period in the MRI-facilities prior to data acquisition. Functional MRI was performed before and after glucose/water administration. The test drink was delivered through a per-oral tube while the subject was still in supine position in the MRI scanner. The pre-ingestion fMRI

scan was performed 10 minutes before, and the post-ingestion scan was started 16 minutes after administration of the test drink. The total MRI procedure lasted 50 minutes.

Blood sampling

Blood samples were taken before and after the entire scanning session by venipuncture. Plasma insulin was measured using a radioimmunoassay kit (Medgenix, Fleurus, Belgium) and plasma glucose was measured using a fully automated Hitachi 704/911 system (Hitachi Medical Systems Europe, Reeuwijk, the Netherlands). Changes in blood levels were statistically analyzed using paired samples t-tests.

Hunger rating

Subjective feelings of hunger were indicated on a Visual Analogue Scale (VAS) which consisted of a 10 cm line, with 'not hungry' and 'extremely hungry' as anchors. Subjects were asked to indicate their score on the line, higher scores indicating a more hungry feeling. Changes in VAS scores were statistically analyzed using the non-parametric Wilcoxon signed-rank test.

MRI data acquisition

MRI scanning was performed on a Philips Achieva 3.0 T scanner using a 32-channel SENSE head coil (Philips Healthcare, Best, The Netherlands). Anatomical high-resolution 3D T1- weighted images of the whole brain were acquired (TR 9.8 ms, TE 4.6 ms, flip angle 8, 140 transverse slices, FOV 224 mm x 177 mm x 168 mm, reconstructed in-plane resolution 0.88 mm x 0.87mm, slice thickness 1.2 mm) along with a high-resolution T2*-weighted EPI scan (EPI factor 35, TR 2200 ms, TE 30 ms, flip angle 80, 84 axial slices, FOV 220 mm x 220 mm, in-plane resolution 1.96 mm x 1.96 mm, slice thickness 2.0 mm) for registration purposes. Resting-state scans were acquired with T2*-weighted gradient echo-planar imaging (EPI factor 35, 160 dynamics, 37 transverse slices scanned in ascending order, TR 2200 ms, TE 30 ms, flip angle 80, FOV 220 mm x 220mm, voxel size 2.75 x 2.75 x 2.50 mm with a 0.25 mm slice gap, total acquisition time: 6 minutes).

MRI data preprocessing

MRI data were preprocessed and analyzed using Functional Magnetic Resonance Imaging of the Brain Software Library (FSL) version 5.0.8. [17], Matlab and Phyton. Of all data sets, structural and functional, non-brain structures were removed using Brain Extraction Tool (BET) tool as implemented in FSL. The T1-weighted images were registered to the 2 mm isotropic MNI-152 standard space image (Montreal Neurological Institute, Montreal, QC, Canada) using non-linear registration with a warp resolution

of 10 mm. The FMRI Expert Analysis Tool (FEAT) was used for motion correction with MCFLIRT, spatial smoothing with a full width at half maximum of 3 mm, and high pass temporal filtering with a cut-off frequency of 0.01 Hz. The functional resting state images were registered to the corresponding T1-weighted images using Boundary-Based Registration (BBR) affine registration, using the high-resolution echo planar images as an additional registration step.

MRI data analysis whole brain BOLD changes

Whole brain BOLD intensities were compared before and after ingestion of glucose and water according to Rombouts et al. [18]. In short, a single volume BOLD signal map was calculated by averaging the time series data. Average cerebrospinal fluid (CSF) signal of the BOLD image was determined by averaging all voxels within the masked CSF. This CSF mask was determined by selecting voxels located in the lateral ventricles on the segmented structural images. This approach decreases the possibility of including unwanted signal of other compartments than CSF. Next, in each subject, a normalized BOLD signal map was calculated by dividing each voxel's signal by the average CSF signal. Voxel-wise comparisons of pre- and post-ingestion normalized BOLD signal maps were done using the Randomize tool of FSL with a paired samples approach and using Threshold-Free Cluster Enhancement (TFCE)[19]. All data were family wise error (FWE) corrected at a level of $p<0.05$.

MRI data analysis Eigenvector centrality changes

For the connectivity analysis the data-driven ICA-based Automatic Removal of Motion Artifacts (ICA-AROMA) was used to identify components in the data related to head motion and to remove these using linear regression [20;21]. Voxel-based connectivity Eigenvector centrality maps were calculated on the ICA-AROMA preprocessed data for each participant using fast-ECM software, which estimates voxel-wise eigenvector centralities from fMRI time series (github.com/amwink/bias/tree/master/matlab/fastECM)[15]. Pre- and post-ingestion Eigenvector centrality maps were compared in a voxel-wise approach in the masked grey matter using the Randomize tool with a paired samples approach and using TFCE [19]. Pre- and post-ingestion scans were compared per condition with paired two-sided contrasts. The same family wise error (FWE) correction at $p<0.05$ as for the whole brain BOLD analysis was used.

MRI data analysis network functional connectivity changes

Functional network analysis was performed on the same ICA-AROMA preprocessed data using the Beckmann resting state functional networks templates [13]. The Beckmann auditory network was used as a template for the salience network as this

standard template encompasses largely the same brain areas [22]. To account for noise, white matter and CSF templates were included in the analyses. Functional connectivity of each network of interest was calculated using the dual regression approach. This results in 3D images for each individual, with voxel-wise Z-scores representing the functional connectivity to each network. The average Z-scores per network were calculated for the pre- and post- ingestion time point. Differences in Z-scores between pre- and post-ingestion were analyzed using paired samples t-tests per functional network. Correction for multiple comparison for eight networks per statistical analysis was done with False Discovery Rate (FDR) correction, FDR corrected $p<0.05$ was deemed significant.

RESULTS

Subject characteristics, blood levels and VAS scores

Subject characteristics for the study group (n=25) are shown in table 1, all participants successfully completed all study visits. Blood levels of glucose, insulin, and VAS scores for feelings of hunger are shown in table 2. As expected, ingestion of glucose solution led to a significant increase in glucose and insulin levels in the blood, whereas water ingestion had no significant effect on blood levels. VAS scores for the feelings of hunger significantly increased after ingestion of water, while glucose ingestion resulted in a minor, non-significant decrease.

Table 1. Subject characteristics

	N = 25
Age (years)	21.2 ± 1.2
Height (m)	1.83 ± 0.06
Weight (kg)	73.6 ± 6.2
BMI (kg/m^2)	22.0 ± 1.2
Average fasted blood glucose	4.8 ± 0.5
Average fasted blood insulin	7.7 ± 5.1

Average fasted blood levels calculated as the average baseline values over two study visits. Values in mean ± standard deviation. Glucose levels in mmol/L , Insulin levels in mU/L

Whole brain BOLD signal changes

Figure 1 shows the changes in BOLD intensity after ingestion of plain water and glucose solution. After ingestion of plain water (Figure 1, left panel), increased BOLD intensity was found in a small cluster in the intracalcarine sulcus and cingulate cortex. No

Table 2. Blood values and VAS scores

	Plain water	Glucose solution
Pre ingestion blood glucose	4.9 ± 0.7	4.8 ± 0.4
Post ingestion blood glucose	4.7 ± 0.7	7.0 ± 1.0
Delta blood glucose	-0.1 ± 0.5	2.2 ± 1.0*
Pre ingestion blood insulin	6.8 ± 4.4	8.6 ± 8.1
Post ingestion blood insulin	5.9 ± 4.2	35.4 ± 20.5
Delta blood insulin	-0.9 ± 2.6	26.8 ± 22.1*
Pre ingestion VAS score hunger	5.2 ± 2.2	5.3 ± 2.4
Post ingestion VAS score hunger	6.1 ± 2.2	5.2 ± 2.6
Delta VAS score hunger	0.9 ± 1.1*	-0.1 ± 2.0

Values in mean ± standard deviation. Glucose levels in mmol/L , Insulin levels in mU/L, * $p<0.05$ tested using paired t-tests for blood values and Wilcoxon signed-rank test for the VAS scores.

decreases in BOLD intensities were found. After ingestion of glucose solution (Figure 1, right panel), BOLD intensity was significantly decreased in large clusters containing the insula, thalamus, anterior cingulate gyrus, orbito-frontal cortex, amygdala, hippocampus and the occipital cortex (intracalcarine cortex, lingual gyrus, and lateral occipital cortex). Additionally, decreases in BOLD intensity were also found in the brainstem and cerebellum.

Eigenvector centrality changes

Figure 2 shows the changes in eigenvector centrality after ingestion of water and glucose solution. After ingestion of plain water (Figure 2, left panel), large clusters of significant bi-lateral increases in eigenvector centrality were found in the post-central gyrus, transverse temporal gyrus, the precuneus and throughout the cingulate gyrus, both in the anterior and posterior division. These increases in Eigenvector centrality after drinking water indicate an increased level and quality of voxel-wise connectivity in these brain areas. After ingestion of glucose solution (Figure 2, right panel), a small significant bi-lateral increase in eigenvector centrality in the thalamus was observed. Neither of the study conditions led to significant decreases in eigenvector centrality.

Network connectivity changes

Figure 3 shows the change in Z-score for the eight functional connectivity networks when comparing pre- and post-ingestion after plain water (Figure 3, top panel) and after glucose solution (Figure 3, bottom panel). Ingestion of plain water led to increased functional connectivity in the medial visual (FDR corrected $p=0.016$) and salience network (FDR corrected $p=0.048$). After ingestion of glucose solution, functional

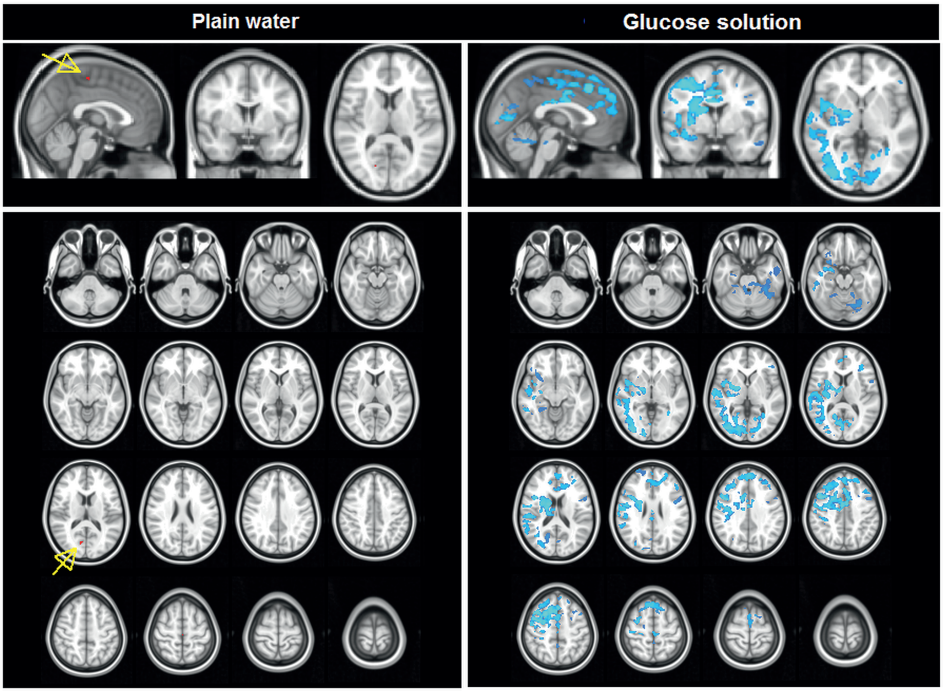

Figure 1. BOLD intensity changes after ingestion of plain water and glucose solution (FWE corrected). Left panel: plain water ingestion, right panel: glucose solution ingestion. Decrease in BOLD signal are shown in blue scale, increases are shown in red-yellow scale (indicate by yellow arrows).

connectivity increased in the medial visual network (FDR corrected p=0.042), whereas functional connectivity was decreased in the sensory motor network (FDR corrected p=0.042), and left (FDR corrected p=0.032) and right (FDR corrected p=0.032) dorsal visual stream network.

DISCUSSION

Our data show that after an overnight fast, ingestion of glucose solution and plain water led to very different brain fMRI responses compared to the pre-ingestion condition. After ingestion of glucose solution, large areas of the brain showed decreased BOLD activity and several functional networks showed decreased connectivity. In contrast, ingestion of plain water led to an increased voxel based and network connectivity.

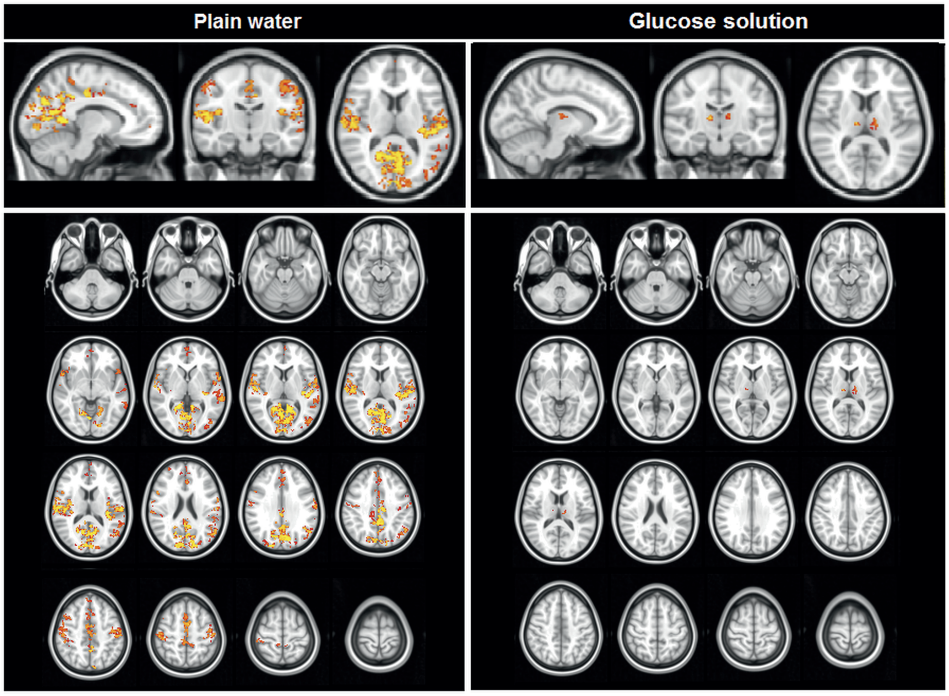

Figure 2. Eigen vector centrality mapping after ingestion of plain water and glucose solution (FWE corrected). Changes in centrality after ingestion of plain water and glucose solution. Increases in ECM are shown in red-yellow scale.

Effects of glucose ingestion on brain activity and connectivity

Ingestion of glucose solution resulted in a decrease in BOLD signal, indicating decreased neuronal activity in many areas of the brain which are known to be associated with reward, reward-learning and feeding behavior [1;23-25]. The occipital clusters that show a decrease in BOLD signal are likely all involved in visual processing of food cues [24]. Our findings combined with earlier studies suggest that brain activity is diminished after receiving energy, whereas in a fasted state these brain regions were active in seeking reward or seeking energy [6;7;26].

On the network level, a decreased connectivity was found in the sensory-motor and right and left dorsal visual stream networks after ingestion of glucose. These decreases in network connectivity are probably correlated with the results of BOLD analyses showing a decreased BOLD signal throughout clusters that overlap with the functional networks. The sensory motor network is involved in visceral perception and reward-based learning [27]. Changes in connectivity in this network in response to glucose could reflect the taste perception of the sweetness of glucose and could influence

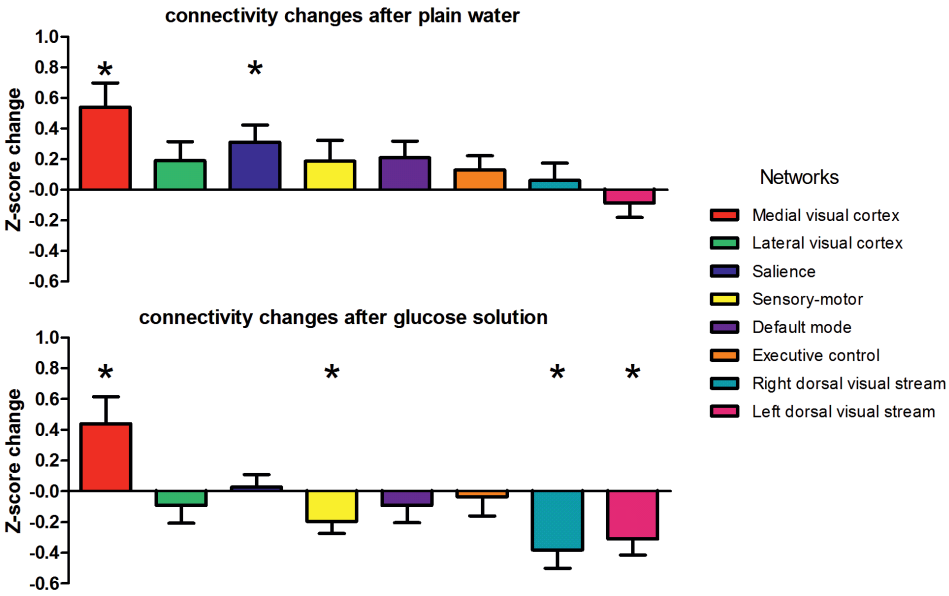

Figure 3. Changes in network functional connectivity before and after ingestion of plain water and glucose solution. Network connectivity changes (mean Z-score per network) are shown for plain water (top panel) and glucose solution (bottom panel). * FDR corrected p<0.05.

subsequent ingestion because of reward learning. In addition, the sensory motor network is involved in visceral perception. Therefore, changes in connectivity could be caused by increases in blood glucose levels after ingestion of glucose [27], which could reflect energy sensing. Indeed, we found a significant correlation between blood glucose levels and Z-scores in the sensory motor network in our study population (data not shown). Furthermore, a recent study has shown disruptions within the sensory-motor network connectivity in patients with type 2 diabetes that were associated with blood glucose levels [28]. Taken together this suggest that the response of sensory motor network found in our study could be important in maintaining glucose homeostasis. Finally, the difference in changes in connectivity found in the dorsal visual stream network could indicate effects on food seeking behavior, since visual processes are involved in determining salience [29], and approach behavior associated with food stimuli has been found to influence visual attention [30]. Additionally, visual food cues, that are processed by these visual networks, have been shown to influence the response in neural circuits involved in energy homeostasis and reward processing [24]. Taken together, the changes in activity and connectivity in visual areas and networks is likely involved in reward processing and decreases in energy seeking.

The effects of drinking glucose solution on the results of ECM was less pronounced. A

small increase in level and quality of voxel-wise connectivity was only seen in a small part of the thalamus. This could be explained by the fact that the thalamus relays signals between the sub-cortical and cortical structures in response to glucose ingestion, leading to a higher connectivity strength even though the average activity of the areas might be decreased. Still the observed effects are relatively small.

Effects of plain water ingestion on brain activity and connectivity

Ingestion of plain water resulted in reversed effects compared with ingestion of glucose solution. In contrast to the ingestion of glucose solution, drinking plain water did not result in any decreases in BOLD signal and led to an increased BOLD signal in small areas within the intracalcarine sulcus and cingulate cortex.

When investigating changes in brain connectivity, both with network analysis and Eigen vector centrality mapping, our data show that on both the voxel-wise and network level connectivity is generally increased after ingestion of plain water. Regions in which increased level and quality of voxel-wise connectivity was found were largely overlapping with functional networks that showed increased connectivity after drinking plain water. The transverse temporal gyrus is a part of the salience network [13], the precuneus and cingulate gyrus are part of the default mode network and the post-central gyrus falls within the sensory-motor functional network [13]. The salience network, which also includes the insular and anterior cingulate cortex [22], is generally considered to be involved in emotional arousal, reward sensitivity, and decision-making [31]. Connectivity changes in this network after plain water ingestion could therefore indicate an increased or continued energy and reward seeking. Indeed, several studies have shown an increased connectivity in the salience network in obesity that was linked to aberrant reward processing and overconsumption of energy [32;33]. In general, ingestion of water after an overnight fast results in an increased BOLD signal and increased functional connectivity on both a voxel-wise and network level. It seems that where glucose decreases brain activity after an overnight fast, ingestion of plain water enhances brain activity, possibly associated with increased or continued reward and energy seeking.

Study limitations and strengths

A limitation of our study is that it was performed only in a very homogenous group of male volunteers and it can be expected that sex differences are present, since it is known that there are several sex-specific differences in responses to satiation [34] and energy metabolism [35], which decreases the generalizability of our findings. A further limitation is that because our participants were fasted during the experiment extrapolation of our results to a non-fasted state might be limited, especially because

we did not take into account the normal eating behavior of the participants when not asked to remain fasted. Furthermore, as the brain consumes large amounts of glucose by default, oral consumption of a glucose dose may have general and non-specific effects on brain activity that are not specifically related to hedonic or energy regulating aspects of glucose intake. Additionally, because we used only a glucose stimulus, to be able to generalize our results to everyday sugar consumption future studies would have to be done using other (more complex) sugars and sweeteners that are commonly used in food and beverages. A strength of our study was our crossover study design, which allowed for a reliable within-subject comparison between interventions as participants were their own controls. A further strength was the hypothesis free approach taking into account the whole brain changes versus a region of interest approach that makes assumptions beforehand.

CONCLUSIONS

In conclusion, our data show that the ingestion of both water and glucose led to changes in BOLD activity and functional connectivity on both a voxel-wise and network level throughout the brain. Our results show that after an overnight fast, drinking of plain water leads to activation and increased centrality of several brain areas, as well as concerted increased connectivity in brain networks that are associated with energy and reward seeking and expectation. On the contrary, ingestion of glucose in a fasted state leads to deactivation and decreased connectivity, which can be associated with satiation and reward effects in the brain and a decrease in energy seeking. Which in turn can stimulate future re-consumption of glucose due to the rewarding effects.
A possible driving factor for the functional brain responses found in our study could be circulating blood insulin levels, increased insulin is generally regarded as a satiety signal and low or decreased insulin as a hunger signal for the brain [36]. This is in line with our findings that glucose ingestion, associated with an increase in blood insulin, leads to changes in activity and connectivity that can be associated with satiation and reward effects. And that water ingestion, with continued low blood insulin, leads to increased activity and connectivity in that can be associated with energy and reward seeking.
Interestingly, the brain areas and networks that show functional responses to consumption of water and glucose in our study have also been implied to function differently in obesity [8;14;32;33;37-41] and type 2 diabetes [28;42-44]. This suggest that maintaining the functional responses found in our study could be important for maintaining energy and glucose homeostasis.

Acknowledgements

Declaration of interests

MH and CB are both employees of Unilever Research and Development Vlaardingen B.V. The Netherlands. The other authors declare no competing interests.

Biographical note

AMvO is PhD student at the department of Radiology of the LUMC. JvdG, AAvdB and AH are senior researchers at the department of Radiology of the LUMC. MH and CB are both researchers employed by Unilever R&D. HP is professor at department of Internal Medicine, Section Endocrinology of the LUMC. SARRB is professor at the department of radiology of the LUMC and the Institute of Psychology, department of Methodology and Statistics of the Leiden University.

Author contributions

AMvO collected the data, did the literature search, wrote the report and made part of the figures. AMvO, AAvdB and AH analyzed the data. AMvO, AAvdB, AH and JvdG interpreted the data. AH prepared part of the figures. JvdG, AAvdB, MH, CB, SARRB and AMvO designed the study. All authors critically appraised and edited the report, and approved the manuscript before submission.

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CHAPTER 3

Dietary sugars and non-caloric sweeteners elicit different homeostatic and hedonic responses in the brain

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ABSTRACT

Objective

The brain is essential in regulating intake of food and beverages, by balancing energy-homeostasis, regulated by the hypothalamus, with reward perception, regulated by the ventral tegmental area (VTA). The main aim of the current study was to investigate the effects of glucose, fructose, sucrose and sucralose (a non-caloric artificial sweetener) ingestion on the magnitude and trajectory of the hypothalamic and the VTA BOLD responses.

Design

In five visits, 16 healthy men between 18-25 years with a BMI between 20-23 kg/m^2 drank five interventions in a randomized order while an functional MRI scan was made. The interventions consisted of: 50g of glucose, fructose, sucrose, or 0.33 grams of sucralose dissolved in 300ml tap water, 300ml of plain tap water was used as the control condition. Blood oxygen level dependent (BOLD) signals were determined in the hypothalamus and the VTA within a manually drawn region of interest. Differences in changes in BOLD signal between stimuli were analyzed using mixed models.

Results

Compared to the control condition, we found a decrease in BOLD signal in the hypothalamus after ingestion of glucose ($p=0.0003$) and a lesser and but delayed BOLD response after sucrose ($p=0.006$) and fructose ($p=0.003$) ingestion. Sucralose led to a smaller and transient response from the hypothalamus ($p=0.026$). In the VTA, sucralose led to a very similar response to the water control condition leading to an increase in VTA BOLD activity that continues over the measured time period. The natural sugars appeared to only lead to a transient increase in VTA activity.

Conclusions

Glucose induces a deactivation in the hypothalamus immediately after ingestion that continued over following 12 minutes, which is correlated with satiety signaling by the brain. Fructose and sucrose are both associated with a delayed and lesser response from the hypothalamus, likely because the sugars first have to metabolized by the body. Sucralose leads to the smallest and transient decrease in BOLD in the hypothalamus and leads to a similar response as plain water in the VTA, which indicates that sucralose might not have a similar, possibly satiating, effect on the brain as the natural sugars.

Keywords: dietary sugar, dietary sweeteners, functional brain responses, homeostasis, reward system, hypothalamus, ventral tegmental area

INTRODUCTION

Our brain is essential in regulating intake of food and beverages, by balancing energy-homeostasis with reward perception [1]. The hypothalamus is an important structure that regulates energy homeostasis by integrating information from glucose and insulin trajectories, with varying levels of hormones and peptides from the gut and stomach [2-5]. The mesolimbic pathway, together with the homeostatic regulation, is responsible for the hedonic response to food. The ventral tegmental area (VTA) and other areas of the limbic system (amygdala, nucleus accumbens) are important parts of the mesolimbic pathway involved in this hedonic response [6]. The VTA is the origin of dopaminergic neurons and dopamine signaling in the mesolimbic system, which is a key substrate for reward prediction and response [7]. The VTA is anatomically and functionally connected to the hypothalamus and integrates homeostatic signals with reward responses [8-10]. Both homeostatic and mesolimbic pathways respond to glucose, which is the natural and preferred source of energy for the body and brain [11, 12]. Glucose concentration in the blood is kept under tight homeostatic control, partly mediated by glucose- sensing neurons in the brain [12]. Glucose intake leads to changes in hypothalamic Blood Oxygen Level Dependent (BOLD) levels, which have been interpreted as a sign of satiety [4, 13]. However, little VTA data are available [14], and further investigation into the VTA may be essential in the understanding of the integration of homeostatic and hedonic responses regulation feeding behavior [6].

In many foods and beverages mono- and di-saccharides or artificial sweeteners are used as sweetener [15]. Increased consumption of these sugars and sweeteners in the modern diet has been speculated to play a role in pathophysiology of obesity, decreased vascular health, metabolic syndrome and type-2 diabetes [15-18]. High fructose consumption has also been hypothesized to have detrimental health effects and is associated with fatty liver disease and has been shown to have greater adverse effects on metabolism and vascular health than glucose in several animal studies [19, 20]. Fructose is used as a sweeter alternative to glucose, allowing for the use of smaller amounts, but the metabolism of both sugars is very different. In contrast to glucose, fructose cannot be used directly as a source of energy [21]. Fructose, which is a naturally occurring saccharide in fruits and vegetables, has to be metabolized by the liver first, before it can be made available as a source of energy [22]. Fructose can be consumed in its free form as a monosaccharide in fruits and high fructose corn syrup but also in the form of sucrose, which is a glucose-fructose disaccharide. In addition to the use of added natural sugars, non-caloric artificial sweeteners are increasingly being used to sweeten foods and beverages. The use of non-caloric sweeteners could be expected to decrease total caloric intake and might therefore be useful to control obesity [23].

However, some epidemiological studies suggest that non-caloric sweeteners might have the opposite effect and might actually lead to increased energy intake [5, 24, 25]. Although these results are not conclusive and the effects of non-caloric sweeteners remain a subject of debate, these result do indicate that these sweeteners could have unexpected effects in the brain. Currently, the homeostatic and mesolimbic effects in the brain of these sweeteners and other common dietary sugars are unknown. The differences in how various sugars are metabolized and made available as energy, and because of the decoupling of sweetness and energy in non-caloric sweeteners, each could lead to different metabolic and physiological responses in the brain [26-29].
To gain a better understanding of the different homeostatic and hedonic responses after ingestion of caloric and non-caloric sweeteners, we investigated the effects of glucose, fructose, sucrose and sucralose (an artificial non-caloric sweetener) on the trajectory and magnitude of BOLD response of the hypothalamus and VTA in normal weight young men.

METHODS

Subjects

Sixteen healthy men aged 18-25 years were recruited by advertisements around Leiden University. Inclusion criteria were: a body mass index (BMI) between 20-23 kg/m^2 and height between 170-190 cm. Exclusion criteria were: diabetes or a history of disturbances of glucose metabolism; genetic or psychiatric disease affecting the brain; renal or hepatic disease; any chronic disease; weight changes of more than 3 kg within the last 3 months or attempts to lose weight; smoking (within the last 6 months); alcohol consumption of more than 21 units per week; use of recreational drugs (within the last 12 months); recent blood donation; recent participation in other biomedical research projects and contra-indications for MRI scanning. Informed consent was obtained from all participants and the study was approved by the local institutional review board and registered at Clinicaltrails.gov under number NCT03247114. Subject characteristics are shown in table 1.

Table 1. Subject characteristics

	Mean ± SD (N=16)
Age (years)	22.4 ± 1.3
Height (m)	1.82 ± 0.06
Weight (kg)	73.0 ± 7.1
BMI (kg/m^2)	22.0 ± 1.2

Study design

In this randomized double blind crossover study the participants received five different conditions, one per occasion with a one week interval. Participants were asked to fast for a minimum of 10hs overnight (except for drinking water), while scans were obtained the next morning. Participants received 1 of the 5 conditions on each occasion in a randomized order. The interventions consisted of: glucose, 50g in 300ml tap water; fructose, 50g in 300ml tap water; sucrose, 50g in 300ml tap water; sucralose, 0.33 gr (matched for sweetness with the glucose solution) in 300ml tap water and 300 ml plain tap water as a control condition, no additional flavoring was added to the stimuli. The 50 grams of sugar in our study stimuli is comparable to sugar amounts found in several high energy beverages and was chosen to provide a strong blood glucose and insulin response. The glucose, sucrose and fructose solutions all contained 200 kilocalories, the sucralose sweetened solution was zero kilocalories. All stimuli were ingested at room temperature. Drinking was performed through an oral tube, lying supine in the scanner during the fMRI scan while the participants were monitored by the investigators. Five minutes after the start of the scan subjects were instructed to drink the total amount in a steady and continuous way. Scanning was continued during and after the start of the ingestion for 16 minutes, making for a total scan time of 21 minutes with five minutes pre-ingestion, four minutes ingestion and twelve minutes post-ingestion.

Blood samples and Visual Analogue scales

Before and after the scanning procedure, 5 ml blood samples were taken by venipuncture in an antecubital vein. Insulin and glucose levels before and 30 minutes after ingestion of the study stimuli were determined by the laboratory for Clinical Chemistry at Leiden University Medical Centre. Plasma glucose was measured using a fully automated Hitachi 704/911 system. Plasma insulin was measured by a radioimmunoassay (Medgenix, Fleurus, Belgium). Participants were asked to rate their feelings of hunger in advance of the scanning procedure and afterwards, using a Visual Analogue Scale (VAS) which consisted of a 10cm line, with 'not hungry' and 'extremely hungry' as anchors. Subjects were asked to indicate their score on the line, higher scores indicating a more hungry feeling. Additionally, 30 minutes after ingestion after finishing the MRI procedure, pleasantness and sweetness of the drink were rated using a similar VAS with 'disgusting' to 'very tasty and 'not 'sweet' to 'extremely sweet' as anchors.

MRI data acquisition

Magnetic Resonance Imaging was performed using a 3 Tesla Achieva whole body MRI scanner (Philips Healthcare, Best, The Netherlands) equipped with a 32-channel head

coil. The protocol for structural MRI comprised a scout view for planning, a high resolution 3D T_1-weighted sequence for registration purposes and a mid-sagittal high resolution single slice for accurate hypothalamus and VTA localization (repetition time 550ms, echo time 10 ms, field of view 208 x 208 mm, voxel size = 0.52 x 0.52 x 14 mm). Mid-sagittal fMRI was performed for 21 minutes in total, by a T_2* weighted, gradient echo-planar imaging (EPI) sequence mid-sagittal single slice that renders BOLD contrast (repetition time 120 ms; echo time 30ms; flip angle 30°; field of view 208 x 208 mm; voxel size = 0.81 x 0.90 x 14 mm; scanning time of one dynamic image 2.52 seconds for a total of 500 data points). A slice thickness of 14 mm was chosen to encompass the hypothalamus in the left to right direction and a single slice technique was used for sufficient signal to noise ratio.

fMRI processing

Imaging data were pre-processed and analyzed using Functional Magnetic Resonance Imaging of the Brain Software Library (FSL) version 5.0.8. [30]. The mid-sagittal fMRI was preprocessed as described before [31]. In short, all 500 dynamic images were motion-corrected by registration to the image that was acquired halfway through the fMRI period (scan 250) by means of Multimodality Image Registration using Information Theory (MIRIT) software in FSL by maximization of mutual information [32]. The complex data were averaged for each set of 4 subsequent volumes after which 125 images were rendered. Regions of interests (ROIs) were drawn manually, using subject-specific T_1-weighted images as a visual aid to define anatomical borders. Three ROIs were drawn: the hypothalamus, the VTA and a reference area. The ROI for the hypothalamus was drawn as described earlier using the optic chiasm, mammillary bodies, thalamus and anterior commissure as anatomical landmarks [31]. Using literature describing the VTA region [33, 34] we defined the ROI for the VTA in the midbrain with half the volume of the hypothalamus. The top of the cerebral aqueduct and the mammillary bodies were used as anatomical landmarks (figure 1). To correct for scanner drift, a third internal reference ROI half the volume of the hypothalamus ROI was selected in the gyrus superior to the genu of the corpus callosum in the grey matter. Hypothalamic and VTA BOLD signals were adjusted linearly to the BOLD signal of this internal reference ROI. Subsequently, to investigate the individual BOLD response after study intervention on the hypothalamus and VTA, the BOLD response after ingestion were all normalized to the BOLD response before ingestion, i.e. the mean BOLD signal before ingestion; the average BOLD signal of the first 4 minutes of the fMRI scan (figure 2).

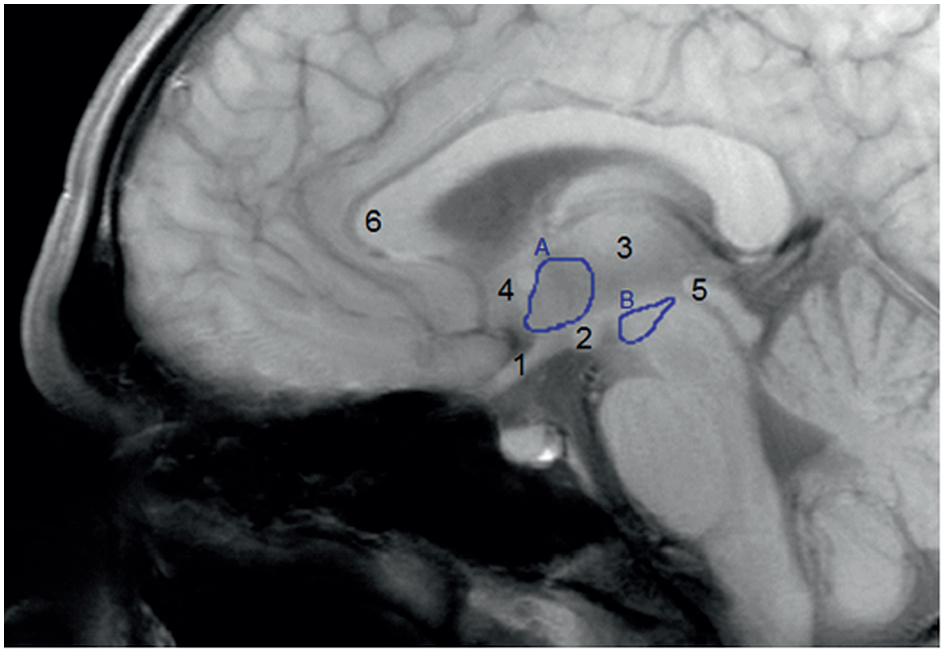

Figure 1. A representative sagittal view with the hypothalamus (A) and the Ventral Tegmental Area (VTA) (B) ROIs. For the hypothalamus ROI (A), the optic chiasm (1), the mammillary bodies (2), the thalamus (3) and the anterior commissure (4) were used as landmarks. For the VTA ROI (B), the top of the cerebral aqueduct (5) and the mammillary bodies (2) were used as landmarks. The reference ROI was drawn superior of the genu to the corpus callosum (6) in the grey matter.

Statistical analysis

Statistical testing of hypothalamus and VTA activations was performed using SPSS version 23. Differences in VAS scores and blood values between pre and post-ingestion and between conditions were tested using repeated measures ANOVA. To test for differences in the post-ingestion BOLD responses between sugars and sweeteners and the water control a mixed model approach was used, with Intervention and Study visit number as fixed effects and Time point as a covariate and a random effect for Subject*Study visit number. To determine the time effects in the BOLD response the entire 12 minute post-ingestion response was divided into four arbitrary 3-minute time blocks. Mixed model analysis for comparison between sugars and sweeteners and the water control was performed per time block. Significances are presented uncorrected. Pearson correlations were used to determine the association between BOLD response and blood levels and VAS scores.

RESULTS

Blood glucose and insulin levels

Blood glucose and insulin level measurements for all participants are shown in table 2. All subjects demonstrated normal fasting glucose levels and the average fasting glucose was not different between conditions. As expected, glucose and sucrose ingestion led to significant increases in both blood glucose and insulin levels. Fructose had no significant effect on blood glucose levels but led to a small significant increase in blood insulin levels. Sucralose had no significant effect on blood glucose levels but led to a small yet significant decrease in insulin levels.

Table 2. blood glucose and insulin levels

	Glucose	Fructose	Sucrose	Sucralose	Water
Pre-ingestion glucose	5.0 ± 0.7	4.9 ± 0.4	4.8 ± 0.3	5.2 ± 1.6	4.7 ± 0.9
Post-ingestion glucose	7.0 ± 1.1*	5.4 ± 0.7	7.0 ± 0.7*	4.7 ± 0.8	4.6 ± 0.9
Delta glucose	2.0 ± 1.4	0.5 ± 0.9	2.3 ± 0.8	-0.5 ± 1.7	-0.1 ± 0.5
Pre-ingestion insulin	6.4 ± 4.7	6.5 ± 4.0	7.5 ± 6.0	6.5 ± 5.3	8.0 ± 5.4
Post-ingestion insulin	32.5 ± 20.3*	12.3 ± 5.9*	26.5 ± 18.9*	5.3 ± 4.6*	6.6 ± 5.1
Delta insulin	26.0 ± 18.5	5.8 ± 3.9	19.0 ± 15	-1.6 ± 1.6	-1.5 ± 3.6

All values in mean± standard deviation. Glucose levels in mmol/L, insulin levels in mU/L. *significantly different between pre and post-ingestion at $p<0.05$.

Visual analogue scores for hunger and subjective rating of the study stimuli

VAS scores for hunger and subjective rating of the stimuli for all participants per study stimulus are shown in table 3. No significant differences were found between the stimuli in hunger score before ingestion. After ingestion of water the VAS score and delta VAS score for hunger was significantly increased compared to the other or stimuli. The taste of sucralose and water was rated significantly less pleasant compared to fructose and sucrose and as expected the water condition was rated significantly less sweet compared to the sugars and sweetener. All other study stimuli were rated similarly for sweetness.

Hypothalamic BOLD response

Figure 2 shows the mean normalized hypothalamic BOLD response curves after ingestion of the five study stimuli. Table 4 shows the corresponding BOLD effect sizes and statistical differences relative to the control condition, both for the entire response

Table 3. Visual Analogue Scale scores for hunger, pleasantness and sweetness

	Glucose	Fructose	Sucrose	Sucralose	Water
Pre-ingestion VAS hunger	5.3 ± 2.5	5.7 ± 1.9	4.8 ± 2.1	5.3 ± 1.9	5.2 ± 1.9
Post-ingestion VAS hunger	5.1 ± 2.4	5.5 ± 1.9	5.0 ± 1.8	5.3 ± 2.3	5.8 ± 2.3*
Delta VAS hunger	-0.2 ± 1.5	-0.2 ± 2.3	0.3 ± 1.8	0.0 ± 1.2	0.6 ±0.9*
VAS pleasantness	5.9 ± 1.7	6.8 ± 1.8	6.4 ± 1.9	5.3 ± 2.1*	5.1 ± 1.1*
VAS sweetness	5.4 ± 2.1	6.4 ± 1.4	6.9 ± 1.4	5.6 ± 2.2	0.8 ± 1.6*

All values in mean± standard deviation *significantly different form other study conditions at p<0.05.

Table 4. Mean difference in post-ingestion hypothalamic BOLD responses to the study stimuli and the water control condition

	Average response	Response min 9-11	Response min 12-14	Response min 15-17	Response min 18-21
Glucose	-1.9 ± 0.4*	-1.4 ± 0.5*	-1.5 ± 0.4*	-1.9 ± 0.5*	-2.0 ± 0.5*
Fructose	-1.2 ± 0.4*	-0.5 ± 0.5	-1.2 ± 0.4*	-1.6 ± 0.5*	-1.5 ± 0.5*
Sucrose	-1.3 ± 0.4*	-0.9 ± 0.5	-1.3 ± 0.5*	-1.3 ± 0.5*	-1.6 ± 0.5*
Sucralose	-0.9 ± 0.4*	-0.9 ± 0.5*	-1.2 ± 0.4*	-1.2 ± 0.4*	-0.9 ± 0.5*

Mean difference in BOLD response indicated in mean % change ± SE. *significantly different form the water control condition at p<0.05.

curve and the 3-minute response intervals. Ingestion of plain water led to an increase in BOLD signal, whereas all sugars and sucralose led to a decrease in BOLD signal. Compared to the control condition, glucose led to an immediate and continues decrease in BOLD response (mean difference 1.8%, p=0.0003). Fructose and sucrose also led to a significant average decrease compared to the water control condition (mean difference 1.2%, p=0.006 and mean difference 1.3%, p=0.003 respectively). However, the response in the first 3 minutes after ingestion of both fructose and sucrose was not significantly different from water, indicating a delayed response to these stimuli. The response to sucralose compared was significantly lower (mean difference 0.9%, p=0.026), and did not increase over time as was the case with the other sugars.

Ventral Tegmental Area BOLD response

Figure 3 shows the mean normalized BOLD response for all participants in the VTA over time after ingestion of the study stimuli. In the VTA, all conditions led to an increase in BOLD response after ingestion. Table 5 shows the mean effect sizes and statistical differences relative to the control condition over the entire 12 minute post ingestion period and during the four 3-minute time blocks. Ingestion of plain water

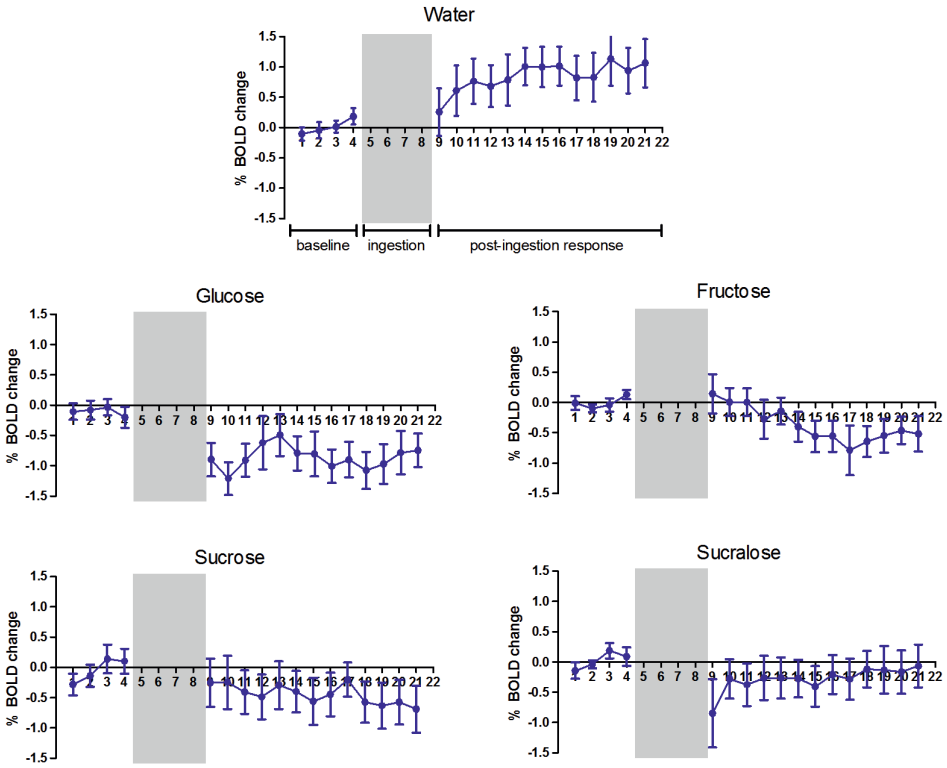

Figure 2. Normalized BOLD response (% change) in the hypothalamus after ingestion of water (control condition), glucose, fructose, sucrose and sucralose stimuli. Data presented as mean response for all participants (n=16) per minute with standard error. Post ingestion response was calibrated to the baseline period. Grey bar represents ingestion period, data recorded during this period was excluded from analysis.

led to an increase in BOLD signal, comparatively all sugars led to a smaller increase in BOLD signal (mean difference 0.4%). However, when comparing the response to the sugars to the response to plain water, no significant differences were found. The response to sucralose showed a close resemblance to the response to water, as indicated by the smallest mean difference between the water and sucralose response (mean difference 0.1%). The response to sucralose, and also to water, remained elevated over the entire 12 minute post-ingestion time period whereas the response to the natural sugars appeared transient. Additionally, glucose ingestion was the only stimulus that led to an initial decrease in VTA BOLD response in the first 3 minutes after ingestion.

Table 5. Mean difference in post-ingestion VTA BOLD responses to the study stimuli and the water control condition

	Average response	Response min 9-12	Response min 13-16	Response min 17-21	Response min 17-21
Glucose	-0.4 ± 0.4	-0.3 ± 0.5	-0.2 ± 0.5	-0.5 ± 0.5	-0.6 ± 0.5
Fructose	-0.4 ± 0.4	+0.2 ± 0.4	-0.4 ± 0.5	-0.6 ± 0.5	-0.6 ± 0.5
Sucrose	-0.4 ± 0.4	+0.2 ± 0.5	-0.5 ± 0.5	-0.5 ± 0.5	-0.5 ± 0.5
Sucralose	-0.1 ± 0.4	+0.3 ± 0.4	-0.3 ± 0.4	-0.2 ± 0.5	-0.1 ± 0.4

Mean difference in BOLD response indicated in mean % change ± SE.

Correlation of hypothalamic and VTA BOLD response with VAS and blood levels

We found a significant negative correlation between the rating of perceived sweetness and the hypothalamic BOLD response (Pearson correlation coefficient= -0.350, p=0.002). This indicates that a sweeter rated stimulus leads to a stronger decrease in hypothalamic response. We found no significant correlations between blood glucose and insulin levels and the BOLD responses of neither the hypothalamus nor the VTA.

DISCUSSION

Our data show that the hypothalamus and VTA demonstrate different BOLD responses to glucose, fructose, sucrose and sucralose ingestion. In contrast to glucose, which has a direct and effect on the hypothalamus, fructose and sucrose ingestion resulted in delayed and smaller hypothalamic BOLD responses. Sucralose ingestion led to a transient response in the hypothalamus. In the VTA, sucralose ingestion led to the same effect as the ingestion of plain water, being a prolonged activation over the measured time period. On the contrary, the natural sugars glucose, fructose and sucrose elicited a smaller VTA activation compared to plain water ingestion.

Our results are in line with earlier studies showing hypothalamic deactivation, which has been associated with satiety signaling, after ingestion of the natural occurring energy-containing sugar glucose [3, 4]. The hypothalamus is important in the homeostatic system regulating energy balance [3, 4], and thus a response to glucose was expected as it is an important energy source for the body and the brain [11]. This is reflected in our results by the almost immediate reaction to glucose, which is almost directly metabolically available and recognized the brain [11]. We found that the hypothalamic response to fructose and sucrose is delayed after ingestion which may be related to the fact that the sucrose dimer first has to be broken down and that

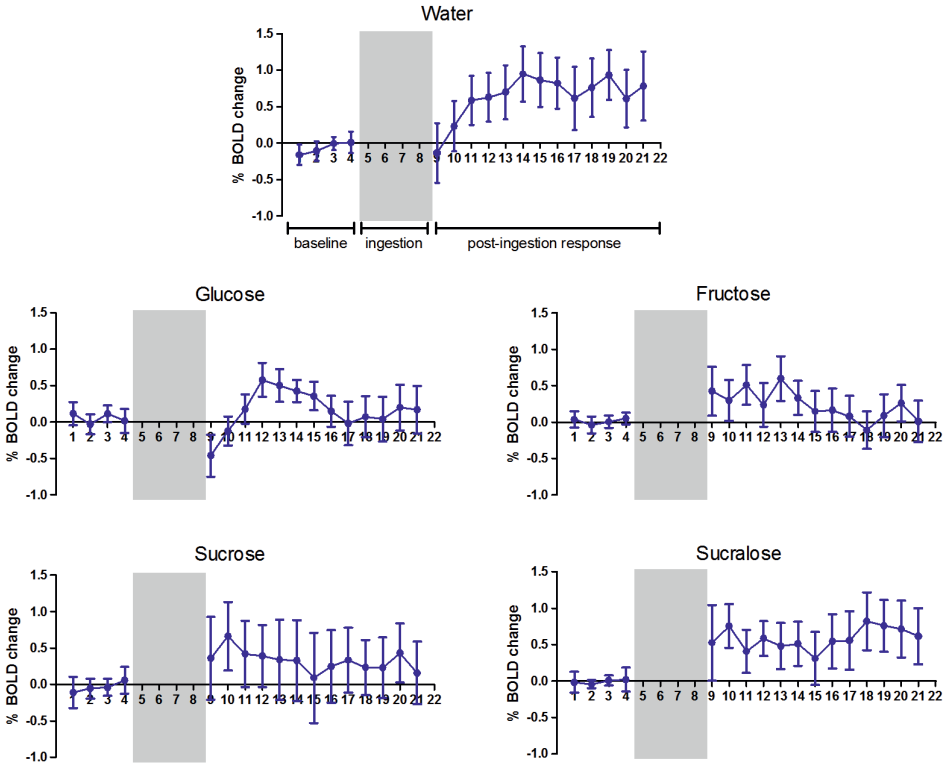

Figure 3. Normalized BOLD response (% change) in the Ventral Tegmental Area after ingestion of water (control condition), glucose, fructose, sucrose and sucralose stimuli. Data presented as mean response for all participants (n=16) per minute with standard error. Post ingestion was calibrated to the baseline period. Grey bar represents ingestion period, data recorded during this period was excluded from analysis.

fructose first has to be metabolized by the liver before it can be detected by energy sensing neurons in the brain [35].

The sweet taste of our study stimuli could be the prediction of the imminent reward of energy content, as sweet taste is deemed a predictor of the post-ingestive consequences of food [6, 36]. The initial response we found immediately after ingestion in the hypothalamus and VTA might therefore be a response to the sweet taste, which creates an expectation of an energetic reward, rather than to gut hormones and/or energy content and increases in blood glucose levels [37, 38]. This is in line with our finding that the artificial sweetener sucralose elicits an initial decrease in BOLD signal in the hypothalamus but does not result in a longer hypothalamic response like the ingestion of natural sugars, possibly because of the absence of energetic content.

The hypothalamus is functionally and anatomically connected to the VTA and influences

the dopamine reward system via several pathways [6, 8-10]. During fasting or while in a hungry state, an activated lateral hypothalamus has been shown to send signals to the VTA that promote reward seeking behavior in mice [39]. Overstimulation of this hypothalamus-VTA axis by a continues high hypothalamic activity, has been shown to lead to compulsive sucrose seeking in mice [39]. Our results show that the hypothalamus becomes deactivated when it senses glucose or energy content of the ingested sweetener, this could then influence the magnitude of the VTA response through the proposed connection between the hypothalamus and the VTA. Our results show that sucralose appears to lead to a prolonged activation of the VTA coinciding with a more transient response from the hypothalamus compared to the natural sugars. Indicating that due to the lack of energy content eliciting a deactivation of the hypothalamus this may result in a prolonged activation of the VTA. This suggests that non-caloric sweeteners do not lead to the same homeostatic and hedonic effects in the brain compared to energy-containing natural sugars, potentially caused by the dissociation between sweet taste and energy content [27].

A limitation of our study is the generalizability, as we only investigated a relatively small sample of male volunteers and it can be expected that sex differences are present, since it is known that there are several sex-specific differences in energy metabolism [40]. Additionally, we only investigated lean subjects and earlier studies have shown different hypothalamic function in obesity [41]. This decreases the generalizability of our findings to the general population. A further limitation is the fact that the sugars in this study were dissolved in water and not part of a broader food matrix. This could also limit the generalizability of our findings to more general, marketable products. On the other hand, by comparing the effect of sugars and sweeteners only, the results are not contaminated by spurious influences of other ingredients of the test products. A further limitation of our experimental design is that it was not representative of normal ingestive behavior of sugar sweetened beverages as these are not usually ingested in a fasted state in the morning or in a supine position. Additionally, as we only found few significant results due to a relatively large variation in VTA responses between participants an increase in sample size would be preferable to be able to better determine the VTA response and the difference in response between stimuli. Furthermore, more extensive VAS scoring of hunger immediately after ingestion, instead of after 30 minutes, and monitoring of energy intake during the rest of the day could provide better insight into the immediate and longer term effects on hunger and satiation caused by our study stimuli. A strength of our study is the measurements of the hypothalamic and VTA response over time, which allowed us to determine the dynamics of these responses to differently metabolized sugars.

Taken together our results show that ingestion of glucose elicits a prolonged decrease in hypothalamus activity with a possible transient response from the VTA, which could be associated with satiety signaling. Sucrose and fructose lead to a delayed hypothalamic response, and a small, if any, response from the VTA. Our data indicate that the brain responds directly and readily to glucose, as the preferred source of energy of the brain, but might not respond as efficiently to other sugars. Ingestion of the non-caloric sweetener sucralose however appears to lead to a more transient deactivation from the hypothalamus and led to the same response from the VTA as plain water ingestion. This could reflect a lack of satiety signaling by the hypothalamus and a different hedonic response to non-caloric sweetener ingestion due to lack of caloric content. Our findings indicate that the responses by the hypothalamus and VTA are mainly driven by sweet taste coupled with caloric content and that sweet taste without caloric content does not seem to elicit a lasting response from these brain areas.

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Trial registration

ClinicalTrails.gov registration number: NCT03247114

Declaration of interests

MH and CB are both employees of Unilever Research and Development Vlaardingen B.V. The Netherlands. The other authors declare no competing interests.

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CHAPTER 4

Brain activity and connectivity changes in response to nutritive natural sugars, non-nutritive natural sugar replacements and artificial sweeteners

Submitted

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ABSTRACT

Introduction

Functional brain responses to energy ingestion targeted to the regulation of energy homeostasis and eating behavior, is increasingly being recognized. The increased ingestion of sugars and sweeteners over the last decades makes investigating the effects of these substances on brain function of particular interest. Understanding of the functional brain responses to sugars or sweeteners could give insight into effects on satiety signaling and feeding behavior involved in maintaining energy homeostasis. In the present study we investigated whole brain functional response to the ingestion of nutrient shakes sweetened with either the nutritive natural sugars glucose and fructose, the low- nutritive natural sugar replacement allulose or the non-nutritive artificial sweetener sucralose.

Methods

Twenty healthy, normal weight, adult males underwent functional MRI on four separate visits. In a double-blind randomized study setup, participants received shakes sweetened with either glucose, fructose, allulose or sucralose. At each visit resting state functional MRI was performed before and after ingestion. Changes in Blood Oxygen Level Dependent (BOLD) signal, functional network connectivity and voxel based connectivity by Eigenvector Centrality Mapping (ECM) were measured.

Results

Glucose and fructose sweetened shakes led to significant decreased BOLD signal. The glucose sweetened shake led to a significant increase in eigen vector centrality throughout the brain and a significant decrease in eigen vector centrality in the midbrain. Sucralose and allulose sweetened shakes had no effect on BOLD signal or network connectivity but sucralose did lead to a significant increase in eigen vector centrality values.

Discussion

Taken together our findings show that even in a shake also containing fat and protein, the type of sweetener can affect brain responses and might thus affect reward and satiety responses and feeding behavior. Glucose had widespread effects on the brain, which might be expected as glucose is the principal source of energy and readily recognized and processed by the brain. Fructose does also affect the brain function, but the effect is limited to the neuronal activity, indicating that the different metabolism of fructose is reflected by the brain response. Both sucralose and allulose lead to very little functional brain responses, even though allulose is a mono-saccharide. The sweet taste without the corresponding energy content of these non-nutritive appears to have much smaller effects on the brain then nutritive sweeteners.

INTRODUCTION

The regulatory role of the brain in directing energy homeostasis and eating behavior is increasingly being recognized [1]. Energy consumption is not only regulated by homeostatic processes, it also has hedonic aspects in which the brain has an important role [2, 3]. Fats, proteins and carbohydrates, all have specific effects on the brain, and ingestion can elicit various functional brain responses [4, 5]. The increased ingestion of sugars and sweeteners over the last decades increases the particular interest of investigating the effects of these substances [6-9]. Understanding of the functional brain responses to sugars or sweeteners could give insight into effects on satiety signaling, feeding behavior involved in maintaining energy homeostasis.

Glucose is a commonly consumed natural sugar. It is the primary source of energy for the brain and its metabolism is kept under tight regulation. The brain responds readily to ingestion of glucose because of its quick absorption [10-12], and it elicits a homeostatic response from the hypothalamus almost immediately after ingestion [13-17]. Glucose has effects on both neuronal activity and functional connectivity throughout the brain in areas involved in reward and feeding behavior [16]. However, many foods and beverages contain other mono- and di-saccharides, such as fructose, sucrose. More and more, low or non-nutritive sweeteners are used to sweeten foods and beverages [6, 18]. The metabolic pathways of these sweeteners are all different. For instance; in contrast to glucose, fructose cannot be used directly as a source of energy but first has to be metabolized by the liver before it can be utilized by the brain [19-21]. The metabolic effects of non-caloric sweeteners are not straightforward. Non-caloric sweeteners are expected to decrease caloric intake and might therefore be useable to control obesity [22]. However, epidemiological studies have found that non-nutritive sweeteners might actually increase energy intake [9, 23]. Earlier findings indicate that the homeostatic and hedonic responses, as measured by BOLD signal changes, by the hypothalamus and ventral tegmental area (VTA) are driven by sweet taste coupled with caloric content. Sweet taste without caloric content, as found in artificial non-nutritive sweeteners, does not seem to elicit a lasting response from these brain areas in this study [17]. Recently, allulose, a low-nutritive natural sugar replacement, has become available. Allulose, or D-psicose, is a very low-energy monosaccharide sugar. Unlike sucralose and other artificial sweeteners, allulose is very similar in molecular structure, taste and texture to fructose and glucose. Allulose has a similar relative sweetness compared to glucose but only 0.3% of its energy content. Additionally, allulose is transported form the gut lumen to the blood via glucose transporters, which has been shown to elicit reward signals [24]. Because

these types of sweeteners are more similar in molecular and metabolism to natural sugars than other artificial sweeteners they could elicit brain response more similar to natural sugars.

Although, it is obvious that regulation and sensing of food in the brain is complex, with many parts of the brain involved, up to now functional MRI studies have mainly focused on single areas, mostly hypothalamus, to study the effect of sugars and sweeteners. However, because of the complexity and involvement of various brain areas in regulation of energy balance [25], fMRI investigations of whole brain responses might be more informative for this regulation. Measurements of local BOLD changes, a measure of neuronal activity [26], can be used to analyses the immediate effects of nutrient ingestion on very specific brain areas[13-15, 27, 28], but can also be used to determine whole brain effects on a voxel based level [29]. Analysis of functional connectivity has been used to provide further insights on network level [16, 30]. In this respect, various functional networks are involved in feeding behavior and energy balance. Networks of special interest are the default mode, salience and executive control network. The default mode network reflects a baseline state of the brain and has been shown to be altered/involved in a disrupted energy balance in obesity [31, 32]. The salience network has been shown to be involved in feeding behavior and reward [33, 34]. The executive control network is involved in decision making and impulse control and is important in maintaining energy balance [34, 35]. A newer method to determine functional brain connectivity is eigenvector centrality mapping (ECM). ECM is used to determine the level and quality of connectivity on a voxel-wise level rather than on a network level [36, 37]. Eigenvector centrality values have been shown to be correlated with states of hunger and satiety [36].

In the present study we aim to investigate the effects of the ingestion of sweetened nutrient shakes containing fats and protein. These shakes were sweetened with either the nutritive natural sugars glucose and fructose, the low- nutritive natural sugar allulose, or the non-nutritive artificial sweetener sucralose, on whole brain neuronal activity and (network) connectivity. Our overall hypothesis is that the different sweeteners, will lead to different responses in the brain.

METHODS

Subject characteristics

Twenty non-smoking, Caucasian men, aged 18 to 25 years were recruited through local advertising. Exclusion criteria were: a history of disturbances in glucose metabolism (e.g. diabetes mellitus), any significant chronic disease, psychiatric disease, BMI below

20 or above 23 kg/m^2, body weight below 70 kg, body height below 170 or above 190 cm, recent weight changes (>3kg gain or loss) within the last 3 months, having smoked within the last 6 months, recent blood donation, alcohol consumption of more than 21 units per week, recent use of recreational drugs, and contra-indications to MRI scanning. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center (NL 55440.058.15) and prospectively registered at clinicaltrails.gov (NCT02745730). All volunteers gave written informed consent before participation.

Study design

The entire study was performed in a double blinded, 4-times crossover design. Resting state functional MRI (rsfMRI) was performed before and after ingestion of the four different conditions in a randomized sequence, using a batch wise randomization procedure, based on a Williams design balanced for first-order carry-over. The randomization was performed by an independent statistician who was not involved in the analysis of the study data. There was an interval of at least one week between each of the four study visits. On all occasions, rsfMRI was performed before and after ingestion of one of the following study conditions; glucose (23 gram of carbohydrate, 93 kcal), fructose (23 gram), sucralose (0.05 gram); matched for sweetness with glucose, or allulose (23 gram); matched for sweetness with glucose. All sugars were dissolved in 165 ml of a sugar free 'milkshake' consisting of water, sodium casseinate 0.20 wt % (0.33 grams of protein, 1 kcal) of protein. coconut oil 3.0 wt % (5 grams of fat, 45 kcal) and guar 0.40 wt %. Cocoa powder was added as flavoring. The milkshake solution was made by Unilever Research & Development Vlaardingen B.V., The Netherlands, and was stored frozen. A full day before examination the frozen shakes were stored in a food-grade-refrigerator with a temperature of 4ºC to defrost. All shakes were consumed at 4ºC. Subjects were asked to fast overnight, i.e. after 10PM no food or drinks were allowed; only water. Drinking of the shakes was performed in the MRI room, sitting in an upright position on the MRI scanner table, outside the magnet bore. Subjects were instructed to taste the shake first with a few nips, then to drink the total amount in a steady and continuous way within 2 minutes. RsfMRI acquisition was continued 15 minutes after ingestion.

Visual analogue score analysis

Subjective feelings of hunger, fullness and wanting a meal at baseline and wanting to continue ingestion after first tasting and full ingestion of the shakes were indicated on a Visual Analogue Scale (VAS) which consisted of a 10 cm line, with 'not at all' and 'extremely' as anchors. Subjects were asked to indicate their score on the line, higher

scores indicating a stronger feeling of hunger/fullness etc. Differences in VAS scores between conditions were analyzed using a repeated measure ANOVA per VAS score using the Statistical Package of Social Sciences (SPSS, version 23.0; SPSS, Chicago, Ill).

MRI data acquisition

MRI scanning was performed on a Philips Achieva 3.0 T scanner using a 32-channel SENSE head coil (Philips Healthcare, Best, The Netherlands). Anatomical high-resolution 3DT1- weighted images of the whole brain were acquired (TR 9.8 ms, TE 4.6 ms, flip angle 8, 140 transverse slices, FOV 224 mm x 177 mm x 168 mm, reconstructed in-plane resolution 0.88 mm x 0.87mm, slice thickness 1.2 mm) along with a high-resolution T2*-weighted EPI scan (EPI factor 35, TR 2200 ms, TE 30 ms, flip angle 80, 84 axial slices, FOV 220 mm x 220 mm, in-plane resolution 1.96 mm x 1.96 mm, slice thickness 2.0 mm) for registration purposes. Resting-state scans were acquired with T2*-weighted gradient echo-planar imaging (EPI factor 35, 160 dynamics, 37 transverse slices scanned in ascending order, TR 2200 ms, TE 30 ms, flip angle 80, FOV 220 mm x 220mm, voxel size 2.75 x 2.75 x 2.50 mm with a 0.25 mm slice gap, total acquisition time: 6 minutes).

MRI data preprocessing

MRI data were preprocessed and analyzed using Functional Magnetic Resonance Imaging of the Brain Software Library (FSL) [38], Matlab and Phyton. Of all data sets, structural and functional, non-brain structures were removed using Brain Extraction Tool (BET) tool as implemented in FSL. The T1-weighted images were registered to the 2 mm isotropic MNI-152 standard space image using non-linear registration with a warp resolution of 10 mm. The FMRI Expert Analysis Tool (FEAT) was used for motion correction with MCFLIRT, spatial smoothing with a full width at half maximum of 3 mm, and high pass temporal filtering with a cut-off frequency of 0.01 Hz. The functional resting state images were registered non-linearly to the corresponding T1-weighted images using Boundary-Based Registration (BBR) affine registration, using the high-resolution echo planar images as an additional registration step.

MRI data analysis whole brain BOLD changes

Whole brain voxel based BOLD intensities were compared before and after ingestion according to Rombouts et al [29]. In short, a single volume BOLD signal map was calculated by averaging the time series data. To decreases the possibility of including unwanted signal of other compartments than CSF an average CSF signal of the single volume BOLD signal map was determined by averaging all voxels within the masked CSF. This CSF mask was determined by selecting voxels located in the lateral ventricles

on the segmented structural images. Next, in each subject, a normalized BOLD signal map was calculated by dividing each voxel's signal by the average CSF signal. Voxel-wise comparisons of pre- and post-ingestion normalized BOLD signal maps were done using the Randomize tool of FSL with a paired samples approach and using Threshold-Free Cluster Enhancement (TFCE) [39]. All data were family wise error (FWE) corrected at a level of $p<0.05$.

MRI data analysis network functional connectivity changes

Functional network analysis was performed on the same ICA-AROMA preprocessed data using the Beckmann resting state functional networks templates for the default mode and executive control network [30]. The Beckmann auditory network was used as a template for the salience network as this standard template encompasses largely the same brain areas [34]. Functional connectivity of each network of interest was calculated using the dual regression approach of FSL [38]. This results in 3D images for each individual, with voxel-wise Z-scores representing the functional connectivity to each network. The average Z-scores per network were calculated for the pre- and post- ingestion time point. Differences in Z-scores between pre- and post-ingestion were analyzed using paired samples t-tests per functional connectivity network, and uncorrected p-value of $p<0.05$ was deemed significant.

MRI data analysis Eigenvector centrality changes

ECM is an assumption- and parameter-free method to determine the level and quality of connectivity on a voxel-wise level which has been shown to be modulated by the physiological state of the subject and could thus be used to investigate states of hunger satiety and changes in response to nutrient ingestion [36, 37]. For the ECM connectivity analysis the data-driven ICA-based Automatic Removal of Motion Artifacts (ICA-AROMA) was used to identify components in the data related to head motion and to remove these using linear regression.[40, 41] Voxel-based connectivity eigenvector centrality maps were calculated on the ICA-AROMA preprocessed data for each participant using fast-ECM software, which estimates voxel-wise eigenvector centralities from fMRI time series [37]. Pre- and post-ingestion eigenvector centrality maps were compared in a voxel-wise approach in the masked grey matter using the Randomize tool with a paired samples approach and using TFCE [39]. Pre- and post-ingestion scans were compared per condition with paired two-sided contrasts. The same family wise error (FWE) correction at $p<0.05$ as for the whole brain BOLD analysis was used.

RESULTS

Subject characteristics and VAS scores

Subject characteristics for the study group (n=20) are shown in table 1. No differences were found in subjective scores of satiation at baseline between conditions (table 2). After ingestion the VAS score for 'wanting to drink more' was similar for all conditions both after first tasting and 20 minutes after full ingestion. All conditions lead to a significant decrease in VAS score for 'wanting to continue consumption' from the first tasting to 20 minutes after full ingestion.

Table 1. Subject characteristics

	N = 20
Age (years)	22.2 ± 1.3
Height (m)	1.84 ± 0.04
Weight (kg)	75.6 ± 4.9
BMI (kg/m^2)	22.4 ± 1.1

Values in mean ± standard deviation.

Table 2. VAS scores for satiation and wanting

	glucose	**fructose**	**sucralose**	**allulose**
Hunger pre ingestion	5.2 ± 2.2	5.7 ± 2.3	5.7 ± 2.2	5.2 ± 2.2
Fullness pre ingestion	3.0 ± 1.2	2.2 ± 1.2	3.1 ± 1.5	3.0 ± 1.5
Wanting meal pre ingestion	5.5 ± 2.2	5.9 ± 2.7	6.5 ± 2.0	5.7 ± 2.1
Wanting to continue consumption after tasting	4.8 ± 1.7	5.4 ± 2.2	4.5 ± 1.8	4.1 ± 2.1
Wanting further consumption after complete ingestion	3.6 ± 1.9	4.2 ± 1.9	3.4 ± 2.2	2.9 ± 2.0

Values in mean ± standard deviation.

Whole brain BOLD signal changes

Whole brain analysis showed significant decreases in BOLD intensity after ingestion of the glucose and the fructose drink (figure 1, blue color). The sucralose and allulose conditions did not lead to any significant changes in BOLD intensity. Brain areas with decreased activity after glucose ingestion include: the posterior cingulate cortex, brainstem, VTA, insula, lingual and fusiform gyrus. Brain areas that showed decreased activity after fructose ingestion include the anterior cingulate cortex, nucleus accumbens, putamen, and insula. The main difference between glucose and fructose is that fructose affects more the frontal parts of the brain, specifically the anterior division of the cingulate cortex instead of the posterior division.

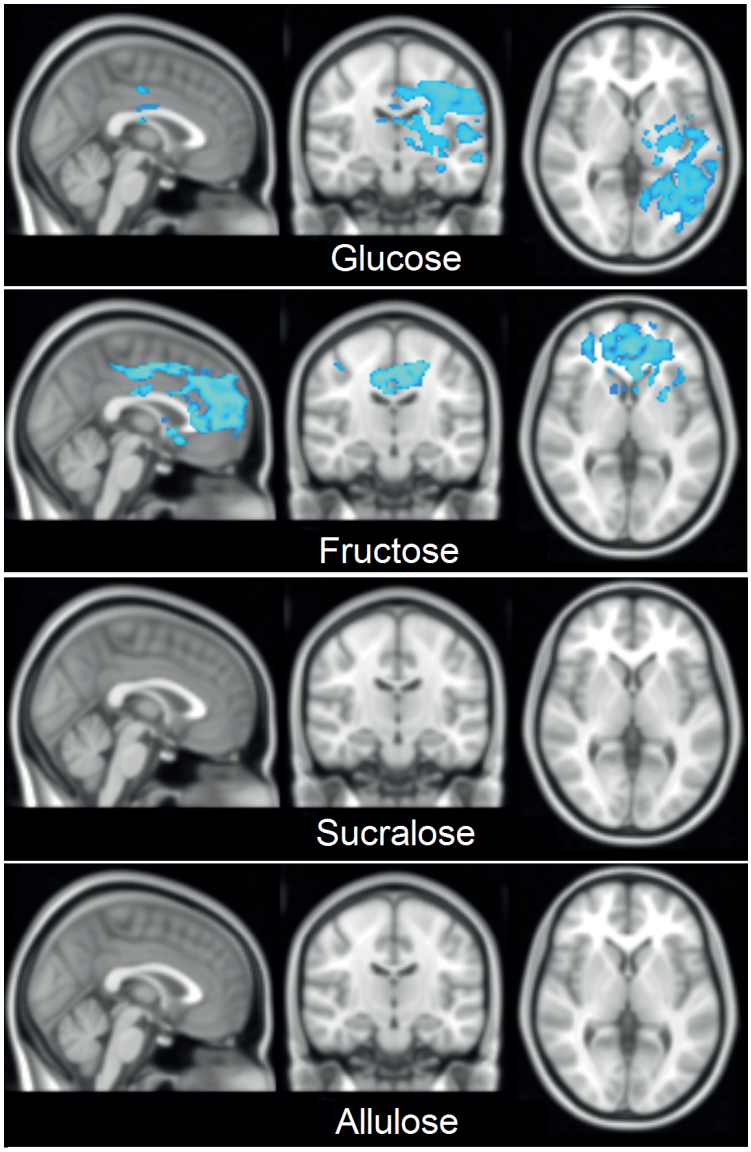

Figure 1. BOLD intensity decreases after ingestion of nutrients shakes with the different sweeteners (FWE corrected). Areas with decreases (P<0.05, TFCE, FWE-corrected) in BOLD signal are shown in blue scale.

Network connectivity changes

Analysis of network connectivity (figure 2) showed that in the salience network, which is associated with feeding behavior and reward, all shakes lead to increased connectivity

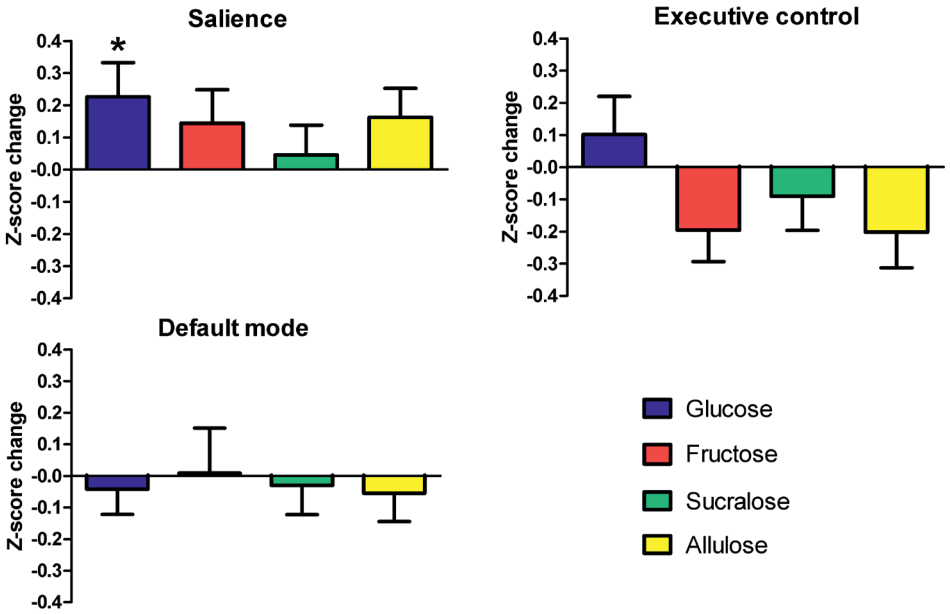

Figure 2. Changes in network functional connectivity before and after ingestion of the nutrient shakes. Network connectivity changes (mean Z-score per network) per condition are shown for the salience, default mode and executive control functional connectivity network. * indicates a significant difference from baseline $p<0.05$.

of which glucose ingestion leads to a significant increase in Z-score ($p=0.048$). The connectivity of the executive control network, involved in decision making, was increased by the glucose sweetened shake while the other shakes led to a decrease in connectivity in this network, although none of these effects were significant. The default mode network seemed not to be affected by any of the shakes

Eigenvector centrality changes

Figure 3 shows the significant brain changes in eigenvector centrality, indicative of voxel based connectivity, after ingestion of the study stimuli. After ingestion of the glucose sweetened shake, an increase in eigenvector centrality values (red-yellow color scale) was found in various brain areas including the pre- and post-central gyri, posterior cingulate cortex, central opercular cortex and insula, lingual gyrus and other gyri in the occipital lobe. A significant decrease (blue color) in eigenvector centrality values was found in a small area in the midbrain in proximity to the hypothalamus and VTA. After ingestion of the sucralose stimulus, a significant increase in eigenvector centrality values was found in the medial part of the cingulate cortex and in the pre-

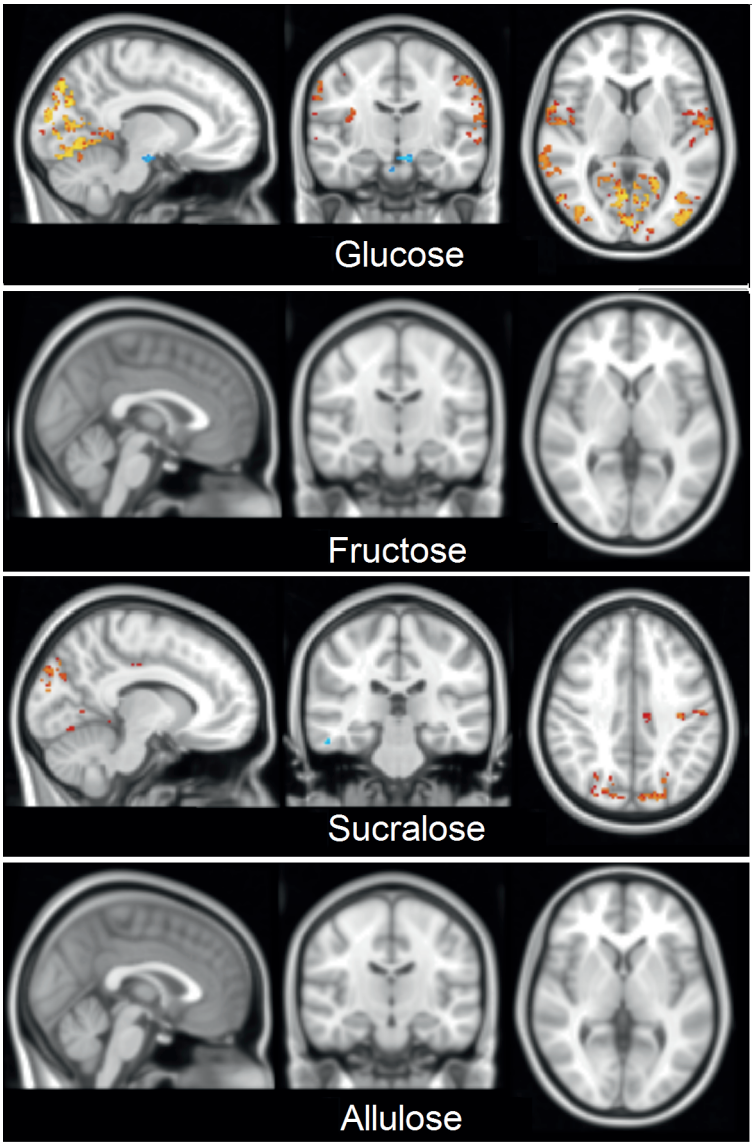

Figure 3. Eigenvector centrality mapping after ingestion of nutrients shakes with the different sweeteners. Increases in Eigenvector centrality values are shown in red-yellow scale, decreases are shown in blue scale (P<0.05 TFCE, FWE-corrected).

and post-central gyrus. A small decrease in eigenvector centrality values was found in the temporal lobe. The allulose and fructose stimuli did not lead to any significant changes in eigen vector centrality values.

DISCUSSION

Our results show that consumption of nutrient shakes sweetened with glucose, fructose, sucralose and allulose led to varied effects on functional brain responses. Of all shakes, only glucose had widespread effects on the brain. After consumption of the glucose shake, decreased brain activity (decreased BOLD signal), and increased neuronal connectivity both on a voxel based and on a network level was observed. Consumption of the fructose shake led to a decrease in BOLD signal, whereas no changes in neuronal connectivity were observed. Consumption of an allulose or sucralose shake did not lead to any changes in BOLD signal nor in network connectivity. In contrast to allulose, consumption of the sucralose shake led to a small decrease in eigenvector centrality.

Consumption of the glucose sweetened shake results in a decreased BOLD signal in basal brain regions like the posterior cingulate cortex, brainstem, VTA and insula, which is concordant with earlier findings [16]. Our data confirm previous findings that brain activity is diminished after receiving glucose, whereas in a fasted state these brain regions were active in seeking reward or seeking energy [13, 14, 42]. Additionally, glucose led to a small decrease in voxel based connectivity in the midbrain comprising the hypothalamus and VTA. The hypothalamus and VTA are both strongly involved in homeostatic and hedonic regulation of energy balance [1, 3]. Although our data are not fully conclusive, it might be that a decrease in eigen vector centrality in specifically these regions are associated with a decreased state of hunger and increased feeling of satiation [36, 43]. More obviously, consumption of the glucose sweetened shake led to a widespread increase of connectivity, both on voxel and network level. Glucose was the only condition which led to a significant increase in connectivity in the salience network. The salience network is involved in feeding behavior and determining reward [33, 34], but also in emotional arousal and decision making [44, 45]. Interestingly, although the effects were not significant, the glucose sweetened shake was the only shake that led to an increased network connectivity in the executive control network, which is involved in decision making [34, 35], where the other stimuli led to a decreased connectivity of this network. The increased voxel based and possibly network connectivity in executive control areas suggest that executive control processes are different in response to glucose than to other sugars and sweeteners. The exact explanation of these differences should still be elucidated.

The fructose sweetened shake led to a decreased BOLD signal in the anterior cingulate cortex which is important in executive control and feeding behavior, in addition, several areas involved in the reward response show decreased activity. The brain areas responding to fructose were not the same as those responding to the glucose shake.

This indicates that not only the hypothalamus and the VTA respond differently to glucose and fructose, as reported previously [17], but that outside these areas, the response of the brain is also different. This indicates that fructose might have lesser satiation effects which might lead to different effects on feeding behavior compared with glucose [21, 46]. We expected that fructose would lead to similar effects on the voxel based connectivity as glucose since both are natural nutritive sweeteners and both showed a decrease in the neuronal activity. However, we found no significant effects of the fructose sweetened shake on the eigen vector centrality. This might be explained by the fact that fructose effects on the connectivity are delayed because fructose is metabolized in the liver and does not deliver energy directly to the brain [47]. Therefore these effects might not be detectable at the time point (15 minutes after ingestion) measured in the current study. None of the sugars or sweeteners seemed to have any effect on DMN network connectivity, indicating that the baseline connectivity of the brain is not immediately effected by ingestion of these sweeteners [31, 32].

The sucralose and allulose sweetened shakes both showed no significant effect on the BOLD signal. This indicates that these shakes, with very little to no energy content in the form of carbohydrates, had no immediate effect on the activity of brain areas involved in feeding behavior, even though the fat and protein in the shake delivered a significant amount of energy. This indicates that sweet taste without presence of carbohydrates does not lead to the activity changes as measured with fMRI that are often associated with satiety, which is in line with earlier findings that sweet taste without energy content does not lead to a lasting decrease in hypothalamic activity [17]. In contrast to the changes in activity, voxel based connectivity changes were not limited to the carbohydrate containing shakes as the sucralose sweetened shake also demonstrated a significant effect. Increase in voxel based connectivity after sucralose could be seen as processing of the opposing effects in the brain caused by the registration of sweet taste but without corresponding energy content [48-51]. This could also explain the absence of a similar effect in response to the allulose sweetened shake, as allulose is absorbed and does have some energy content. Additionally, the changes in ECM to the sucralose sweetened shake could also indicate a response caused by the fats and protein in the shake as these are the only source of energy in this stimulus and all the other shakes have at least some carbohydrate content. In addition to glucose sensing neurons, the brain has nutrient sensing neurons for fatty acids and amino acids [4, 5, 52]. Sensing of the different macronutrients separately leads to different brain and downstream peripheral responses [11]. Responses to ingestion of combinations of macronutrients differ from the response to individual substances as the body and the brain responds differently to sugars when ingested in context of other nutrients [5]. Sugars are metabolized differently in the context of other

nutrients, fructose for example has been shown to be metabolized differently when ingested combined with glucose in a mixed meal than when ingested in isolation [47], which could also alter the response of the brain to its ingestion. The effects of the combination of macronutrients could also explain why we found different changes in connectivity in response to glucose in the context of a mixed meal then when dissolved in water in our earlier study [16].

A limitation of our study is that it was performed only in a very homogenous group of male volunteers. Since it is known that there are several sex-specific differences in energy metabolism [53], it can be expected that sex differences in the brain responses measured in this study are present. Strengths of our study were the crossover design and the hypothesis free approach taking into account the whole brain changes versus a region of interest approach that makes assumptions beforehand.

Taken together our findings show that even in a mixed meal, different types of sweeteners can elicit different brain responses. Therefore the type of sweetener used might differentially affect feeding behavior. Of the sweeteners used, glucose had the most widespread effect on the brain. Fructose does affect the brain function, but the effect is limited to neuronal activity. Additionally, the neuronal activity of different areas was affected, possibly related to the different metabolism of fructose affecting the brain response. Both sucralose and allulose lead to very little functional brain responses even though allulose is a mono-saccharide, indicating that sweet taste without the corresponding energy content in these non-nutritive appears to have much smaller effects on the brain then nutritive sweeteners and especially glucose.

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Conflict of interest

M. Hoeksma and T.P.J. Mulder are both employees of Unilever Research and Development Vlaardingen B.V. The Netherlands. All other authors have no conflict of interest to disclose.

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CHAPTER 5

The effect of consumption temperature on the homeostatic and hedonic responses to glucose ingestion in the hypothalamus and the reward system

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ABSTRACT

Background

Excessive consumption of sugar sweetened beverages (SSBs) has been associated with obesity and related diseases. SSBs are often consumed cold, both the energy content and temperature might influence the consumption behavior of SSBs.

Objective

The main aim of this study is to elucidate whether consumption temperature and energy (i.e., glucose) content modulate homeostatic (hypothalamus) and reward (VTA) responses.

Design: Sixteen healthy men participated in our study (aged 18-25, BMI 20-23). High resolution fMRI data was collected after ingestion of four different study stimuli; plain tap water at room temperature (22°C), plain tap water at 0°C, a glucose containing beverage (75g of glucose dissolved in 300 ml water) at 22°C, and a similar glucose drink at 0°C. BOLD changes from baseline (7 mins pre-ingestion) were analyzed over time in the hypothalamus and VTA for individual stimulus effects and for effects between stimuli.

Results

In the hypothalamus, water at 22°C led to a significantly increased BOLD response, all other stimuli resulted in a direct, significant decrease in BOLD response compared to baseline. In the VTA, a significantly decreased BOLD response compared to baseline was found after ingestion of stimuli containing glucose at 0°C and 22°C. These responses were not significantly modulated by consumption temperature. Drinking plain water did not have a significant VTA BOLD effect.

Conclusions

Our data show that glucose at 22°C, glucose at 0°C, and water at 0°C lowered hypothalamic activity, which is associated with increased satiation. On the contrary, drinking of water at room temperature increased activity. All stimuli led to a similar VTA response suggesting that all drinks elicited a similar hedonic response. Our results indicate that in addition to glucose, the low temperature at which sweetened beverages are often consumed also leads to a response from the hypothalamus and might strengthen the response of the VTA.

Keywords: Hypothalamus, glucose, energy sensing, ventral tegmental area, temperature, reward system.

INTRODUCTION

The global prevalence of obesity has risen over the past three decades [1]. An important contributor to this increase, among other factors, is an escalation of consumption of added sugars, especially in the form of sugar sweetened beverages (SSBs)[2-4]. Several factors are involved in the palatability and rewarding aspect of consumption of SSBs, including the energy content because of the sugars and refreshment and thirst quenching because of the low consumption temperature. Consumption of sugars has been shown to have an effect on reward areas in the brain in rodents [5-7], which could lead to continued overconsumption of sugar, although these effects are still unclear in humans [8]. In addition to the high sugar content, the relatively low temperature at which SSBs are generally consumed could intensify the reward responses as cold drinks are more thirst quenching than drinks consumed at room temperature.[9]

In the brain, the hypothalamus regulates both energy expenditure and intake and thirst [10,11]. Glucose containing beverages have been shown to alter the functional hypothalamic BOLD (Blood Oxygen Level Dependent) response proportionally with the energy (glucose) content of the beverage consumed [12]. It is currently not known how the temperature of a consumed beverages or sugar sweetened beverage relates to regulation of energy and fluid homeostasis. In addition to these homeostatic interactions, hedonic aspects of SSBs are important determinants of consumer behavior. Brain areas involved in this hedonic response are the ventral tegmental area (VTA) and areas of the limbic system (amygdala, nucleus accumbens). Although BOLD responses in these regions are consistently linked to reward [13], little is known about the effects of the BOLD response in these reward centers caused by energy content and drinking beverages at different temperatures. In addition to the unknown interaction of energy and temperature in the hypothalamus, the relationship between the temperature at which beverages are consumed and hedonic brain responses is unknown. As mentioned previously, such interactions can be expected since SSBs are less appreciated at room temperature, than cooled [14].

Taken together, there are reasons to believe that consumption temperature has an effect on brain responses regarding energy regulation and hedonic reward, and hence may impact energy intake in humans. Furthermore, it seems likely that energy homeostasis and hedonic aspects are interdependent. In the present study we aim to elucidate the effects of two aspects of SSB ingestion, namely the energy content and ingestion temperature, on homeostatic and reward response. To this end, we studied the effects of plain water and glucose containing water at both room temperature and at 0^{o}C, on brain reward (VTA) and homeostatic (hypothalamus) centers, in a double blind, four-way crossover study.

METHODS

Subjects

Sixteen healthy, normal-weight, Caucasian young adult men participated in our study. Subjects were recruited via local advertisements and through the use of mailing lists. A participant flow chart for the recruitment of subjects is include as Supplemental Figure 1. All subjects were between the ages of 18 and 25 and had a body mass index (BMI) ranging from 20 to 23. Exclusion criteria were the following: a history of diabetes or disturbed glucose metabolism; any genetic, psychiatric, renal, hepatic or other chronic or current disease; recent fluctuations in weight > 3 kg; current smoking; current alcohol consumption >21 units/week and use of recreational drugs; recent blood donation or participation in other biomedical research (within the last 3 months); use of medication affecting glucose or lipid metabolism; contra-indications for MRI-scanning. Written informed consent was obtained from all subjects and the protocol was approved by the Medical Ethics Committee of the Leiden University Medical Centre. The study protocol is registered at ClinicalTrails.gov under registration number NCT03181217.

Experimental procedure

We used a randomized, controlled, crossover study design. Randomization was based on a Williams Design for four study stimuli in four periods, balanced for periods and carry over, randomization was performed by an independent person at Unilever R&D by generating a randomized list of stimulus order per subject. The four different study stimuli used were; water at room temperature (22°C) and 0°C, and glucose containing beverages at room temperature (22°C) and at 0°C. All beverages consisted 300 ml of water with or without 75 g of glucose added. Before all study visits participants were fasted from 10.00 pm the previous day but were allowed to drink water. During each study visit after 7 minutes of baseline fMRI scanning the study stimuli were administered during the fMRI scan through a peroral tube, lying supine in the scanner, in the 32-channel head coil. Subjects were instructed to drink the total amount in a steady and continuous way. Scanning was continued during and after start of ingestion. The hypothalamus was used as a seed region to determine the homeostatic response to ingestion of the study stimuli and as a post-hoc analysis the VTA was used as a seed region to determine the hedonic response.

Blood sampling

Blood samples were used to ascertain a normal glucose metabolism in each subject. On every test day, 2 blood samples (5 ml each) were drawn by venipuncture in an

antecubital vein; one sample was taken before scanning and the other one after the scanning procedure, 30 minutes after ingestion of the study stimuli. Sample handling and analysis was performed by the laboratory for Clinical Chemistry at Leiden University Medical Center. Plasma glucose was measured using a fully automated Hitachi 704/911 system. Plasma insulin was measured by a Radio Immuno Assay (Medgenix, Fleurus, Belgium). A total amount of 10 ml blood was taken on each study day.

MRI data acquisition and analysis

MRI was performed on a 3.0 Tesla Achieva clinical scanner (Philips Healthcare, Best, the Netherlands). A mid sagittal (x=0) T1 structural hypothalamus scan (single slice scan, repetition time 550 ms, echo time 10 ms, field of view 208x208 mm, voxelsize = 0.52x0.52x14 mm, scan time 1.14 min) and a mid-brain single slice (x=0) T_2* weighted, gradient echo-planar imaging (EPI) fMRI scan that renders BOLD contrast (repetition time 120 ms, echo time 30 ms, field of view 208x208 mm, voxelsize = 0.81 x 0.81 x 14 mm, scan time 21.2 min, 500 dynamics) were made. Imaging data were pre-processed and analyzed using Functional Magnetic Resonance Imaging of the Brain Software Library (FSL) version 5.0.8. Data was pre-processed as described in earlier studies [15]. Data were averaged for each set of 4 subsequent volumes, reducing the 500 dynamic scans to 125. The hypothalamus was segmented manually on the middle volume of the single slice MRI scan according to anatomical landmarks as previously described [15] (average number of voxels: 155), the VTA ROI was also segmented manually in similar manner using the mammillary bodies and the cerebral aqueduct as anatomical anchor points (average number of voxels: 51) (example ROIs shown in figure 1). To correct for scanner drift, all hypothalamic BOLD values were corrected for the BOLD signal obtained in an internal reference ROI. This internal reference ROI was drawn in grey matter, superior of the genu of the corpus callosum. To establish the post-ingestion hypothalamic BOLD response to the stimuli, the mean baseline signal (first 7 minutes of scan time) was measured for contrast. All data points (n=125) were divided by the mean baseline value and converted to percentages, yielding the percentage signal change relative to baseline.

Statistical analysis

Statistical analysis was performed using SPSS version 23.0.0.2. To analyze differences in blood values between the different study stimuli we used repeated measures ANOVA. All fMRI results are reported as percentage BOLD change relative to baseline (0-7 minute pre-drinking BOLD response). For presentation purposes, data were pooled into 'minute' data points: The first seven minutes were considered baseline BOLD response. Data between minute 8 and 11 were omitted from the analysis due to non-

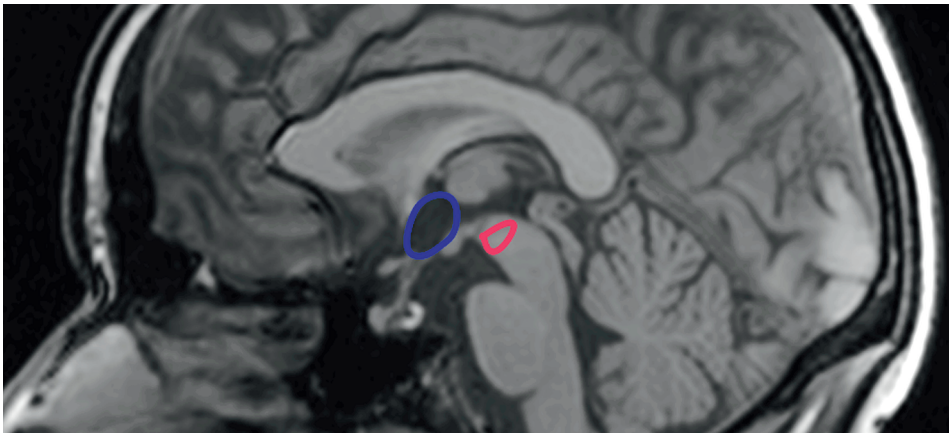

Figure 1. Region of interest (ROIs) used for the BOLD response analysis. Hypothalamus ROI indicated in blue and ventral tegmental area (VTA) ROI indicated in red.

physiologic excessive BOLD changes caused by swallowing of the test drink (BOLD change of more than 3.5% at 3T)[16]. Data from minute 11 and up (11-21) were considered the post drinking BOLD response. To determine whether study stimuli led to a significant BOLD change, repeated measures ANOVA was used per study stimulus, in which the data for each stimulus was pooled per minute (by averaging bins of 6 consecutive fMRI volumes for each minute). Statistical analysis for the comparison of the stimuli effect between the study stimuli was performed by a mixed model analysis using the study stimulus as a fixed effect, time point (125 time points per subject per treatment) as a covariate and subject per occasion as a random factor. Additionally, to analyze potential differences in timing of the response of the individual stimuli, the post drinking BOLD response was analyzed per minute using the same mixed model but with the per minute time points as a factor instead of a covariate. Uncorrected p-values, due to small sample size, of <0.05 are deemed significant for all tests.

RESULTS

Subject characteristics

Baseline subject characteristics are shown in Table 1. Blood levels for glucose and insulin showed a normal glucose metabolism for all participants with a normal response to the glucose stimuli. Furthermore, all participants had normal fasting insulin and glucose blood levels, confirming that all participants were fasted on the study days.

Table 1. Subject characteristics

	n=16
Age (years)	20.2 ± 1.7
Height (m)	1.81 ± 0.05
Weight (kg)	71.8 ± 4.9
BMI (kg/m^2)	22.0 ±1.15
Glucose Fasted[1]	5.0 ± 0.3
Post glucose ingestion[1]	7.0 ± 1.0
Insulin Fasted[1]	4.8 ± 2.4
Post glucose ingestion[1]	25.2 ± 17.0

Values in mean ± standard deviation. Glucose levels in mmol/L, Insulin levels in mU/L.
[1] average blood levels for the two visits with glucose stimuli per subject (n=16), post ingestion levels measured 30 minutes after ingestion.

No significant differences (p>0.05) in absolute and relative blood values were found between the different glucose stimuli.

Changes in hypothalamic activity

Figure 2 shows the BOLD response of the hypothalamus over time per study stimulus. General inspection of the response curve shows that the hypothalamic BOLD response after ingestion of water at 22ºC remained positive during the entire experiment, whereas the BOLD response after ingestion of water at 0º, or glucose at 22ºC or 0ºC normalized after a few minutes. Table 2 shows the average hypothalamic post ingestion BOLD response relative to baseline for all study stimuli. Water at 22°C led to a significant average increase (1.2%, p=0.027) in BOLD response. Water at 0°C, glucose at 22°C and 0°C all resulted in a decrease in BOLD response relative to baseline. Figure 2 shows that directly after ingestion, a significant decrease in BOLD that returned to baseline was observed for all three of these stimuli, as indicated by the asterisks (maximum difference water 22°C: 1.5% p=0.009, and at 0°C: -2.0% p=0.014, glucose at 22°C: -1.9% p=0.011 and at 0°C: -2.2% p=0.005), although the average decrease over the entire post ingestion period was not significant. When comparing the BOLD responses (after intervention) to the water response at 22ºC, water at 0°C, glucose at 22°C and 0°C all showed an significant average BOLD decrease (mean difference of -1.6%, -1.6% and -1.8% respectively, all p<0.05) compared to water at 22ºC (table 3).

Changes in Ventral Tegmental Area activity

Figure 3 shows the BOLD responses of the VTA over time for all stimuli. Both glucose stimuli (glucose at 22°C and 0°C) showed a significantly decreased BOLD signal relative

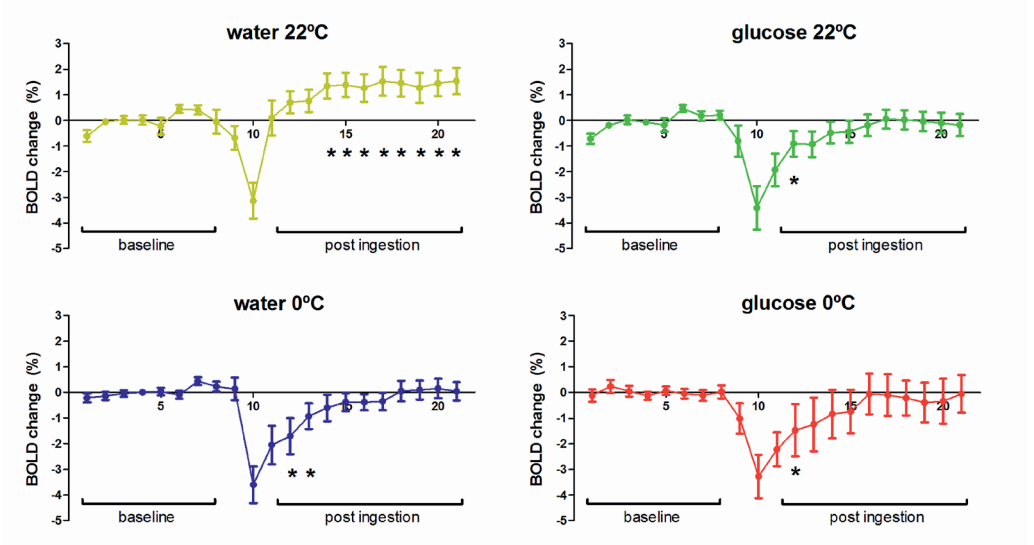

Figure 2. Hypothalamic BOLD response. BOLD responses to all four study stimuli as group average (n=16) response per minute with standard errors. Study stimuli were ingested from minute 8-10, overall response period post ingestion of study stimulus from minute 11-21. * Post ingestion BOLD response significantly different from baseline period at p<0.05 (repeated measures ANOVA).

Table 2. Average post-ingestion BOLD response relative to baseline (n=16)

	Hypothalamic response (%)		**VTA response (%)**	
	Mean ± SE	p-value[1]	*Mean ± SE*	p-value[1]
Water 22°C	+1.2 ± 0.5	0.027	-0.6 ± 0.4	0.150
Water 0°C	-0.5 ± 0.3	0.133	-0.7 ± 0.5	0.203
Glucose 22°C	-0.4 ± 0.4	0.300	-0.6 ± 0.6	0.348
Glucose 0°C	-0.7 ± 0.8	0.387	-1.5 ± 1.0	0.154

[1] Determined by repeated measures ANOVA analysis

Table 3. Average post-ingestion BOLD response relative to Water 22°C control condition (n=16)

	Hypothalamic response (%)		VTA response (%)	
	Mean difference ± SE	p-value[1]	*Mean difference ± SE*	p-value[1]
Water 0°C	-1.6 ± 0.7	0.028	-0.2 ± 0.9	0.831
Glucose 22°C	-1.6 ± 0.8	0.040	-0.1 ± 1.0	0.898
Glucose 0°C	-1.8 ± 0.7	0.019	-0.8 ± 0.9	0.388

[1] Determined by linear mixed model analysis

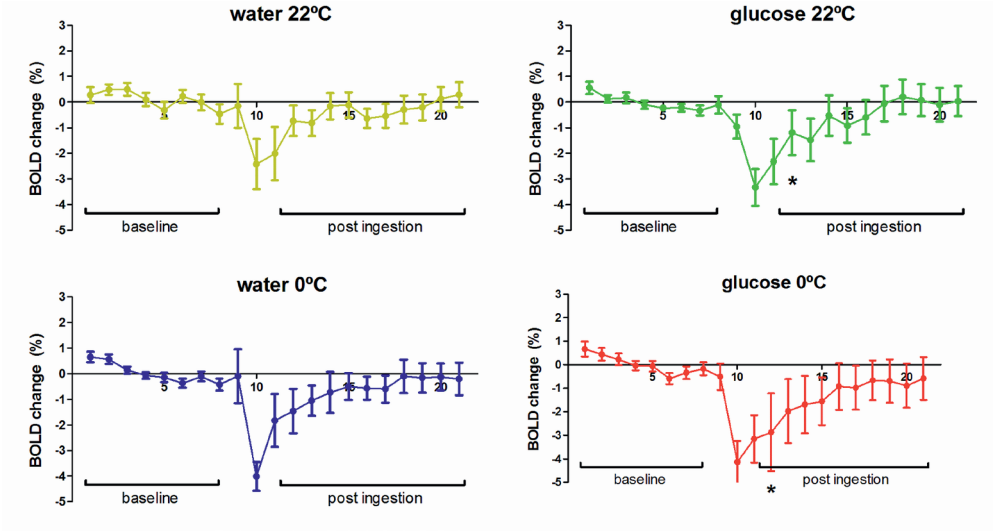

Figure 3. Ventral tegmental area BOLD response. BOLD responses to all four study stimuli as group average (n=16) response per minute with standard errors. Study stimuli were ingested from minute 8-10, overall response period post ingestion of study stimulus from minute 11-21. * Post ingestion BOLD response significantly different from baseline period at p<0.05 (repeated measures ANOVA).

to baseline directly after ingestion (maximum difference: glucose at 22°C -0.5% p=0.0 and at 0°C -0.5% p=0.0) but did not result in an average significant VTA BOLD change compared to baseline over the entire post-ingestion time (p>0.1, table 2). Both water stimuli did not have a significant effect on the VTA BOLD signal, over the entire post-ingestion time (p>0.1, table 2) or directly after ingestion (maximum difference: water at 22°C: -2.2% p=0.055 and at 0°C: -1.6% p=0.093). Although the VTA BOLD response after ingestion of glucose at 0°C appears most pronounced, no statistically significant differences were found between stimuli when all four responses were compared with mixed model analysis (p>0.3 for all stimuli, table 3). In all cases, the VTA BOLD response normalized after a few minutes.

DISCUSSION

Our data show that drinking water at 0°C, even in the absence of glucose, resulted in a significant decrease in hypothalamic BOLD response. Drinking of glucose at both ambient and cold temperature conditions also resulted in a significant decrease in the hypothalamic BOLD signal. This finding is in line with previous research indicating that the hypothalamus is a key brain structure for energy sensing [12]. On the contrary, when compared to the pre-drinking baseline, drinking water at 22°C resulted in a significant increase of hypothalamic BOLD response that lasted for at least 12 minutes. In the VTA, all stimuli led to an immediate decrease in BOLD signal. Although both glucose stimuli (glucose at 22°C and 0°C) showed a significantly decreased BOLD response in the VTA immediately after ingestion, this response was not significantly modulated by consumption temperature. All VTA BOLD responses normalized to baseline within the time frame of the study.

The hypothalamus plays a central role in the regulation of temperature, fluid homeostasis and thirst and control of energy intake and feeding behavior [11,17,18]. To regulate energy intake and feeding behavior the hypothalamus relies on various complex neural systems that together regulate satiety signaling and hunger cues [19]. In a fasted and thus "hungry" state, these neural systems are highly active to drive feeding behavior [20,21]. When activity in these systems is suppressed, the drive for feeding is decreased, indicating that a decrease in activity in the hypothalamus could be a marker of satiation. Similar response are found during thirst and thirst quenching [10,22]. Our data show that hypothalamic activity decreases in response to both the low temperature stimulus and glucose stimulus in a similar manner. Water at room temperature was seen to increase hypothalamic activity. Our results indicate that the low temperature at which SSB are most often consumed in and of itself can lead to a satiety, or more likely thirst quenching, response from the hypothalamus. Although, water at low temperature elicits a response from the hypothalamus, no temperature effects were found on consumption of glucose containing stimuli, therefore our data do not suggest that temperature has an additive effect.

The VTA is known to be a core structure involved in hedonic motivation for energy intake [23]. Earlier studies have shown that in a fasted state, food cues lead to VTA activation [24,25]. In addition, intravenous infusion of glucose modulates the reward response in the VTA [26]. Our data show that the VTA also responds directly to the ingestion of glucose. In addition to the response to energy intake, the activation of the VTA by oral temperature might be important since it is known that the hedonic dimension of thermal sensation in humans also motivates energy intake in order to maintain core temperature [27]. The response to ingesting glucose at 0°C seemed to

strengthen the average response in the VTA by 0.7% compared to drinking glucose at 22°C. Although the response was not significantly different between both stimuli at might suggest an interplay between temperature and energy content in the response of the reward system.

A limitation of our study is that it was performed in male volunteers only and it can be expected that sex differences are present, since it is known that there are several sex-specific differences in energy metabolism [28]. A second limitation of our study design is the lack of subjective ratings of hunger, thirst and satiety and regular consuming habits. Combining our more objective fMRI measurements with these subjective measures would give more insight into the satiety- and hedonic response. Thirdly, our participants were fasted, which may limit the extrapolation of results to a non-fasted state. Also, we used a high dose glucose stimulus, to be able to generalize our results to everyday consumption future studies have to be done using other sweeteners more commonly used in SSBs in a lower dose. Additionally, as we only found few significant results due a relatively large variation in VTA responses between participants an increase in sample size would be preferable to be able to better determine the VTA response and the difference in response between stimuli. Furthermore, it should be noted that various publications have reported large changes in BOLD in the reward system resulting from visual food cues and listening to music enjoyable music, indicating that ingestion of energy is not necessary for a neurophysiological reward responses [24,29]. A strength of our study was the use of the specific high resolution mid brain fMRI scan, which allowed us to accurately determine ROIs and perform BOLD measurements in the hypothalamus and VTA [12,13].

In conclusion, our results indicate that low temperature of consumption can elicit a homeostatic response. In the hypothalamus, we observed a decrease in BOLD signal immediately after intake of cold and glucose containing drinks, whereas the signal was increased after intake of room temperature water. Although suppression of the hypothalamic BOLD signal is often associated with satiation, and increased BOLD signal with hunger and drive for feeding, a lack of subjective hedonic and satiety measures prevents us from analyzing this further. Future research should focus on investigating the effects of cold SSB on the VTA and hypothalamus in an extended, more heterogeneous study population with males and females and including groups at risk for becoming obese from regular consumption of SSBs, such as children and adolescents [30].

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Author contributions JvdG, AvdBH, MH and CB designed research; AvO and JvdG conducted research; AvO and AvdBH analyzed data; AvO and JvdG wrote the paper; JvdG had primary responsibility for final content. All authors read and approved the final manuscript

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CHAPTER 6

Effect of flavor on homeostatic and reward responses of the hypothalamus and ventral tegmental area

Submitted

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ABSTRACT

Introduction

Although it is well known that food intake is affected by the palatability of food, the actual effect of taste and smell on regulation of energy- homeostasis and reward perception by the brain, remains unclear. To gain a better understanding of these effects we investigated the effect of ethyl-butyrate (EB), a common non-caloric food flavoring, added to plain water or a glucose solution, on the blood oxygen level dependent (BOLD) response in the hypothalamus (energy- homeostasis) and ventral tegmental area (VTA; reward).

Methods

Sixteen normal weight, healthy men (18-25 years, BMI 20-23 kg/m^2) drank various study stimuli during an fMRI setup in a randomized order. The stimuli reported in the current study were; plain water, water with EB flavoring, a glucose solution (50gram/300ml) and a glucose solution with EB flavoring. BOLD responses to ingestion of the stimuli were determined in the hypothalamus and VTA.

Results

In both the hypothalamus and VTA, glucose had a significant effect on the BOLD response (p=0.006, p=0.007) but EB flavoring did not (p=0.430, p=0.612).). No significant interaction effect was found between glucose and EB flavoring. Compared to the water control, glucose with and without EB led to a similar decrease in hypothalamic BOLD response (p=0.008, p=0.017), and glucose with EB resulted in a decrease in VTA BOLD response (p=0.028).

Discussion

Our results suggest that the changes in activity in the hypothalamus is mainly driven by energy ingestion and the addition of EB to a beverage in this study does not influence the homeostatic response. Changes in VTA activity are mostly elicited by energy combined with flavor. Flavoring without energy content showed no effects, indicating a lack of reward by flavor alone.

Trial registration: This study is registered at clinicaltrails.gov under number NCT03202342

INTRODUCTION

In understanding the complexity of eating behavior, the brain in is increasingly recognized as an essential organ. Energy balance and intake is regulated by the brain by two major regulatory systems: the homeostatic system and the reward systems [1-3]. The hypothalamus is known to control energy homeostasis through glucose and energy sensing and appetite regulation [1, 3]. The hypothalamus has been shown to respond directly to the ingestion of glucose and plays a pivotal role in central glucose sensing [4-8]. This central glucose sensing and metabolism have been established as an essential part of control of feeding and hunger [9]. In addition to the hypothalamus hunger and appetite are also controlled in conjunction with the orbitofrontal cortex, insula and the reward system [1, 10]. The reward, or mesolimbic, system is responsible for the hedonic response to food. An important brain area involved in this hedonic response is the ventral tegmental area (VTA). The VTA forms the basis of dopamine signaling in the mesolimbic system, which is a key substrate for reward prediction and response [11]. Furthermore, the VTA is anatomically and functionally connected with the hypothalamus and integrates homeostatic signals with reward responses and therefore plays a pivotal role in regulating palatable feeding [12-14]. Indeed, the VTA has been shown to have a (reward) response to both taste stimuli and the ingestion of energy [8, 15].

Pleasantness of food, affected by factors such as flavor and texture, has a direct influence on feeding behavior by making food palatable and attractive and thereby eliciting a reward response in the brain [10, 12, 16]. In this respect, pleasant flavoring might cause overeating and cause an energy misbalance [17]. Taste and flavor of food might also be involved in energy balance as taste and flavor are an important part of the palatability of food and plays a role in satiation and the rewarding effects and consumption volume of food [16, 18]. In this respect, pleasantness of food enhances the satiating effect of high-fat and high-carbohydrate meals [19]. Additionally, taste and aroma of food without added energy have been shown elicit responses from the brain and the periphery. Ingestion of a fat aroma with low fat content has been shown to elicit and modulate responses from the gustatory system [20]. Also, sweet taste that is rated as pleasant without caloric intake has been shown to elicit an insulin response when applied only to oral cavity [21]. Although the evidence for the effect of taste on the cephalic phase insulin response is limited, these results do suggest that the taste of food, independent of fuel content, might influence the central regulation of the peripheral energy metabolism in addition to activation of the reward pathway in the brain. Furthermore, when glucose is paired with a congruent flavoring the perception of flavor is enhanced, and results in a greater feeling of satiety [22-24]. Therefore added

flavor may lead to changes in glucose sensing and homeostatic responses in both the VTA and hypothalamus. Additionally, the added perceived sweetness and satiation by flavoring is interesting to investigate as this might be used as a strategy towards reduction of energy content [24].
Taken together, taste and flavor may affect both homeostatic and hedonic aspects of energy intake, and even may have an interactive effect. To gain a better understanding of the differences in homeostatic and hedonic brain responses to flavoring and energy content and the interaction of the two, we investigated the BOLD response after ingestion of glucose, Ethyl-Butyrate (EB) a common food flavoring with a fruity taste, and a combination of both glucose and EB in the hypothalamus and VTA in normal weight young men.

METHODS

Study participants

Sixteen healthy, normal-weight men participated in our study. Participants were recruited via local advertisements and through the use of mailing lists. All participants were between the ages of 18 and 25 and had a body mass index (BMI) ranging from 20 to 23. Exclusion criteria were the following: a history of diabetes or disturbed glucose metabolism; any genetic, psychiatric, renal, hepatic or chronic disease; recent fluctuations in weight > 3 kg; current smoking; current alcohol consumption >21 units/week and use of recreational drugs; recent blood donation or participation in other biomedical research (within the last 3 months); use of medication affecting glucose of lipid metabolism; contra-indications for MRI-scanning. Written informed consent was obtained from all participants and the protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center (LUMC) and registered at clinicaltrails.gov under number NCT03202342.

Experimental procedure

We used a randomized, controlled, crossover study design. A batch-wise randomization procedure, respecting 1st order, was followed. The randomization was performed by an independent person. Participants arrived at MRI facilities of the Leiden University Medical Centre (LUMC) after an overnight fast on a separate study occasion for each stimulus. After blood sampling and recording pre scanning visual analogue scores (VAS), participants were positioned in the MRI scanner where the study stimuli were ingested within the scanner bore through a per oral tube during fMRI scanning. Study procedures are illustrated in figure 1. The different stimuli used and investigated were;

plain water, water with ethyl-butyrate (EB), glucose solution and glucose solution with ethyl-butyrate (EB) (glucose solutions consisted of 50 grams of glucose dissolved in 300 ml water),stimuli were ingested at room temperature. In the present trial, an additional fifth stimulus was investigated, consisting of a glucose solution with EB ingested at 0ºC, to investigate the effects of temperature. As the aim of the current study was to determine the effects of flavoring only and the effects of temperature have been described in an earlier study by our group [8] the results of this condition are therefore only briefly shown and not discussed further in the current study. The stimuli were ingested five minutes after the start of the fMRI scan. Water was plain, non-chlorinated tap water. Ethyl-butyrate is a commonly used food flavoring with a fruity flavor and is an FDA and EU approved food flavoring under title 21 section 182.60 and EU Regulation 1334/2008 & 178/2002.

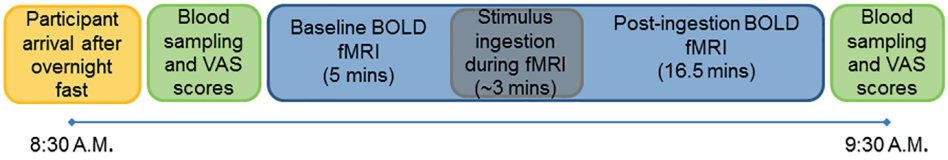

Figure 1. Study procedures. Illustrative depiction of the study procedures and timing during the study visits.

Blood sampling and VAS scores

Blood samples were used to ascertain a normal glucose metabolism in each participant. On every test day, 2 blood samples (5 ml each) were drawn by venipuncture in an antecubital vein; one sample was taken before scanning and the other one after the scanning procedure, 30 minutes after ingestion of the study solution. Sample handling and analysis was performed by the laboratory for Clinical Chemistry at LUMC. Plasma glucose was measured using a fully automated Hitachi 704/911 system. Plasma insulin was measured by a Radio Immuno Assay (Medgenix, Fleurus, Belgium). A total amount of 10 ml blood was taken on each study day. Participants were asked to rate their feelings of hunger in advance of the scanning procedure and afterwards, using a visual analogue scale (VAS) which consisted of a 10cm line, with 'not hungry' and 'extremely hungry' as anchors. Additionally, after the MRI scan pleasantness of the drink was rated using a similar VAS using 'very unpleasant' to 'very pleasant' as anchors.

MRI data acquisition

The MRI was performed using a 3 Tesla whole body MRI scanner (Philips Achieva, Philips Healthcare, Best, The Netherlands) equipped with a 32-channel head coil. The protocol for structural MRI comprised a scout view for planning, a high resolution 3DT_1-weighted sequence for registration purposes and a mid-sagittal high resolution single slice for accurate hypothalamus and VTA localization (repetition time 550ms, echo time 10 ms, field of view 208 x 208 mm, voxel size = 0.52 x 0.52 x 14 mm). Mid-sagittal fMRI was performed for 21 minutes in total, by a T_2* weighted, gradient echo-planar imaging (EPI) sequence mid-sagittal single slice that renders BOLD contrast (repetition time 120 ms; echo time 30ms; flip angle 30°; field of view 208 x 208 mm; voxel size = 0.81 x 0.90 x 14 mm; 21 k-line excitations for each dynamic volume, scanning time of one dynamic image 2.52 seconds for a total of 500 data points). A slice thickness of 14 mm was chosen to encompass the hypothalamus in the left to right direction and a single slice technique was used for sufficient signal to noise ratio.

High resolution mid brain functional MRI analysis

Data was pre-processed as described previously [7]. In short data was averaged for each set of 4 subsequent volumes, reducing the 500 dynamic scans to 125. Regions of interests (ROIs) were drawn manually, using subject-specific T_1 images to define anatomical borders. Three ROIs were drawn: the hypothalamus, the VTA and a reference area. The ROI for the hypothalamus was drawn as described earlier using the optic chiasm, mammillary bodies, thalamus and anterior commissure as anatomical landmarks [7]. Using literature describing the VTA region[25, 26] we defined the ROI for the VTA in the midbrain using the top of the cerebral aqueduct and the mammillary bodies as anatomical landmarks (example ROIs shown in figure 2). To correct for scanner drift, a third internal reference ROI was selected superior to the genu of the corpus callosum in the grey matter. BOLD signals derived from the hypothalamus and the VTA were corrected for BOLD signals obtained from this reference ROI by dividing the ROI BOLD signals by the reference BOLD signal. Percentage BOLD change from corrected baseline was calculated by dividing the post ingestion BOLD signal by the average baseline BOLD signal for the individual post ingestion dynamic scans/ measurement time points (n=125) and for the total post ingestion period (minute 8-21 of the fMRI scan).

Statistical analysis

Statistical analysis was performed using SPSS version 23. Differences in blood levels and VAS scores between pre- and post-ingestion and between study stimuli were tested with repeated measure ANOVA. All fMRI results are reported as BOLD change

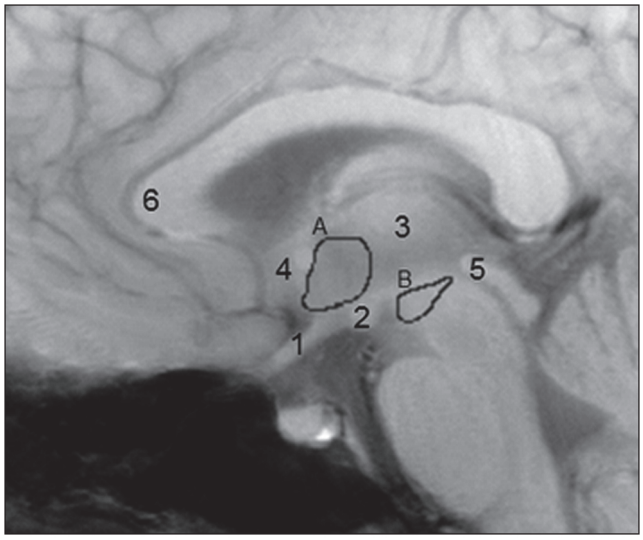

Figure 2. A representative sagittal view with the hypothalamus (A) and the VTA (B) ROIs. For (A), the optic chiasm (1), the mammillary bodies (2), the thalamus (3) and the anterior commissure (4) were used as landmarks. For (B), half the volume of (A), the top of the cerebral aqueduct (5) and the mammillary bodies (2) were used as landmarks. The reference ROI was drawn superior of the genu to the corpus callosum (6) in the grey matter.

relative to baseline (0-5 minutes pre-drinking BOLD response). To determine the main effect of energy content (presence of glucose yes or no) and flavor (presence of EB yes or no) on the study stimuli linear mixed model analysis was performed using energy content and the presence of flavoring and the study occasion as fixed effects and subject*study occasion as a random factor and the BOLD measurement time points as a covariate. To determine the interaction between energy content and flavoring the same mixed model was performed with an interaction term of both factors as a fixed effect. Statistical analysis of the difference in treatment effect between the study stimuli was performed by similar linear mixed model analysis using the study stimulus and study occasion as fixed effects, time point as a covariate and subject*occasion as a random factor using plain water as a negative and glucose solution as a positive reference stimulus. To determine the association between blood levels and the subjective ratings of hunger and flavor of the study stimulus and the BOLD response of the hypothalamus and VTA additional similar mixed models were used where these variables were added as covariates. All mixed model analyses were applied on the n=125 point dataset.

RESULTS

Subject characteristics

Sixteen participants were enrolled in the study. All participants successfully completed the all study visits. Table 1 shows the subject characteristics of the study participants. All showed normal fasting glucose (3.9-5.5 mmol/L) and insulin levels (< 20 mIU/L) and these levels were not different between visits.

Hunger and flavor scores

Subjective VAS scores for feelings of hunger and for rating of flavor of the study stimuli are shown in table 2. Before consumption, no significant differences were found between visits. The plain water and water flavored with EB stimuli initiated a significant increase in VAS scores for hunger. The flavor VAS score for water with EB was significantly lower compared with the other three study stimuli. The VAS score for flavor for the combined stimulus of glucose and EB was not significantly different from water and glucose stimuli but was rated significantly more pleasant than EB alone.

Hypothalamic activity changes

Group average hypothalamic BOLD responses per study stimulus are shown in figure 3 (top panel). Compared to baseline BOLD signal(before ingestion), ingestion of water led to a 0.63% average increase in BOLD signal. Ingestion of water flavored with EB led to an average increase of 0.18%. Both water with glucose and water with glucose combined with EB led to an average decrease in BOLD signal of 0.54% and 0.38%, respectively.

Table 1. Subject characteristics

		n=16
Age (years)		20.6±1.3
Height (m)		1.83±0.05
Weight (kg)		72.4±5.5
BMI (kg/m^2)		21.7±1.1
Glucose	Fasted*	4.7 (4.2-5.4)
	Post glucose ingestion*	6.9 (5.7-8.1)
Insulin	Fasted*	6.5 (3.5-15.0)
	Post glucose ingestion*	25.0 (9.5-80.0)

Values in mean ± standard deviation. Glucose and insulin levels in median and range. Glucose levels in mmol/L, normal fasted range: 3.9-5.5 mmol/L, Insulin levels in mU/L, normal fasted range: <20 mU/L. *average fasted blood levels over all four visits and the average post ingestion levels for two visits with glucose stimuli per subject measured 30 minutes after ingestion.

Table 2. Visual analogue scale (VAS) scores for hunger and flavor

	Water	EB	Glucose	Glucose + EB
VAS hunger pre-ingestion	5.4±2.3	5.8±2.1	5.4±2.2	5.9±2.1
VAS hunger post-ingestion	6.6±1.8	6.8±1.6	5.5±2.4	5.7±2.3
Delta VAS hunger	1.2±1.2	0.9±1.6	0.2±2.2	-0.3±1.6
VAS flavor	5.2±1.4	3.9±1.5	5.4±1.6	5.3±2.5

Values in mean ± standard deviation. VAS consisted of a 10cm line scored from 0 to 10, anchors for the VAS score for hunger 0: 'not hungry' and 10: 'extremely hungry', anchors for the VAS score for flavor 0: 'very unpleasant' and 10: 'very pleasant'

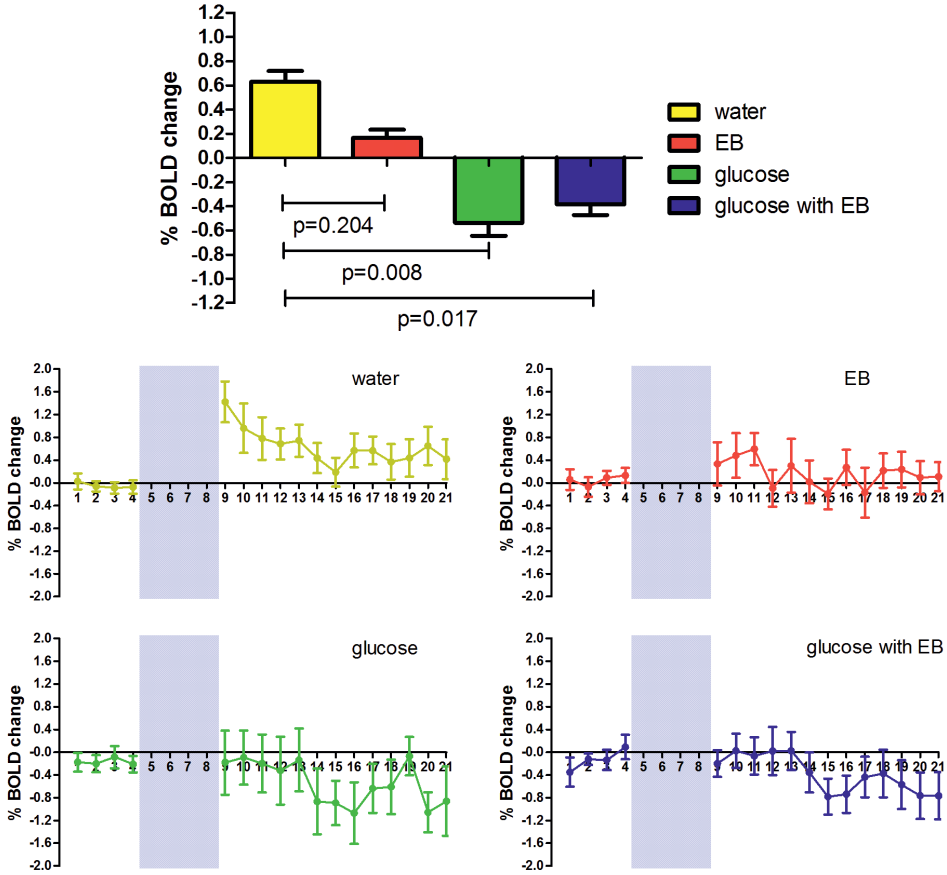

Figure 3. Hypothalamic average BOLD signal change and BOLD time courses. BOLD responses to all four study stimuli (Mean % change with SEM). Percentage change from pre-ingestion (0-5 minutes) to post-ingestion (minute 9-21 post ingestion) were calculated. Differences in percentage change between study stimuli were tested against the water intervention with linear mixed model analysis. Grey areas in the time course graphs indicate period during which the stimuli were consumed, data points were excluded from the analysis.

When looking at the response over time after ingestion (figure 3 bottom panel) the stimuli without energy content (water and water flavored with EB) led to an initial increase in BOLD signal that became smaller over the measured time period. In contrast, the stimuli with energy content led to a decrease in signal that became stronger over the measured time period.
When determining the effect of energy content and the presence of EB flavoring on the hypothalamic BOLD response we found that energy content had a significant effect on hypothalamic activity (p=0.006), however the EB flavoring did not have a significant effect (p=0.430). Energy content and EB flavoring did not have a significant (p=0.310) interactive effect on the BOLD response, possibly due to the lack of effect of EB alone. Compared to the plain water response, water flavored with EB did not lead to an altered BOLD signal (p=0.204). On the contrary, both ingestion of glucose with and without EB resulted in a significant decrease in BOLD response (glucose only p=0.008, and glucose flavored with EB p=0.017). The addition of EB to glucose did not lead to significantly additive effect compared to glucose without flavoring (p=0.870). Ingestion of glucose flavored with EB ingested at 0°C did not lead to different response compared to ingestion at room temperature.

Ventral tegmental area activity changes

VTA group average BOLD responses per study stimulus are shown in figure 4 (top panel). Compared to baseline BOLD signal (before ingestion), ingestion of water led to a 0.38% increase in BOLD signal. Ingestion of water flavored with EB led to an increase of 0.59%. Similar to the hypothalamic response, both water with glucose and water with glucose combined with EB led to a decrease in BOLD signal of 0.31% and 0.93%. When looking at the response over time after ingestion (figure 4, bottom panel) the stimuli without energy content (water and EB) led to an initial increase in BOLD signal that became smaller over the measured time period for water alone but remained increased for the water flavored with EB. Similar to the response in the hypothalamus, the stimuli with energy content also led to a decrease in signal in the VTA that became stronger over the measured time period.
When determining the effect of energy content and the presence of EB flavoring on the overall BOLD response we found that energy content had a significant effect on VTA activity (p=0.007), however the EB flavoring did not have a significant effect (p=0.612). Energy content and EB flavoring did not have a significant (p=0.363) interactive effect on the BOLD response, possibly due to the lack of effect of EB alone. Compared to the plain water response, water flavored with EB did not lead to an altered BOLD response (p=0.786). Ingestion of glucose only, resulted in a non-significant decrease in BOLD response (p=0.187). On the contrary, glucose flavored with EB mean

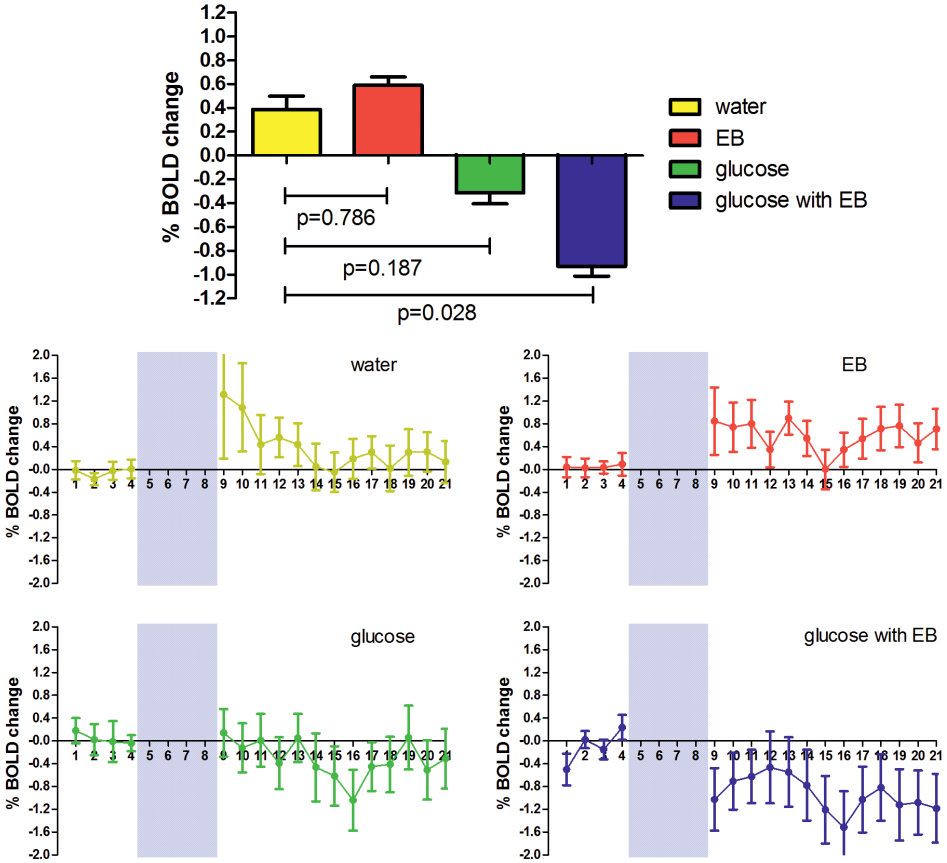

Figure 4. Ventral Tegmental Area (VTA) average BOLD signal change and BOLD time courses. BOLD responses to all four study stimuli (Mean % change with SEM). Percentage change from pre-ingestion (0-5 minutes) to post-ingestion (minute 9-21 post ingestion) were calculated. Differences in percentage change between study stimuli were tested against the water intervention with linear mixed model analysis. Grey areas in the time course graphs indicate period during which the stimuli were consumed, data points were excluded from the analysis.

difference led to a significant decrease in BOLD response (p=0.028), although this BOLD response was not significantly stronger compared to glucose only (p=0.317). Ingestion of glucose flavored with EB ingested at 0°C did not lead to different response compared to ingestion at room temperature.

Associations between blood values, subjective ratings of hunger and flavor and the hypothalamic and VTA activity changes

Mixed model analysis showed that increased ratings of hunger before stimulus

ingestion were associated with a positive shift of the BOLD response in both the hypothalamus (+0.2%, p=0.043) and VTA (+0.2%, p=0.012). Moreover, ratings of hunger after stimulus ingestion were also associated with a positive shift of the BOLD response in both the hypothalamus (+0.2%, p=0.042) and VTA (+0.03%, p=0.001). Subjective rating of pleasantness of flavor did not have a significant association with the BOLD response in either the hypothalamus (p=0.512) and VTA (p=0.573). Blood glucose levels were not significantly associated with the BOLD response of the hypothalamus or VTA. Insulin levels after ingestion were associated with the BOLD response in the VTA (+0.2%, p=0.009).

DISCUSSION

The results of this study show that in the VTA the combination of glucose with EB flavoring led to a significant decrease in activity, which was threefold stronger than to glucose alone. Adding EB flavoring to plain water (no caloric content) did not have any effect on the VTA. In the hypothalamus only the ingestion of glucose, and not EB, was associated with changes in neuronal activity.

The ingestion of a sweet taste, with and without energy content, has been shown to elicit a response from the reward system [15]. Sweet taste is regarded by the brain as a predictor of energy content of the ingested food or drink [15, 27, 28]. VTA is active in expectation of reward, sweet taste could therefore lead to an increase in VTA activity because of the expectation of energy content. Interesting in this context are non-nutritive sweeteners ,which are increasingly use in foods and beverages, because they deliver sweet taste without any caloric content.[29, 30] However, the flavoring we used in our study is not necessarily a sweet flavor on its own without the addition of glucose or another sweetener [15]. This could explain why we did not find a significant response from the VTA, although the activity did appear to increase, with just the EB flavoring added to plain water. Adding flavoring to sugars amplifies the sweet taste and palatability which could increase the rewarding response [18, 19]. Additionally, when glucose is paired with a congruent flavoring, the perception of flavor is enhanced [22, 23], and could subsequently lead to a stronger reward response. This is in line with our finding that EB combined with glucose elicited the most pronounced response from the VTA. Furthermore, energy coupled with taste has been shown to lead to stronger subjective feelings of satiation and short term satiety compared to either stimulus separately [24], which can also be linked to a stronger reward response by the brain. The strongest response to the combined stimulus in the VTA could be explained by the VTAs role in regulating palatable eating by integrating energy content

with reward via the VTAs anatomical and functional connections with the hypothalamus [12-14]. Finally, the strongest combined effect of flavoring and glucose on the VTA could indicate that added flavoring can be used as strategy towards reduction of energy content, as our results indicate that the reward response to lower (but not zero) energy content could be strengthened by added flavoring.

In addition to reward effects, flavor might affect the homeostatic response by the hypothalamus as sweet taste without caloric intake has been shown to elicit an insulin response [21]. However, other studies indicate that taste only elicits or modulates a hormonal response in combination with caloric content, as regulated by the hypothalamus [5, 30, 31]. Earlier studies by our group have shown that the ingestion of glucose solution leads to a decreased activity throughout the brain and specifically in the hypothalamus [8, 32]. The results from the current study confirm once again that hypothalamic activity decreases after glucose ingestion and show that the driving force of the hypothalamic response is indeed the ingestion of energy and not the flavor indicated by the lack of response to flavoring without energy content. Our result are also concordant with the results of other previous studies showing that taste without energy does not affect the hypothalamus [4-7].

Our data show that stronger feelings of hunger, both before and after stimulus ingestion, had a positive effect on activity change adjusted for treatment in both the hypothalamus and VTA. This indicates that a stronger feeling of hunger lessened the satiety effect in the hypothalamus. In a state of hunger, the brain - and specifically the hypothalamus and VTA - is actively seeking energy and reward and therefore shows a high activity that decreases after energy intake [5, 6, 12, 33]. Our finding that a higher subjective score for hunger leads to a larger decrease in activity in the hypothalamus and the VTA support this theory. This suggests that these responses might reflect an objective evaluation of (perceived) energy content and reward.

A limitation of this study is the generalizability as we only investigated a relatively small sample of male volunteers and it can be expected that sex differences are present, since it is known that there are several sex-specific differences in energy metabolism [34]. Additionally, we only investigated lean subjects and earlier studies have shown different hypothalamic function in obesity [35]. A strength of our study was our crossover study design, which allowed for a reliable within-subject comparison between interventions as participants were their own controls.

Taken together, our results suggest that the reward response is strongest to a combination of both flavor and energy and that the homeostatic satiety response is mainly driven by energy ingestion. Although neither response seemed dependent on subjective ratings of pleasantness in the basal brain areas investigated here, the levels of hunger do influence the responses in these areas. This suggests that the responses

by the hypothalamus and VTA might be mostly involved in subconscious rather the conscious regulation of satiety, appetite and feeding behavior and is mainly driven by energy demand and intake.

Acknowledgements and author contributions

JG, AB, MH and CB designed research; AO and JG conducted research; AO and AB analyzed data; AO and JG wrote the paper; JG had primary responsibility for final content. All authors read and approved the final manuscript.

Conflicts of interest

This study was funded by Unilever Research and Development Vlaardingen B.V. The Netherlands. M. Hoeksma and C. Blonk are both employees of Unilever Research and Development Vlaardingen B.V. The Netherlands. All other authors have no conflict of interest to disclose.

Data availability statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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CHAPTER 7

Effects of intranasal insulin application on the hypothalamic BOLD response to glucose ingestion

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ABSTRACT

Background

The hypothalamus is a crucial structure in the brain that responds to metabolic cues and regulates energy homeostasis. Patients with type 2 diabetes demonstrate a lack of hypothalamic neuronal response after glucose ingestion, which is suggested to be an underlying cause of the disease. In this study, we assessed whether intranasal insulin can be used to enhance neuronal hypothalamic responses to glucose ingestion.

Methods

In a randomized, double-blinded, placebo-controlled 4-double cross-over experiment, hypothalamic activation was measured in young non- diabetic subjects by determining blood-oxygen-level dependent MRI signals over 30 minutes before and after ingestion of 75g glucose dissolved in 300ml water, under intranasal insulin or placebo condition.

Results

Glucose ingestion under placebo condition lead to an average 1.4% hypothalamic BOLD decrease, under insulin condition the average response to glucose was a 2.2% decrease. Administration of water did not affect the hypothalamic BOLD responses.

Conclusions

Intranasal insulin did not change circulating glucose and insulin levels. Still, circulating glucose levels showed a significant dampening effect on the BOLD response and insulin levels a significant strengthening effect. Our data provide proof of concept for future experiments for testing the potential of intranasal application of insulin to ameliorate defective homeostatic control in patients with type 2 diabetes.

INTRODUCTION

Worldwide, there has been a rapid increase in the number of patients who have type 2 diabetes mellitus. Type 2 diabetes, found in >90% of all cases of diabetes, is characterized by insulin resistance and a defective secretion of insulin. For several decades, glucose homeostasis was assumed to be regulated primarily via peripheral mechanisms and centered around insulin produced by pancreatic islet cells and its effects on suppression of hepatic glucose production and, stimulation of glucose uptake by adipose tissue and muscle [1-3]. Recent data however demonstrated that the brain also plays a pivotal role in glucose regulation [4, 5]. A key brain area that is involved in glucose homeostasis is the hypothalamus, which regulates metabolic homeostasis via its reciprocal projections to other brain regions and the periphery [6]. The hypothalamus senses, integrates and co-ordinates circulating metabolic cues, thereby aiding regulation of body weight and feeding behavior [6, 7]. Insulin, a signal reflecting energy availability and reserves, provides direct negative feedback to hypothalamic nuclei that control energy and glucose homeostasis [8]. Previous studies have clearly shown that insulin receptors and insulin signaling in the hypothalamus is vital in the regulation on glucose and energy metabolism [8-11]. Studies on the role of the brain in glucose regulation in humans have been facilitated by non-invasive techniques such as measurement of blood oxygen level dependent (BOLD) brain responses, which allows quantification of brain activities over time during functional MRI studies. BOLD MRI contrast is a reflection of hemodynamic changes associated with neuronal activity due to changes in local concentrations of oxygenated and deoxygenated hemoglobin [12, 13]. These BOLD changes can be measured in the hypothalamus, in response to glucose or to other stimuli [14]. In healthy men, a prolonged, dose-dependent decrease in hypothalamic BOLD signal was observed in response to glucose ingestion, showing adaptability of the hypothalamus to nutrient ingestion [14, 15]. Conversely, in patients with type 2 diabetes, a lack of significant decrease in BOLD signal was observed in response to glucose ingestion, implying diabetes- induced failure to inhibit hypothalamic neuronal activity in response to glucose ingestion [16]. Interestingly, in patients with type 2 diabetes, this absent hypothalamic response normalized after a 4-day very-low-calorie diet, indicating that hypothalamic dysfunction to glucose sensing is potentially reversible [17].

We hypothesize that deficits in central insulin action play a role in defective hypothalamic gluco-regulatory responses and that increasing central insulin levels may potentiate brain responses to glucose. Earlier studies have shown that insulin, and other compounds, can be non-invasively delivered to the CSF and cross the brain barrier via

the intranasal route, without spilling over to the periphery, allowing for a specific increase in central insulin levels [18, 19]. Several studies have shown that intranasal insulin has an effect on various hypothalamic functions, indicating that the hypothalamus is a target for and is reached by intranasal aplicaition of insulin [20, 21]. In a randomized, double blind, placebo-controlled cross-over experiment, we aimed to investigate the hypothesis that intranasal insulin application may potentiate the BOLD response to glucose ingestion in the hypothalamus in young healthy volunteers. First, we applied insulin or placebo using a customized nasal atomizer. Thereafter hypothalamic BOLD signals were measured before and during 30 minutes after ingestion of 75 g glucose dissolved in water, or plain water being the control condition.

METHODS

Ethics statement

The study was approved by the Medical Ethical Committee of Leiden University Medical Centre under protocol P13.164 and the Dutch competent authority (Centrale Commissie Mensgebonden Onderzoek (CCMO)) under protocol code number NL45043.058.13. The study is registered in the European Clinical Trials Database under number 2012-005650-29 on the 9th of April 2014. All investigations have been conducted according to the principles expressed in the Declaration of Helsinki. All participants provided written informed consent after complete written and verbal description of the study was given.

Experimental subjects

Our study population consisted of 8 healthy, normal weight (20 kg/m^2 < BMI < 25kg/m^2) adult male volunteers from the general population. Exclusion criteria included fasting plasma glucose above 7 mmol/l, anaemia (haemoglobin < 7.1 mmol/l), any significant endocrine, neurological and cardiovascular diseases, or use of medication known to influence lipolysis, thyroid function, glucose metabolism, GH/IGF-1 secretion or any other hormonal axis. Furthermore, persons with a history of smoking addiction, alcohol addiction or substance abuse were excluded as well as persons with characteristics that might interfere with effective intranasal application, including presence of anatomic deviations of the nose or chronic colds, running nose or allergies.

Experimental protocol

We performed a randomized, double-blinded, placebo-controlled cross- over experiment. For each participant, the study was carried out on four study visits, each spaced apart by one week. The participants were randomized over two treatment

arms, with half of the participants having intranasal application of placebo first and the other half intranasal application of insulin first. Concealment of treatment allocation was ensured by delivery of experimental medication in same type of vial that was prepared and re-labelled by the hospital pharmacy. De-blinding was done at the end of the study. After a 10 hour overnight fast, participants arrived at the MRI facilities at 8AM. First, a baseline venous blood sample was collected in a serum-separator (SST)-tube and thereafter, every 10 minutes, 4 ml of blood was collected into a SST-tube and 2 ml into K3-EDTA tube. Ten minutes after the first blood withdrawal, the study intervention was applied, which consisted of intranasal application of 40 IU insulin (Actrapid; Novo Nordisk, Mainz, Germany) or placebo (Normal saline), applied using the ViaNase Electronic Atomizer (Kurve Technology inc) 20 minutes before the start of the MRI scanning procedure. At 30 minutes after application of intranasal insulin/placebo, single slice BOLD mid-brain fMRI scans were performed, during which participants drank either the glucose solution or water (75gr of glucose dissolved in 300ml of tap water or 300ml of plain tap water). This timing of ingestion of the glucose and/or water stimulus was chosen as we expected the greatest effect of the intranasal treatment at this time. As previous studies have shown that it takes 30 minutes for insulin to reach maximal concentrations in the CSF [22], and a similar time window has been used previously in other studies where brain – related effects of intranasal insulin were studied [21, 23, 24]. The total time for the BOLD scans (baseline measurement, ingestion and post-ingestion measurements) was 30 minutes.

Processing of blood samples

After blood withdrawal, K3-EDTA tubes were immediately placed on ice before centrifugation. Serum tubes were kept at room temperature and centrifuged at 3520 RPM at 4°C for 10 minutes when the samples were clotted, usually between 30–60 minutes. EDTA plasma and serum samples were stored in two aliquots of 500 µl during the rest of the study day at −20°C. After completion of sample collection, these were stored at −80°C. All laboratory measurements were performed with fully automated equipment and diagnostics from Roche Diagnostics (Almere, The Netherlands). Glucose levels were measured using Hitachi Modular P800 from Roche (Almere, the Netherlands), with coefficient of variation (CV) for measurement less than 1%. Insulin levels were measured using the Immulite 2500 from DPC (Los Angeles, CA), with CV less than 6%. HOMA-IR index was calculated according to the formula: fasting insulin x fasting glucose / 22.5.

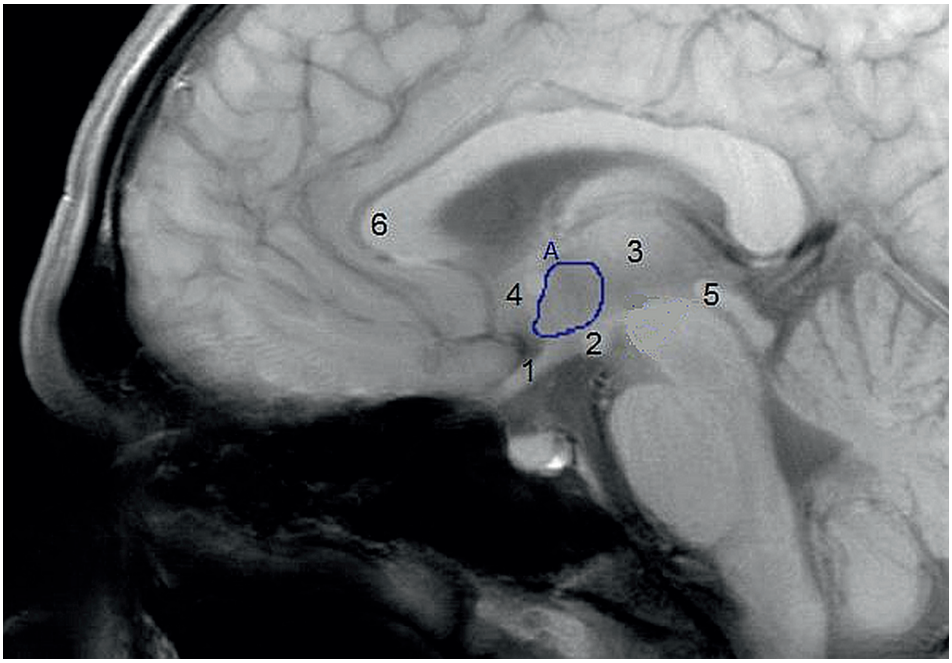

Figure 1. Segementation of the hypothalamus region of interest (ROI). For the hypothalamus ROI (A), the optic chiasm (1), the mammillary bodies (2), the thalamus (3) the anterior commissure (4) and the top of the cerebral aqueduct (5) were used as landmarks. The reference ROI was drawn superior of the genu to the corpus callosum (6) in the grey matter.

MRI acquisition and analysis

MRI was performed on a 3.0 Tesla clinical MRI scanner (Achieva Philips Healthcare, Best, the Netherlands). We performed a structural mid brain scan (single slice scan, repetition time 550 ms, echo time 10 ms, field of view 208 x 208 mm, voxel size= 0.52 x 0.52 x 14 mm, scan time 1.14 min) and a high resolution mid brain single slice fMRI scan (repetition time 120 ms, echo time 30 ms, field of view 208 x 208 mm, voxel size= 0.81 x 0.81 x 14 mm, scan time 30 min, 700 dynamic volumes). Both of these single slice mid-sagittal scans were planned using third ventricle, the corpus callosum and brain midline as landmarks. Data was pre-processed as described in previous studies [17]. Data was averaged for each set of 4 subsequent volumes, reducing the 700 dynamic scans to 175. The hypothalamus was segmented manually, using the mid brain single slice anatomical image as a guide for delineation. Manual segmentation was performed on the middle dynamic volume of the fMRI scan as described previously using the anterior commissure, the optic chiasm and the mammillary bodies as anatomical landmarks [14, 17]. Example delineation of the hypothalamus, with the

anatomical landmarks used for delineation, is shown in *figure 1*. To correct for scanner drift, all hypothalamic BOLD values were corrected using an internal reference ROI in the grey matter superior of the genu of the corpus callosum.

Statistical analyses

For statistical analyses, Statistical Package for Social Sciences (SPSS) software for windows (version 20.0) was used. Graphs were made using GraphPad Prism version 5 (GraphPad, San Diego, CA). Descriptive statistics were used to summarize the characteristics of both study groups. All fMRI results are reported as percentage BOLD change, relative to baseline; 0-8 minute BOLD response before drinking of the test solution. For presentation purposes, the individual 175 dynamic scans time points were pooled into 'per minute' data points: The first 8 minutes were considered baseline BOLD response. Data from minute 8 through 11 were omitted from the analysis due to non-physiologic excessive BOLD changes caused by swallowing of the drink. Statistical analysis of the differences in effect between the study stimuli on the overall BOLD response was performed by a single linear mixed model analysis using the study stimulus as a fixed effect, time point as a covariate and subject per occasion as a random factor using the total time series. Differences in treatment effect was deemed significant at $p<0.05$. To test for treatment effects over time the same model was used per two minute intervals instead of the entire series. P-values for the analysis per two minute intervals are reported corrected for multiple comparison with a Bonferonni correction of eight ($p<0.00625$). To test for the influence of blood glucose and insulin levels and the HOMA-IR index on the response to the study stimuli, additional mixed model analyses were performed, in which these values were added as covariates. These effects of glucose and insulin on the BOLD response are reported as estimated effects in percentage BOLD change.

RESULTS

Subject characteristics

The characteristics of study subjects are summarized in *table I*. The participants had a mean age of 22 years and mean BMI of 23.6 kg/m^2. The mean fasted glucose was 4.8 mmol/L , median insulin level was 0.84 μU/ml and the median HOMA-IR index was 1.2.

Blood glucose and insulin trajectories after intranasal insulin application

The mean serum glucose and insulin trajectories of all participants over 2.5 hour encompassing the time period before, during and after the intranasal application of

Table I. Characteristics of study subjects

Demographics	
Age in years	22.3 (1.8)
BMI in kg/m^2	23.6 (2.2)
Alcohol intake (units/week)	14.4 (2.2)
Systolic BP (mmHg)	125 (9.3)
Diastolic BP (mmHg)	74 (7.2)
Metabolic	
Fasted glucose in mmol/L	4.8 (0.3)
Fasted insulin in μU/ml, median (IQR)	0.84 (0.69, 0.95)
HOMA-IR index, median (IQR)	1.2 (1.0, 1.5)

Unless otherwise stated, values are means (standard deviation).
BMI: body mass index.

placebo or insulin and MRI scanning procedure are shown in *figure 2*. Serum glucose levels after drinking water were stable throughout the experimental period and were not significantly different between insulin and placebo conditions (*figure 2a*). After glucose ingestion, the serum glucose levels increased in both intranasal insulin and placebo conditions, with the rise in glucose levels starting approximately 15 minutes after glucose ingestion. A peak glucose level of 7.0 mmol/l was reached 50 minutes after glucose ingestion under the intranasal insulin condition. For the placebo condition, a peak of 6.3 mmol/l was reached 60 minutes after glucose ingestion. Thereafter, glucose levels for both insulin and placebo conditions declined to 5.2 mmol/l at the end of the experinment.

Serum insulin levels after drinking water were stable throughout the experimental period and were not significantly different between the intranasal insulin and placebo conditions (*figure 2b*). After glucose ingestion serum insulin levels increased under both intranasal insulin and placebo conditions. After intransal insulin application, the serum insulin levels reached a peak of 48.4mU/L at 80 minutes after glucose ingestion. Under intransal application of placebo, a peak in serum insulin of 40.65 mU/L was reached 90 minutes after ingesting the glucose drink.

Changes in hypothalamic BOLD response

Figure 3 shows the group averaged hypothalamic BOLD responses before, during and after ingestion of study stimulus (glucose or water). The water and glucose responses under placebo condition are shown in panel A. The corresponding responses under insulin condition are shown in panel B.

Over the entire 22- minute post- drinking period, the BOLD response after water ingestion under placebo condition, was 0.2% lower compared with baseline, and after

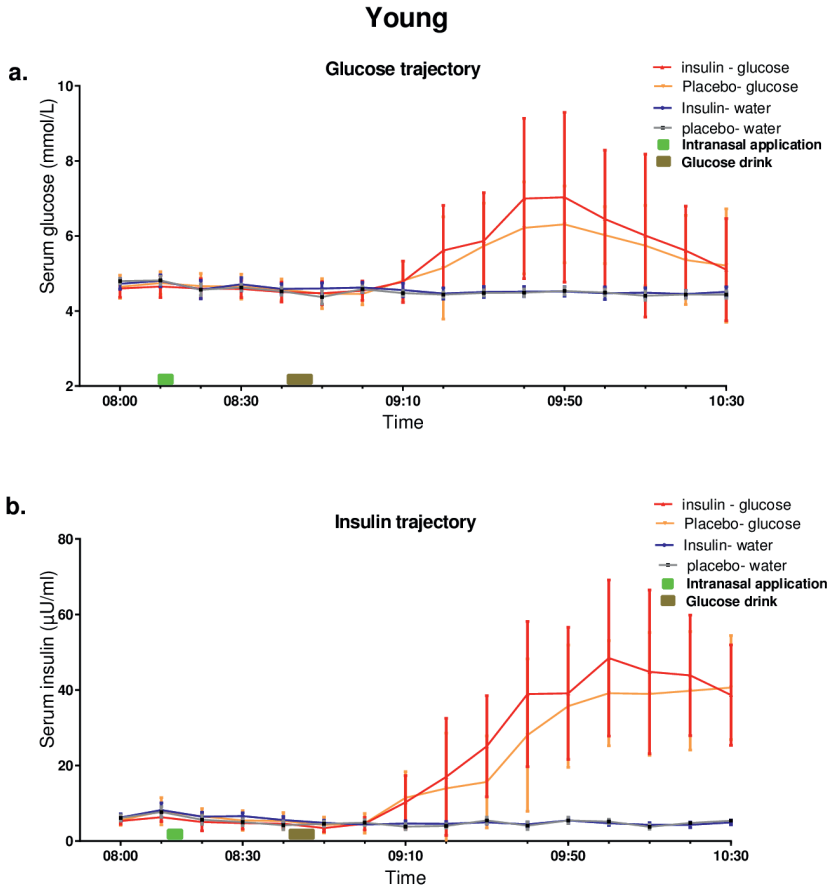

Figure 2. Glucose and insulin trajectories during the experimental period. Concentration of A. glucose and B. insulin in serum before and after intranasal insulin application (40 IU Insulin actrapid, blue line) or placebo (saline, red line) using Vianase nasal atomizer. Data are presented as mean (Standard error) of A. glucose and B. insulin levels measured every 10 minutes. The green bar represents the timing of intranasal application, with the brown bar represent the timing of ingestion of a glucose drink or water during the fMRI scanning.

glucose ingestion 1.4% lower compared with baseline. Although the response was lowered after ingestion of glucose compared to water ingestion no significant difference between the two conditions was found. Also, no differences between these conditions for the two minute time intervals were found.

Panel B shows the water and glucose responses under insulin condition. Over the entire period, the BOLD response after water ingestion was 0.1% lower compared with baseline, and after glucose ingestion 2.2% lower compared with baseline. For the entire post drink period, the glucose BOLD response was significantly (p=0.028, whole time

series mixed-model analysis) lower than the water response. Especially between 20 and 23 minutes after ingestion this difference becomes most prominent ($p<0.05$ Bonferroni corrected, 2-min window mixed model analysis).

Effects of plasma glucose and insulin levels on the BOLD response

Circulating glucose levels showed a significant effect on the BOLD response (estimated effect: +0.3% BOLD change, $p=0.001$). Indicating that a higher plasma glucose level leads to a dampened decrease in the BOLD signal of the hypothalamus. Circulating plasma insulin levels had a significant, albeit small, decreasing effect on the BOLD response (estimated effect: -0.03% BOLD change, $p=0.005$). The HOMA-IR index did not have a significantly effect on the BOLD response.

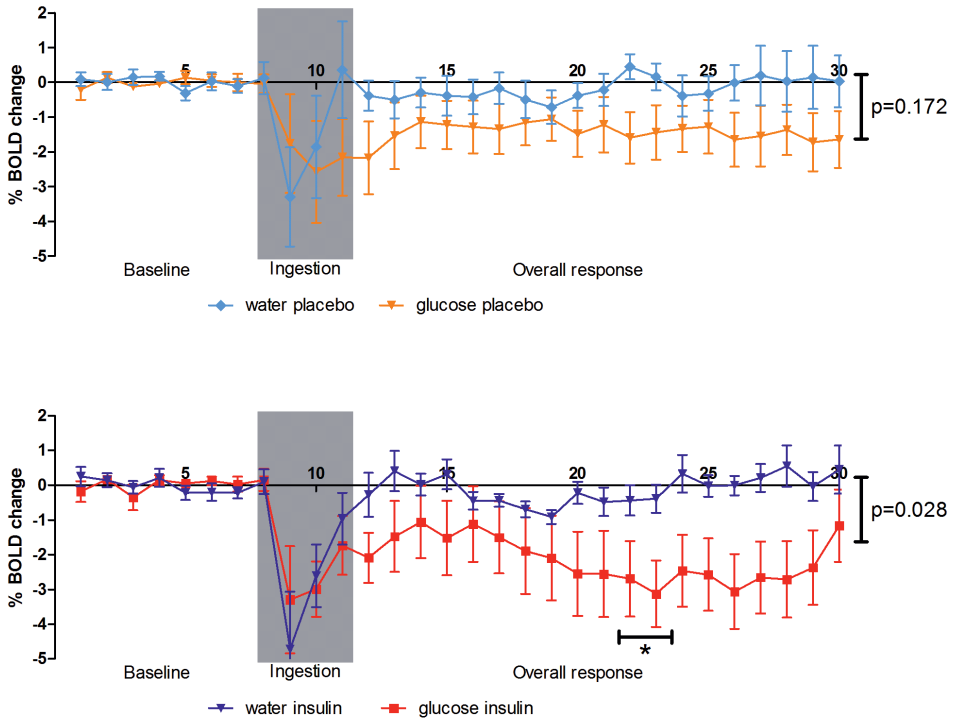

Figure 3. Hypothalamic BOLD responses under placebo and insulin conditions. Baseline BOLD signal measured from minute 1 to 8, ingestion of either glucose solution or water from minute 8 through 11 (indicated by grey box), overall response period after ingestion of glucose solution or water from minute 12 through 30. Panel A: light blue line: response to water ingestion under placebo condition, orange line: response to glucose ingestion under placebo condition. Panel B: dark blue line: response to water ingestion under insulin condition, red line: response to glucose ingestion under insulin condition. * Indicates significant differences at $p<0.05$ Bonferroni correct for multiple comparison for separate time window analysis.

CONCLUSIONS

To study the effects of intranasal insulin application on neuronal activity in the hypothalamus after glucose ingestion in young healthy volunteers, we performed a randomized, double blind, placebo-controlled cross-over proof of concept experiment and applied insulin or placebo via the nose using a customized nasal atomizer. The major new finding of our study is that the decrease in fMRI BOLD response in the hypothalamus after glucose ingestion was augmented after intranasal application of insulin. Furthermore, we found that circulating glucose and insulin levels did not change after intranasal application of insulin compared to placebo indicating that there was no spill over to the systemic circulation and we were able to target the CNS specifically. Nevertheless, in line with the absence of reduction in hypothalamic BOLD response to glucose previously observed in patients with diabetes type 2 with increased circulating glucose [16, 17], we found that in healthy volunteers higher plasma glucose level also lead to reduced hypothalamic BOLD responses.

The hypothalamus plays a key role in the central regulation of food intake and energy metabolism, and specific hypothalamic nuclei are critical for the control of peripheral metabolism by insulin [6, 8]. We found intranasal insulin administration augmented the decrease in hypothalamic BOLD response to glucose ingestion, with an average decrease in the BOLD signal of 1.2% after intranasal placebo applications and 2.1% when the glucose ingestion was preceded by intranasal application of insulin. Indicating that central insulin can affect the hypothalamic function to maintain glucose homeostasis as is suggested by earlier studies [8, 10, 25]. Among patients with type 2 diabetes, the glucose-induced reduction in hypothalamic BOLD signal was absent, suggesting that glucose ingestion failed to inhibit hypothalamic neuronal activity [16, 17]. Considering that we found the decreased BOLD signal to ingested glucose was potentiated after intranasal insulin, future experiments might be aimed at testing the potential of intranasally applied insulin to rescue the defective BOLD response that was observed in diabetic patients. Future studies could also be targeted at performing these experiments under conditions of increased adiposity, such as obesity, which has also been implicated to have effects on the hypothalamic response [26].

One limitation of this study is that it was done only in male volunteers and it can be speculated that a sex dimorphism is present since it is known that there are several sex-specific differences in energy metabolism [27]. This decreases the generalizability of our findings. Another limitation is the indirect measurement of neural activity in the targeted brain area using BOLD measurement technique, as it is unclear what direct functional consequences these signals reflect without additional measurements of downstream functional effects. Furthermore, other pathways could also affect the

hypothalamic response to glucose besides the direct sensing of glucose by the hypothalamus. Other studies have shown that dopaminergic pathways also respond to energy ingestion and could thus have an effect on the hypothalamic response [22]. To determine this interplay BOLD measurements in the limbic system might thus be useful in future studies. The strength of this study is the methodological rigor and the fact that the participants were their own controls, thus ruling out possible dilution of effects that could have been introduced due to differences in individual phenotypes.

Summarily, using a randomized, double blind, placebo-controlled cross- over proof of concept experiment, we studied the effects of intranasal insulin application on blood oxygen level dependent (BOLD) signals as proxy for neuronal activity in the key brain area of the hypothalamus that has been implicated in responses to metabolic cues. We found that intranasal insulin augmented the decrease in fMRI BOLD signal in response to glucose ingestion in the hypothalamus. Further research is needed to investigate whether intranasal application of insulin has similar effects on hypothalamic BOLD responses in patients with type 2 diabetes, and what the functional consequences are.

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Author contributions

A.vO. collected and analyzed the data, made the MRI figures and contributed to the intellectual content of the report. A.A.A. collected, analyzed and interpreted the data, and wrote the report. MvE collected the data. R.W. and H.P. designed the study and critically appraised the report. D.vH. and J.vdG. designed the study, analyzed and interpreted the data and contributed to the intellectual content of the report. All the authors edited and approved the manuscript before submission.

Competing Financial Interests

The authors declare no competing financial interests.

Data availability

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

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CHAPTER 8

Hypothalamic BOLD response to glucose intake and hypothalamic volume are similar in anorexia nervosa and healthy control subjects

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ABSTRACT

Background

Inconsistent findings about the neurobiology of Anorexia Nervosa (AN) hinder the development of effective treatments for this severe mental disorder. Therefore the need arises for elucidation of neurobiological factors involved in the pathophysiology of AN. The hypothalamus plays a key role in the neurobiological processes that govern food intake and energy homeostasis, processes that are disturbed in anorexia nervosa (AN). The present study will assess the hypothalamic response to energy intake and the hypothalamic structure in patients with AN and healthy controls.

Methods

10 women aged 18-30 years diagnosed with AN and 11 healthy, lean (BMI <23 kg/m^2) women in the same age range were recruited. We used functional magnetic resonance imaging (MRI) to determine function of the hypothalamus in response to glucose. Structural MRI was used to determine differences in hypothalamic volume and local grey volume using manual segmentation and voxel-based morphometry.

Results

No differences were found in hypothalamic volume and neuronal activity in response to a glucose load between the patients and controls. Whole brain structural analysis showed a significant decrease in grey matter volume in the cingulate cortex in the AN patients, bilaterally.

Conclusions

We argue that in spite of various known changes in the hypothalamus the direct hypothalamic response to glucose intake is similar in AN patients and healthy controls.

INTRODUCTION

Anorexia nervosa (AN) is a severe mental disorder mostly seen in teenage girls and young women. Individuals with AN are preoccupied with food and eating rituals to the point of obsession, engage in relentless restrictive eating and compulsively over-exercising, often become severely emaciated and have a distorted body image [1]. The disorder is characterized by high rates of chronicity, morbidity and mortality [2]. Although it was long thought that AN was primarily caused by psychosocial factors, recently many different neurobiological factors have been shown to be involved in the pathology of AN. Inconsistent findings hinder our understanding of this complex disorder and thereby the development of effective treatments.[3] Therefore it is important to gain a better understanding of the neurobiological factors underlying AN.

A brain region that seems to be an important point of focus for neurobiological AN research is the hypothalamus, as it plays a key role in the intricate and complex neuroendocrine interactions that govern food intake and energy homeostasis. Hypothalamic functioning is known to be affected in AN; the hypothalamic-pituitary-adrenal (HPA) axis is hyperactive [4] and there is a dysregulation of neuropeptides involved in the regulation of food intake and satiety [5, 6]. The function of the HPA axis in AN has been extensively studied. However, to the best of our knowledge, the hypothalamic response to energy intake has not been investigated in AN. Using functional magnetic resonance imaging (fMRI) it is possible to investigate the hypothalamic response to energy intake by measuring the direct activity of the hypothalamus during nutrient ingestion. Using this technique in healthy humans, an oral glucose load shows a decrease in hypothalamic neuronal activity [7, 8], whereas no such response was observed in patients with type 2 diabetes [8]. Furthermore, a delayed and attenuated response is found in obese participants [9]. This indicates that in these patients, the hypothalamus inappropriately perceives and/or processes signals in response to a nutrient load, possibly reflecting an abnormal perception of the current metabolic status. Due to the known changes in hypothalamic functioning, patients with AN might also show different hypothalamic neuronal activity in response to an oral glucose load compared to healthy participants. Earlier studies assessed hypothalamic activation using fMRI before and after a meal in AN patients, but not directly during a nutrient load [10]. As the hypothalamic response to nutrients and subsequent feeding behavior is quite fast, it will be even more informative to see the direct response to a nutrient load. In addition to changes in the function of the hypothalamus several studies on psychiatric disorders also show abnormalities in hypothalamic structure [11]. A decreased volume of the hypothalamus is also found in other disorders with a similar dysfunction of the HPA axis as in AN [12].

The aim of the present paper is to further elucidate hypothalamic functioning and structure in AN by fMRI and volume calculations of the hypothalamus together with a voxel-wise analysis to detect differences in local grey matter volume. We hypothesize that the hypothalamic neuronal activity after a glucose load might be different in female AN patients compared to healthy female control participants. Subsequently, we hypothesize that AN patients might have a smaller hypothalamic volume compared to healthy controls.

METHODS

Participants

We recruited 10 Caucasian women aged 18-30 years from Centre for Eating Disorders Ursula, diagnosed with AN according to the Diagnostic and Statistical Manual of Mental Disorders Fourth edition criteria [1], with a body mass index (BMI) <17.5 kg/m^2. 11 Healthy and lean (BMI <23 kg/m^2) Caucasian women in the same age range were recruited as control participants via advertisements at the University of Leiden. A standard MRI screening form and a health and lifestyle questionnaire were used to assess eligibility. Exclusion criteria for both patients and controls were: diabetes mellitus (DM) or a history of DM in first grade relatives, chronic diseases, genetic or somatic disease affecting the brain, substance abuse or addiction according to DSM-IV criteria (except smoking) and MRI contraindications. In addition, control participants who were on a weight reducing diet, lost or gained more than 3 kg in weight over the last 2 months and/or who did not use oral contraceptives were excluded from participation. The latter criterion was set to minimize influence of menstrual cycle and to ensure stable levels of female hormones. The study protocol was approved by the local institutional review board, and written informed consent was obtained from all participants. To compare demographic data between the AN patients and controls independent means t-tests were used.

Data acquisition

Participants were asked to fast (no food or beverage except water) from 10 p.m. the night before the MRI scanning. The MRI was performed between 8.30 and 10.45 a.m.. To overcome the feeling of anxiety and to increase willingness to participate in our study, AN patients were allowed to skip breakfast and their morning snack on the study day in exchange for drinking the glucose solution. All participants received a glucose solution during the scanning session consisting of 50 grams of glucose dissolved in 200 ml water. This was administered during the hypothalamus fMRI scan via a tube

attached to the head coil of the scanner. After the scanning session participants' height and weight were measured.
MRI was performed at our institution using a 3.0 Tesla Achieva clinical scanner (Philips Healthcare, Best, The Netherlands). The protocol comprised of a whole brain 3DT1 (repetition time 9.8 ms, echo time 4.6 ms, field of view 224 x 177 x 168 mm, voxel size 1.17 x 1.17 x 1.20 mm, scan time 4.55 min) for planning and two single-slice, mid-sagittal scans: a T1-weighted Turbo Spin Echo sequence for imaging mid-sagittal anatomical structures (repetition time 550 ms, echo time 10 ms, field of view 208 x 208 mm, voxel size 0.52 x 0.52 x 14 mm, scan time 1.15 min) and a T2*-weighted BOLD-fMRI echo-planar imaging sequence (repetition time 120 ms, echo time 30 ms, flip angle 30°, field of view 208 x 208 mm, voxel size 0.81 x 0.81 x 14 mm, scan time 30 min, 707 time points). A slice thickness of 14 mm was chosen to incorporate the hypothalamus in the left to right direction and to ensure an adequate signal-to-noise ratio and modulus and phase images were acquired to enable complex averaging in post-processing. Eight minutes after the start of the fMRI scan, participants were signaled to drink the glucose solution.

MRI data analysis

Both the fMRI and anatomical scans were processed and analyzed using different tools of FMRIB Software Library (FSL) [13-15]. Statistical analyses were performed using SPSS version 20 (SPSS Inc., Chicago IL, USA). All 707 functional images of each time series were motion corrected by registration to the image that was taken in the middle of the post-drink period (± image 450) with the Multimodality Image Registration using Information Theory software by maximization of mutual information [16]. The resulting registration matrix was applied to the real and imaginary images that were calculated from the original phase and modulus images. Complex data were averaged for each set of four subsequent volumes to reduce phase artefacts caused by head motion or swallowing. Modulus images were then recalculated rendering 177 movement and phase-error corrected images.
Delineation of the hypothalamus region of interest (ROI) was performed manually, based on predefined anatomical borders with use of the anatomical image as an aid to delineate the following anatomic landmarks: anterior commissure, optic chiasm, and mammillary body. Two orthogonal axes were used to divide the hypothalamus into four sub regions as shown in Figure 1 according to Matsuda et al. [9]. In addition, an ROI was delineated in grey matter, superior of the genu of the corpus callosum, as a reference region. The mean signal within every ROI was established for each time point. The mean baseline signal for each ROI was calculated by averaging the signal over all measurements up to half a minute before drinking of the glucose solution started. To obtain the percentage signal change relative to baseline, the post-drink

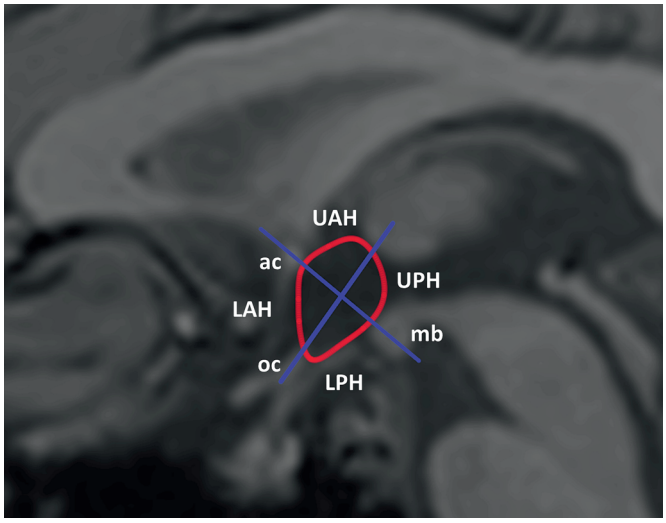

Figure 1. Segmentation and subdivision of the hypothalamus into four ROIs. For analysis of the hypothalamic BOLD response the hypothalamus was manually segmented using several anatomical landmarks. For further analysis the hypothalamus was divided in four spate subdivisions. LPH/UPH lower/upper posterior hypothalamus; LAH/UAH, lower/upper anterior hypothalamus; mb, mammillary body; oc, optic chiasm; ac, anterior commissure.

measurements were normalized to the mean baseline signal. The signal in the reference ROI was used to correct for scanner drift. To test for differences in average hypothalamic BOLD response before and after drinking the glucose solution participant data were pooled per time point. First a student's t-test was used to compare the baseline response between groups. A paired t-test was then used to compare the average BOLD response post glucose ingestion to the average baseline response before glucose ingestion. To compare the BOLD response over time between patients and controls mixed model analysis was performed. General linear mixed model analysis was performed using study group as a fixed effect, time point as a covariate and participant as a random factor.

To analyze differences in hypothalamic and overall brain structure we performed manual segmentation of the hypothalamus to calculate hypothalamic volume and we performed VBM analysis to determine voxel-wise differences in volume between groups according to Holle et al. [17]. Manual segmentation of the hypothalamus was done based on morphological anatomical landmarks as described earlier [11, 12, 18]. A coronal view of an example segmentation of the hypothalamus is shown in Figure 2. Differences in hypothalamic volume between patients and controls were tested using an independent samples t-test.

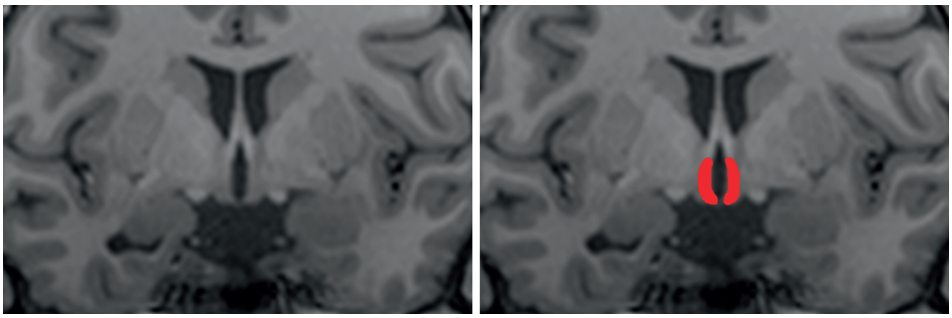

Figure 2. Coronal T1 weighted image of the hypothalamus. For structural analysis of the hypothalamus a region of interest was manually drawn on T1 scans. The left panel shows an representative coronal view of a T1 scans, the right panel shows an example hand delineated region of interest overlaid over the original image.

For the voxel-wise comparison, standard VBM analysis was performed using the FSL-VBM tool [19]. To analyze the potential differences, voxel-wise general linear model (GLM) analysis was carried out, using permutation-based non-parametric testing, correcting for multiple comparisons across space [20].

RESULTS

Population characteristics

Characteristics of patients and controls are shown in Table 1. Structural MRI data was obtained for all participants. As two patients resisted drinking the glucose solution at the last moment during the scanning session, valid fMRI data was obtained for 8 patients.

Functional hypothalamic response to glucose ingestion

Figures 3:A-E show the results of the hypothalamic response to glucose ingestion for patients and controls, in the total hypothalamus and the four sub regions. The percentage BOLD-signal change from baseline value is shown over time, averaged per study group. At around 8/9 minutes a peak and drop in signal was observed, which is associated with head movement during drinking. The baseline response used for normalization was not significantly different between groups (average baseline signal in patient was 0.9327, s.d. 0.064 and 0.9017, s.d. 0.063 in controls, p=0.89). Controls showed a significant average hypothalamic BOLD-signal change of -1.0% compared to the baseline in response to the oral glucose load. Patients showed a significant average signal change of -1.4% compared to baseline. There was no significant difference in

Table 1. Participant characteristics.

	Patients (n=10)	Controls (n=11)	p-value
Age (years)	22.1 (3.3)	20.8 (0.52)	.217
Weight (kg)	45.1 (4.06)	59.3 (3.05)	<.001
BMI (kg/m^2)	15.6 (1.02)	20.3 (1.5)	<.001
Duration of AN (years)	3.54 (2.30)	NA	
AN subtype R/BP (%)	56/44	NA	

Values are reported as means with s.d. (standard deviation). NA, not applicable; BMI, body mass index; kg/m^2, kilogram per square meter; R, restricting; BP, binge-eating purging.

total hypothalamic BOLD response between patients and controls (estimated difference between groups 0.4%, standard error 1.2%, p=0.721). The LPH, LAH and UPH segments showed similar responses that were not significantly different between groups either (estimated difference 0.4%, standard error 1.3%, p=0.762, estimated difference 0.5%, standard error 1.4%, p=0.736 and estimated difference 0.6%, standard error 1.3%, p=0.634 respectively). In the UAH a somewhat different response to glucose ingestion was observed; here the response of the patients was smaller than in the other sub regions, however this was roughly the same for the controls (estimated difference 0.5%, standard error 1.0%, p=0.601). In addition, no significant response difference in BOLD signal after glucose intake for each individual hypothalamic sub region was observed between subjects with AN and control subjects.

Structural brain differences

No differences in hypothalamic volume was observed between the AN patients (mean hypothalamic volume of 0.482cm^3, s.d. 0.064 cm^3) and healthy control participants (mean volume of 0.477 cm^3, s.d. 0.057 cm^3). VBM was used to assess voxel-wise differences in brain volume. VBM results revealed two clusters (one in each hemisphere) where the volume of the cortex was significantly different between the patients and controls (see Figure 4). Compared to controls, AN patients showed significantly ($p<0.05$) reduced grey matter volume in the right cingulate gyrus, anterior division (number of voxels: 231; MNI x, y, z: 8, 22, 30) and reduced grey matter volume in the left cingulate gyrus, posterior division (number of voxels: 237; MNI x, y, z: -14, -16, 48).

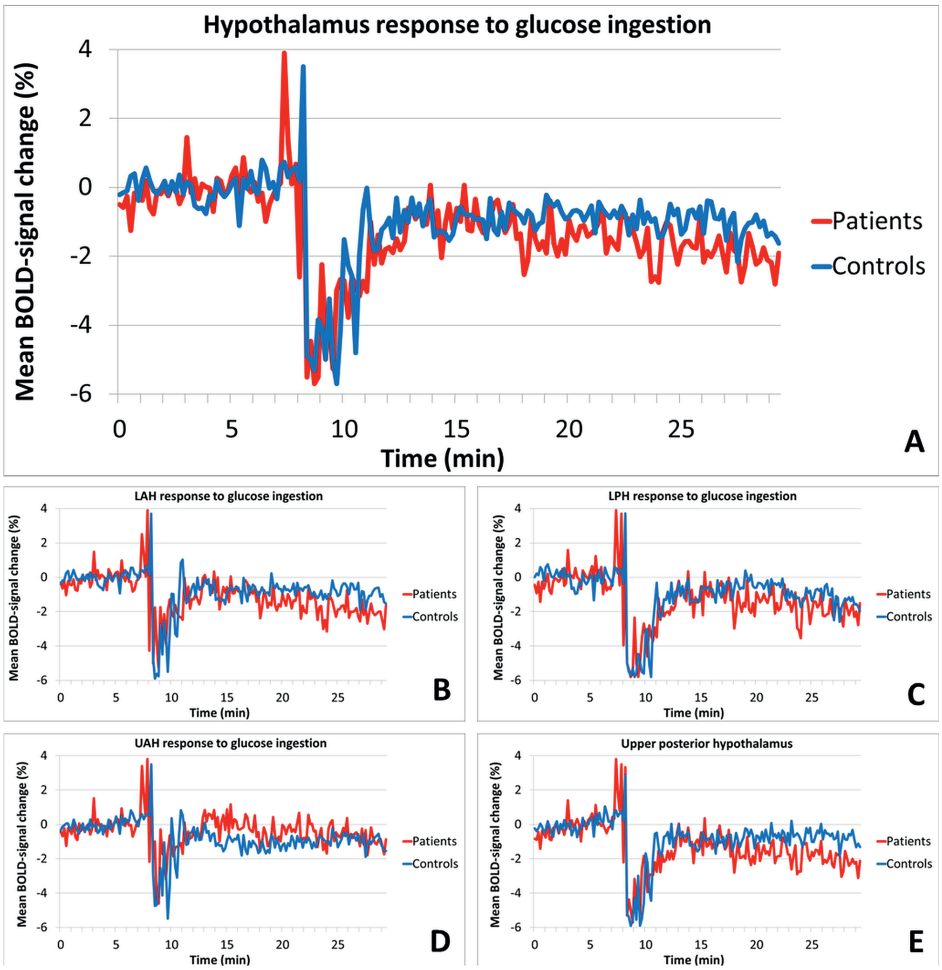

Figure 3. Relative BOLD fMRI signal change of the hypothalamus in response to glucose. The hypothalamic BOLD response showed a decrease of 1.4% in patients and a decrease of 1.0% in controls after glucose ingestion, no significant differences in response were found between groups. Segmentation of the hypothalamus into four subdivisions showed similar results. A: Total hypothalamus B: lower anterior hypothalamus (LAH), C: lower posterior hypothalamus (LPH), D: upper anterior hypothalamus (UAH), E: upper posterior hypothalamus (UPH).

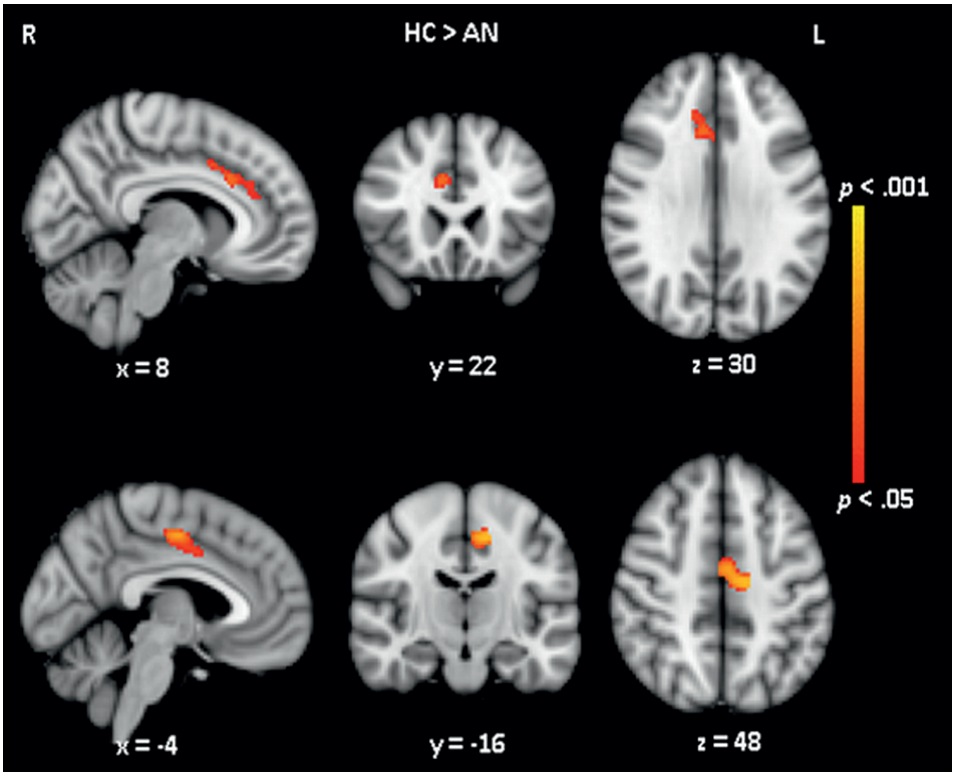

Figure 4. Voxel-wise structural differences between healthy controls and anorexia nervosa. Voxel based morphometry analysis showed a decreased grey matter volume of the cingulate cortex in AN compared to controls. The upper row shows the area of decreased grey matter in the right hemisphere the lower row shows the area of decreased grey matter in the left hemisphere.

DISCUSSION

The results presented in this study shows that the average hypothalamic BOLD response to glucose ingestion is not different between patients with AN and healthy controls. In addition we show that the hypothalamic volume is not different but the volume of the cingulate cortex, a higher-order cortical structure involved in cognitive processes, is decreased in AN.

To the best of our knowledge, this is the first study to investigate the direct response of the hypothalamus following glucose ingestion in patients with anorexia nervosa. The hypothalamus plays a key role in the intricate and complex neuroendocrine interactions that govern food intake and energy homeostasis. Glucose sensing neurons that are present in several hypothalamic nuclei communicate with other neuronal

systems involved in these intricate processes. Receptors that are responsive to several neurotransmitters involved in food intake are present in the hypothalamus as well [21]. In addition, the hormones leptin and insulin signal the hypothalamus to reduce food intake [22]. The levels of these neuropeptides become abnormal with weight loss, signaling the body that it needs food, as there is not enough fuel to maintain body processes. Despite the fact that these levels are abnormal in AN patients [6], they still manage to refrain from eating, indicating that AN patients might be overriding the hypothalamic signaling.

In healthy participants glucose ingestion decreased hypothalamic neuronal activity [8, 23, 24]. We observed a similar decrease in overall hypothalamic neuronal activity after glucose ingestion in the AN patients compared to the healthy controls. The AN patients showed a slightly lower response on average, but this difference was not statistically significant. Within the four hypothalamic subdivisions no significant differences were detected either, indicating that more specific areas in the hypothalamus also showed no significantly different response between groups. The observed glucose responses are in line with previous hypothalamus glucose studies in healthy controls. Vidarsdottir et al. [8] observed signal decreases up to 3%, and Smeets et al. [25] observed decreases up to 2.5%. However, since these studies both used 75 grams of glucose instead of the 50 grams used in the present study, the observed signal changes are slightly higher, as the response of the hypothalamus to glucose is dose-dependent [23]. In addition, the observed decreases in BOLD signal are within the expected measurable range with 3.0 Tesla MRI. At a field strength of 3.0 Tesla, a 3% change in BOLD signal is estimated to be the maximal BOLD effect that can be measured [26]. This indicates that we measured a true effect size, as the mean signal changes were within this 3% range. An earlier study by Holsen et al. assessed the hypothalamic activity in AN patients in response to pictures of high-calorie food objects in a fasted and satiated state [10]. When comparing hypothalamic activity changes between AN patients and the control group this study did not report any significant differences, both in a fasted and a satiated state. On the other hand, when fasted activity changes were analyzed within the different groups, a significant hypothalamic activation was found in the controls, but not in the AN patients. When comparing this study to ours, Holsen et al. assessed cognitive processes, as food pictures trigger neural responses that activate appetite regulating regions including the hypothalamus [27], whereas we assessed the physiological hypothalamic response, as an oral glucose load actually changes blood glucose levels, signaling the glucose sensing neurons in the hypothalamus [28]. Together these findings indicate that in AN direct hypothalamic nutrient sensing is not different but higher-order cognitive processes might indeed be influencing and/or suppressing the hypothalamic signaling.

Our structural findings are in line with the intact function of the hypothalamus in response to energy intake as we did not find an different hypothalamic volume in the AN patients by manual segmentation and voxel based analysis compared to healthy control subjects. However, the VBM analysis did reveal significantly smaller volumes in parts of the cingulate cortex in the AN patients (bilaterally). The cingulate cortex is a region known to be involved in emotion, attention, pain, and memory [29]. This combination of processes gives the cingulate cortex an important role in various psychiatric disorders, such as schizophrenia and major depressive disorder [30, 31]. In a recent systematic review about the neurobiology of AN, it was shown that differences in cingulate cortex structure and function are often observed in AN as well [32]. Using VBM, an earlier study observed decreased grey matter volume in the anterior cingulate cortex in acute AN patients compared to healthy controls, and also in long-term weight-restored AN patients compared to healthy controls [33]. A further study used resting state brain activity to assess intrinsic connectivity within the cerebellar network in AN and bulimia nervosa patients. In AN patients, the cerebellar network was more connected with the posterior cingulate cortex compared to healthy controls, whereas BN patients showed greater connectivity in the anterior cingulate cortex compared to both healthy controls and AN patients [34]. These and many more studies indicate that the cingulate cortex may be involved in the pathophysiology of AN.

A technical limitation of our study is that the hypothalamus is a complex region for automated segmentation due to the inherent lack of contrast on T1-w and T2-w MRI. Therefore, we segmented the hypothalamus manually by using clear anatomic landmarks: anterior commissure, optic chiasm, and mammillary body. These landmarks were used for functional and structural analysis. Possible small variations in size between AN patients and controls are difficult to detect using this manual segmentation, especially on the spatial resolution available with MR imaging. Additionally, the available spatial resolution of the BOLD fMRI also prevents us to further determine which specific nuclei in the hypothalamus respond to the glucose ingestion. Therefore, we cannot conclude that there are no differences in activity between AN patients and controls in all nuclei of the hypothalamus but we can only conclude that the overall neuronal activity in the hypothalamus is unaffected. A further potential limitation of the present study is that it was not designed as a randomized oral water/glucose crossover design. To limit the study load especially for anorexia nervosa patients, we have chosen not to use a water condition as an additional control reference. It should be realized that the intrinsic reference values, or calibration, of our experiments is the BOLD signal before glucose administration (i.e., the BOLD signal in the first 8 min). In the AN patients the anticipation of the glucose ingestion could perhaps influence this baseline BOLD

signal due to increased stress. However, our analysis of the baseline signal between groups shows that the signal level is comparable between patients and controls, indicating that an external water control is not necessary to compare the relative BOLD response between groups. In addition, several experiments in both control subjects and patients with type 2 diabetes have clearly shown that water in itself without glucose does not elicit a BOLD response and would add very little information [8, 9, 23, 24].
A general major limitation and methodological issue in all AN research is the question of cause and effect between the impact of malnutrition on neurobiological factors and pathological eating. Therefore caution is warranted when interpreting research findings in AN patients. A possible strategy to avoid the confounding effects of abnormal nutrition is studying weight-restored AN patients [3]. However, structural differences of the cingulate cortex have also been observed in weight-restored AN patients [33,35], indicating that these findings are not merely a consequence of malnutrition. The fact that we did not observe other structural differences that are often reported, such as the amygdala, hippocampus and overall reduced grey matter volumes, might be explained by our rather heterogeneous patient group regarding AN subtype and duration of disease at the time of participation [36]. Despite this heterogeneity we did observe decreased cingulate cortex volume, indicating that this region plays a key role in the pathophysiology of AN. Our study used a modest sample size of participants, mostly caused by the difficulty of recruiting AN patients willing to participate in the drinking of the glucose solution. Our sample size could be a problem in detecting subtle differences in hypothalamic response between patients and controls. However several other case-control studies using the same fMRI technique for determining hypothalamic function have detected significant differences using similar sample sizes [8,9]. In addition, power calculations for our study showed that each group would need to consist of 23 participants. Despite of our small sample size we do find significant structural differences, further suggesting that the effect size, and possibly the pathophysiological role, of structural differences is much larger than functional differences.

Disclosures

All authors reported no biomedical financial interests or potential conflicts of interest.

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CHAPTER 9

Changes in brain activity after weight loss in obesity

Submitted

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ABSTRACT

Objectives

The regulatory role of the brain in directing glucose homeostasis, energy homeostasis, eating behavior, weight control and obesity is increasingly recognized. Brain activity in (sub)cortical neuronal networks involved in homeostatic cognitive control and hedonic responses is generally increased in obese persons. Currently it is not known whether and which of these functional changes are reversible after dieting. The aim of the current study was to investigate whether prolonged fasting and/or weight loss influences neuronal brain activity in obese persons.

Methods

We included 14 obese participants (2 males, 12 females, BMI 35.2 ± 1.2 kg/m^2) at baseline. Whole brain resting state functional magnetic resonance imaging (fMRI) was performed after an overnight fast (baseline) and after a prolonged 48-h fast in all obese participants. Measurements were repeated after an 8 week weight loss intervention in 10 of the obese participants.

Results

An 8 week weight loss intervention decreased BOLD signal in areas of the brain involved in salience, sensory-motor and executive control. BOLD signal in these areas correlated with leptin levels and BMI. No whole brain voxel-wise changes were observed after the 48-h fast.

Conclusions

An 8 week weight loss intervention decreased activity in brain areas involved in feeding behavior and reward processing, but less in homeostatic regulation of energy balance. Furthermore, our data indicate that these obesity associated alterations in neuronal activity are directly related to excessive bodyweight and might return to more normal level as found in lean subjects after weight loss.

INTRODUCTION

Obesity is an important public health concern [1]. Obesity is marked by a disruption of energy balance leading to increased storage of energy, which can lead to the disruption of a glucose homeostatic and often coincides with disrupted eating behavior [2]. The regulatory role of the brain in directing energy homeostasis, eating behavior, weight control and thus obesity is increasingly recognized [3-5]. Energy homeostasis is centrally regulated by the brain through several interacting neuronal systems. The so-called homeostatic system and the reward and executive control systems are both involved in energy balance [3, 6]. The homeostatic system, consisting, of the hypothalamus and several nuclei in the brainstem, regulates energy intake by combining satiety signals, metabolic and hormonal cues [7]. This is then integrated with signals from the reward system and the executive control system [8]. Hedonic processes however can (completely without awareness) override the homeostatic system and lead to obesity via an excessive energy intake relative to metabolic needs [9, 10].

A large body of work has shown that in obese persons brain function is altered in areas and neuronal networks involved in energy regulation, both the homeostatic and hedonic areas have been shown to be affected [11-15]. Generally, in obese persons brain connectivity and activity in specific (sub)cortical brain areas and neuronal networks is increased compared to lean subjects [11, 14-16]. Furthermore, responses to fasting and food intake have also been shown to be different between obese and lean subjects, predominantly demonstrating an increased activity in brain areas involved in reward systems [11-13, 15]. Therefore, brain alterations in obesity might be involved in an increased energy intake versus the amount of energy needed for a stable body weight [3].

Although these various changes in brain function have been found in obesity in cross sectional studies, it is not known whether and which of these altered brain functions are reversible after significant weight loss. Therefore it remains unknown whether these alterations in brain function are cause or consequence of obesity. The aim of the current study was to investigate whether prolonged fasting and/or weight loss influences ('normalizes') neuronal brain activity in obese participants and if this correlates with alterations in metabolic markers. To distinguish between differences induced by feelings of hunger or weight loss, we examined the effect after prolonged fasting (48h) and after an 8 week diet intervention.

METHODS

Study population and design

For detailed description; methods have also been described in an earlier study by Wijngaarden et al. [15]. We included 14 obese but otherwise healthy (e.g., non-diabetic) participants at baseline. Inclusion criteria were: Caucasian, healthy, weight stable, with a normal fasting plasma glucose (≤5.6 mmol/L). Exclusion criteria were: a positive family history of diabetes type 2, smoking, medication use affecting glucose homeostasis and/or brain function,. The study was conducted in accordance with the Declaration of Helsinki, informed consent was obtained from all participants and the study was approved by the local institutional review board of the Leiden University Medical Center and registered in the Netherlands Trial Register under number NTR2401. Functional magnetic resonance imaging (fMRI) was performed after an overnight fast (baseline) and after a prolonged 48-h fast in all participants. Measurements were repeated after an 8 week weight loss intervention in 10 of participants.

MRI data acquisition

MRI scanning was performed on a Philips Achieva 3.0 T scanner using an 8-channel head coil (Philips Healthcare, Best, The Netherlands). For registration purposes anatomical high-resolution 3D T1- weighted images of the whole brain were acquired (ultra-fast gradient echo acquisition, repetition time (TR) 9.78 ms, echo time (TE) 4.59 ms, flip angle 8, 140 axial slices, FOV 224 mm x 244 mm, reconstructed in-plane resolution 0.875 mm x 0.875 mm, slice thickness 1.2 mm) along with a high-resolution T2*-weighted EPI scan (EPI factor 29, 84 axial slices scanned in ascending order, TR 2200 ms, TE 30 ms, flip angle 80, FOV 220 mm x 220 mm, voxel size 1.96 x 1.96 x 2.0 mm). Resting-state scans were acquired with T2*-weighted gradient echo-planar imaging (EPI factor 29, 160 volumes, 38 axial slices scanned in ascending order, TR 2200 ms, TE 30 ms, flip angle 80, FOV 220 mm x 220mm, isotropic voxel size 2.75 mm with a 0.25 mm slice gap).

MRI data preprocessing

MRI data were preprocessed and analyzed using Functional Magnetic Resonance Imaging of the Brain Software Library (FSL) version 5.0.8. [17]. Of all data sets, structural and functional, non-brain structures were removed using Brain Extraction Tool (BET) tool as implemented in FSL. The T1-weighted images were registered to the 2 mm isotropic MNI-152 standard space image (Montreal Neurological Institute, Montreal, QC, Canada) using non-linear registration with a warp resolution of 10 mm. The FMRI Expert Analysis Tool (FEAT) was used for motion correction with MCFLIRT, spatial

smoothing with a full width at half maximum of 3 mm, and high pass temporal filtering with a cut-off frequency of 0.01 Hz. The functional resting state images were registered to the corresponding T1-weighted images using Boundary-Based Registration (BBR) affine registration, using the high-resolution echo planar images as an additional registration step.

MRI data analysis BOLD changes

Whole brain BOLD signal intensities were compared between study visits according to Rombouts et al. [18]. In short, a single volume BOLD signal map was calculated by averaging the time series data. Average cerebral spinal fluid (CSF) signal of the BOLD image was determined by averaging all voxels within the masked CSF. This CSF mask was determined by selecting voxels located in the lateral ventricles on the segmented structural images. This approach decreases the possibility of including unwanted signal of other compartments than CSF. Next, in each subject, a normalized BOLD signal map was calculated by dividing each voxel's signal by the average CSF signal. Voxel-wise comparisons of normalized BOLD signal maps between study visits were done using the Randomize tool of FSL with a paired samples approach and using Threshold-Free Cluster Enhancement (TFCE) [19]. After voxel-wise comparisons mean BOLD signal was calculated for the clusters showing a significant change in BOLD signal compared to baseline. The statistical clusters were binarized and used as masks/region of interest to calculate mean BOLD signal in these clusters per subject for every study time point.

Statistical analysis of quantitative data

Changes in subject characteristics between study time points were analyzed by repeated measures ANOVA. Associations between average BOLD signal in the extracted clusters and metabolic markers were determined using Pearson's correlation coefficient. Associations were determined for glucose, insulin, leptin, weight and BMI. A $p<0.05$ was considered significant.

RESULTS

Subject characteristics at baseline and after diet intervention

We included 14 obese (2 males, 12 females, BMI 35.2 ± 1.2 kg/m^2) at baseline. Measurements were repeated after a 48-h fast in all participants and after an 8 week diet intervention in 10 of the participants (4 exclusions due to drop-outs and motion artifacts during scanning). Subject characteristics for the study group at the different study time points are shown in table 1.

Table 1. Subject characteristics

	baseline (n=14)	after 48-h fast (n=14)	after diet (n=10)
Age (years)	30 ± 3		
Height (m)	1.74 ± 0.08		
Weight (kg)	107 ± 16	104 ± 4*	95 ± 4*
BMI (kg/m^2)	35.2 ± 4.3	34.4 ± 1.2*	31.5 ± 1.3*
Fasted insulin (mU/L)	19.6 ± 7.9	3.2 ± 3.0*	19.9 ± 8.6
Fasted glucose (mmol/L)	5.0 ± 0.7	4.0 ± 0.6*	4.6 ± 0.7*
Fasted leptin (µg/L)	36.2 ± 13.3	20.6 ± 11.8*	13.7 ± 8*

Values in mean ± standard deviation, *significantly different from baseline at p<0.05 (repeated measure ANOVA)

Weight and BMI decreased significantly after the weight loss intervention with a mean weight loss of 12.7%. Leptin and glucose levels decreased significantly compared to baseline after both the 48-h fast and the weight loss intervention. Insulin levels were significantly decreased after the 48-h fast.

Qualitative and quantitative BOLD signal changes

The 48-h fast intervention did not lead to significant voxel-wise changes. The dietary weight loss intervention resulted in a significant voxel-wise decrease in BOLD signal in parts of the insula, orbito-frontal cortex and in areas of several frontal gyri (figure 1). The quantitative BOLD signal were determined in the clusters showing significant changes after weight loss. The mean quantitative BOLD signals for all three measured time points are shown in figure 2 for the three main clusters containing the OFC, the insula and middle and inferior frontal gyri. The mean BOLD signals in the OFC cluster and the insula clusters were significantly decreased after the diet intervention (p<0.0001) but not show changes in signal after the 48-h fast (p=0.252 and p=0.687 respectively). The mean BOLD signal in the middle and frontal gyri cluster was significantly decreased after weight loss (p<0.0001) but also showed a significant decrease in BOLD signal after the 48-h fast (p=0.043).

Correlation between BOLD activity and metabolic markers

Leptin - At all the three different time points (overnight fast, 48 hour fast and after weight-loss intervention), leptin showed a significant positive correlation with the activity in the orbitofrontal cortex (OFC), insula and frontal gyri. At baseline (overnight fast) the correlation coefficient for the BOLD signal in the OFC was 0.625 (p=0.017), for the insula it was 0.590 (p=0.026) and for the frontal gyri it was 0.745 (p=0.002). After the diet intervention the correlations were similar or stronger; the correlation coefficient

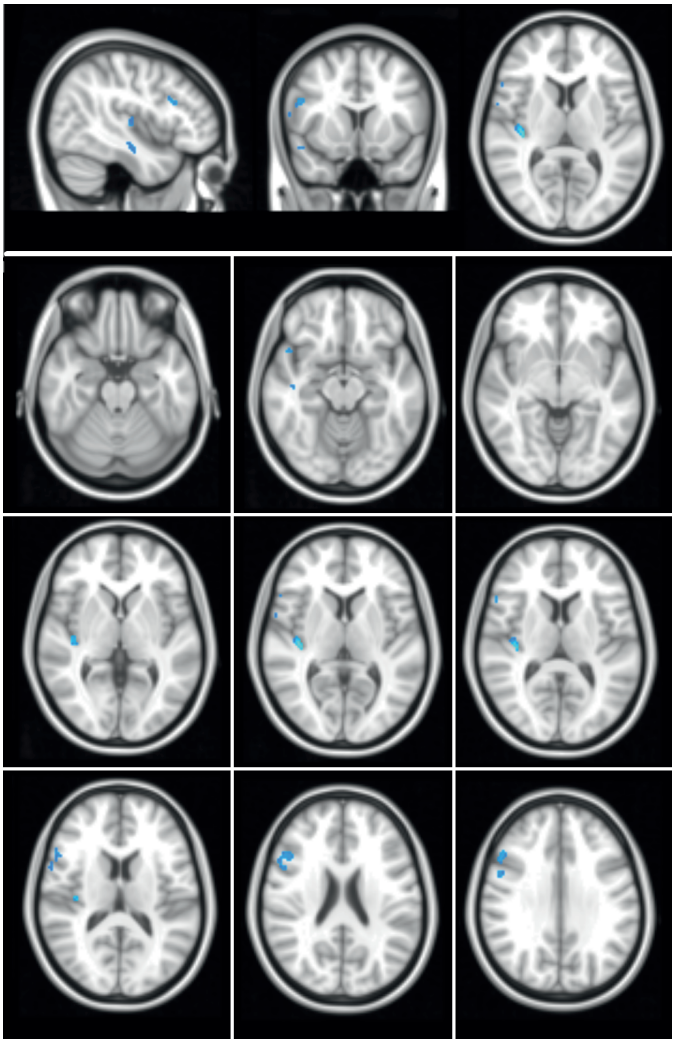

Figure 1. Brain areas with significant changes in BOLD signal/neuronal activity after diet intervention. Decreases in BOLD signal are shown in blue scale (FWE corrected).

for the BOLD signal in the OFC was 0.639 (p=0.047), for the insula it was 0.768 (p=0.009) and for the frontal gyri it was 0.742 (p=0.014).

BMI - BMI showed a significant positive correlation with the activity in the OFC and frontal gyri, but not the insula, at several but not all of the measured study time points. At baseline the correlation coefficient for the BOLD signal in the OFC was 0.433 (p=0.122) and 0.683 (p=0.007) for the frontal gyri. After the diet intervention the

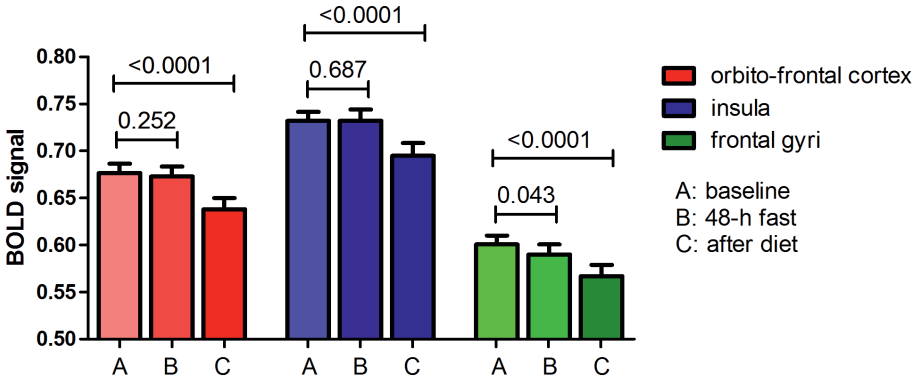

Figure 2. Quantitative BOLD signal in significantly affected clusters per study timepoint. Data depicted as mean with standard error.

correlation coefficient for the BOLD signal in the OFC was 0.684 (p=0.029) and 0.696 (p=0.026) for the frontal gyri. Scatter plots for the correlation at baseline between leptin and BMI with the BOLD signals in the clusters are shown in figure 3. Insulin/glucose - At baseline no association was found between insulin and glucose with the BOLD signal. After the 48-h fast only, insulin levels showed a positive correlation with the activity in all three clusters. The change in leptin, glucose, insulin and BMI after weight loss did not show a correlation with the change BOLD signal in the affected clusters.

DISCUSSION

In this study we compared fMRI data from obese individuals after an overnight fast with a 48-h fast and an 8-week weight loss intervention (again after an overnight fast). Our data show that an 8-week weight loss intervention, but not a 48-h fast, significantly decreased BOLD signal in parts of the insula, orbito-frontal cortex and in areas of several frontal gyri, indicating a decrease in neural activity in these brain areas. Additionally, we found that the neuronal activity in these areas was correlated with leptin and BMI.

The areas of the brain we found showing decreased neuronal activity after weight loss are brain areas that have previously been shown to be affected in obesity [11-15]. Most of these studies show that the activity and/or connectivity in these areas is increased in obesity compared to lean subjects. Therefore it might be that the decreases in activity

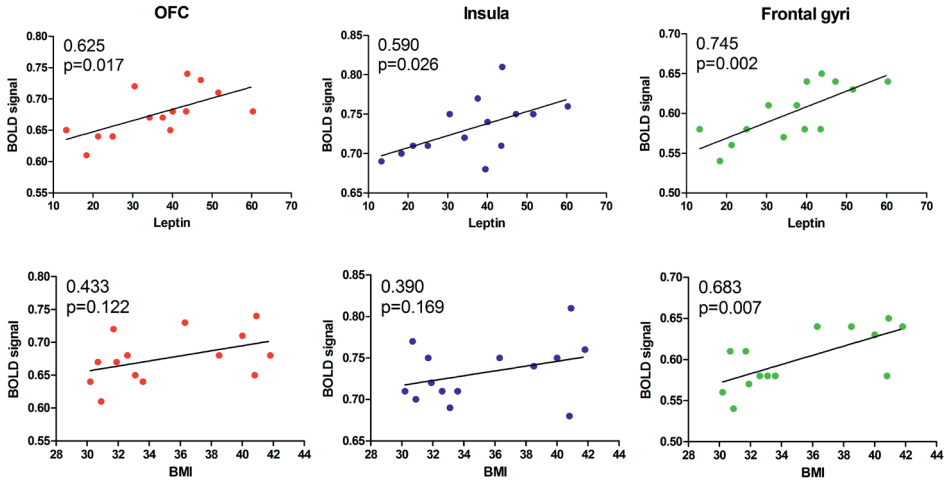

Figure 3. Scatter plots indicating the correlation between leptin and BMI and BOLD signal in the significantly affected clusters. Leptin in µg/L, BMI in kg/m

we found after weight loss in these areas is an indication of normalization of brain activity in these areas. However, one could also speculate that these alterations are the result of weight-loss that might even drive food intake and cause the so-called yo-yo effect. Interestingly, our findings are in line with a recent study showing alterations in the same brain areas (insula and frontal cortex) upon a weight loss intervention [20]. This study did look at changes in functional connectivity instead of BOLD activity, which is a different measure of brain function, but the areas affected by the weight loss intervention are nonetheless very similar. We found no voxel wise changes in BOLD signal after the 48-h fast in the current study. This suggests that the decreases in neuronal activity might not be caused by short term metabolic changes or changes in feelings of hunger and satiety caused by fasting, but are more likely related to actual decreases in body weight. However, in an earlier we study we did find several changes in functional connectivity between the hypothalamus and the insula and anterior cingulate cortex after an 48-h fast in obese participants using a seed-based approach [15]. In the current study, although we did not find whole brain effects of the 48-h fast, when looking at the mean BOLD signal in the affected clusters we did find a significant decrease in the frontal gyri cluster after the 48-h fast. A possible explanation for the results in our current and previous study is that the effects of fasting lead to more subtle changes in brain function, which can be detected with more specific region of interest analysis but do not lead to whole brain effects.

The brain areas showing decreases in activity after weight loss are known to be involved in regulation of energy balance and feeding behavior [6]. As mentioned, both the homeostatic control and hedonic and executive control systems work in concert to regulate energy balance [3, 6]. The areas we find showing a decrease in activity after weight loss are mainly involved in the hedonic and executive control systems and less in the homeostatic system. The insula is involved in determining salience of incoming stimuli [21] and in taste perception [22]. Due to the combination of these functions the insula is therefore involved in palatable feeding. The insula has been shown to have a stronger BOLD response to ingestion of sucrose in obese adolescents compared to lean subjects [23]. Similar to the insula, the orbito-frontal cortex is also involved in determining salience due to its role in the limbic system and executive functioning [24, 25]. The orbito-frontal cortex is important in determining reward and affective value of food via stimuli including taste [26]. Furthermore, the orbito-frontal cortex has been indicated as an important structure in termination of food intake and this function has been shown to be disrupted in obesity [27]. The decreases in neuronal activity in both the insula and orbito-frontal cortex found in our study could indicate a change in reward and salience response after weight loss. Furthermore, the areas we found responding to weight loss showed overlap with brain areas that responded to glucose ingestion in an earlier study by our group [28]. In this study we found the areas that decrease in activity in the insula, thalamus, anterior cingulate gyrus, orbito-frontal cortex, amygdala, hippocampus and the occipital cortex in response to glucose, these areas are involved in hunger and satiety, indicating a satiety response after energy ingestion. This indicates that the decrease neuronal activity in these brain areas might lead to a different satiety response after weight loss.

A main limitation of our study is the study population. The sample size of our study is limited and consist mostly of female participants due to a low response to our advertisement, especially by male participants. Furthermore, it would be an interesting – but probably unethical – experiment to compare the effects of weight loss we found in our obese participants to the effects of weight loss in lean subjects to determine if the effects are caused by loss of excess weight or weight loss in general. Additionally it would be interesting to follow up our participants after the weight loss to determine if the effects on the brain activity are maintained when participants return to a less restrictive diet.

In addition to our finding of significant changes in neuronal activity after weight loss we found that the neuronal activity in the responsive areas was correlated with hormonal and metabolic markers of obesity. Central regulation of energy balance is known to be influenced by peripheral hormonal signal, both in response to energy intake but also in response to long term signals of energy balance such as leptin.[7,

29] Earlier research has shown that various functional brain responses are affected by hormonal signals such as insulin and leptin.[30, 31] Our data show a positive correlation between leptin and BMI and the BOLD signal in the affected brain areas, indicating that high BMI and high leptin levels are associated with a high activity in these brain areas. The change in leptin, glucose, insulin and BMI after weight loss did not show a correlation with the change BOLD signal in the affected clusters, perhaps due to our small sample size and therefore limited statistical power. However, weight loss did lead to decreases in BMI and leptin levels and to decreases in neuronal activity in our study. This further substantiates that the activity in these brain areas might be related to body weight instead of as leptin, insulin and BMI are all correlated with fat mass and body weight.

Taken together, we studied brain activity before and after a weight-loss intervention. We found a decreased activity in brain areas important for feeding behavior and reward processing. Furthermore, the neuronal activity in these brain areas was correlated with metabolic markers of obesity. These results might indicate that alterations in neural activity are reversible, although we did not compare our data with lean individuals in this study. Besides, since it is well known that a lot of obese patients cannot maintain weight-loss for a longer period of time, the exact connotations of these findings still need to be established.

Acknowledgments and conflicts of interest

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CHAPTER 10

Summary, conclusions and future perspectives

The primary aim of this thesis was to gain more insights into the brain function in the maintenance of energy balance by determining the effects of several aspects of our modern day diet and the effects of a disrupted energy balance on functional brain responses. With the goal to further elucidate both normal and disrupted central regulation of energy consumption and feeding behavior to find neurophysiological markers aiding in the management of obesity, metabolic syndrome and eating disorders. Various factors, both internal and external, influence the maintenance of energy balance by the brain. In this thesis we focused on the effects of several of these factors. First we investigated the effects of the ingestion of different types of sugars and sweeteners on the brain. Next we determined the effects of food characteristics that play a role in the palatability of food, namely consumption temperature and flavoring. Furthermore, we determined if the functional brain response to energy ingestion could be influenced by central insulin signaling. Also, we studied which effects changes and disruption of energy balance had on the functional brain responses by investigating brain responses after glucose ingestion in patients with Anorexia Nervosa (AN) and the effects of weight loss in obese participants.
In this chapter the main findings of this thesis are summarized and overall conclusions and future research perspectives following this thesis are discussed.

Summary of findings

In **chapter 2, 3 and 4** we investigated the response of the brain to the ingestion of glucose and various other sugars and sweeteners using several different approaches. In **Chapter 2** we used an assumption free whole brain approach to study the normal response of the brain to the ingestion of glucose in healthy normal weight persons (the effects are summarized in figure 1). The results of this chapter show that after an overnight fast, ingestion of glucose leads to neuronal deactivation and decreased functional network connectivity in brain areas involved in homeostatic and reward responses, which can be associated with satiation and reward effects in the brain and a decrease in energy seeking. However, these rewarding effects could in turn also stimulate and increase future re-consumption of glucose.
As a follow up to **Chapter 2** where we found a response to glucose in homeostatic and reward areas in the brain, in **Chapter 3** we determined the effects of glucose and additional other sugars and sweeteners on the homeostatic and hedonic systems specifically. To this end, instead of investigating the whole brain, we used a region of interest based approach focusing on the hypothalamus and ventral tegmental area (VTA). Our data show that, like in **Chapter 2**, ingestion of glucose elicits a prolonged decrease in hypothalamic activity with a possible transient hedonic response from the VTA, which is associated with satiety signaling. Our data indicate that the hypothalamus

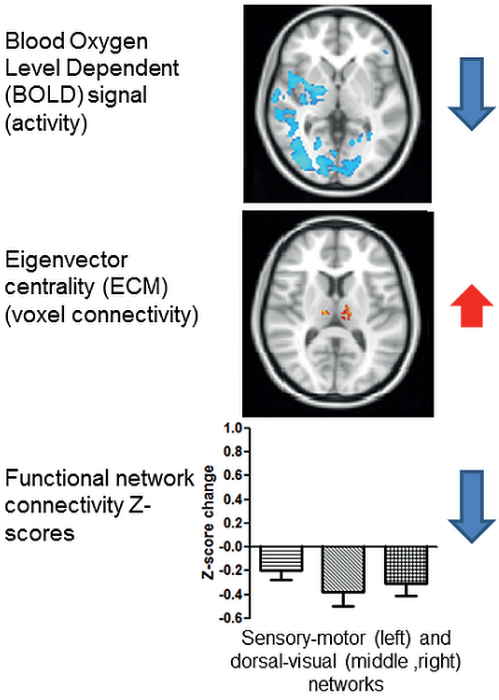

Figure 1. Whole brain functional responses to ingestion of glucose. Glucose dissolved in water leads to various functional changes in activity and connectivity throughout the brain in fasted, normal weight, healthy participants.

and VTA respond directly and readily to glucose but do not respond as efficiently and quickly to sucrose and fructose. Ingestion of the non-caloric artificial sweetener sucralose does not lead to homeostatic satiety signaling. Our findings indicate that the responses by the hypothalamus and VTA are mainly driven by energy content and that sweet taste without the corresponding energy content does not seem to elicit a lasting response from these brain areas.

Because sugars and sweeteners are most often consumed in the context of other nutrients in **Chapter 4** we determined brain responses to ingestion of different sugars and sweeteners consumed in a mixed meal together with other nutrients (fat and proteins). We again found that glucose had widespread effects on neuronal activity and connectivity in the brain and that while fructose also affects brain function, these effects are not as pronounced. The non-/low-nutritive sweeteners sucralose and allulose lead to very little functional brain responses. Similar to our findings in **Chapter 3**, we found that even in the context of other nutrients the sweet taste without the corresponding (carbohydrate) energy content of these non-nutritive sweeteners appears to have much smaller effects on the brain than nutritive sweeteners. The results of this chapter show that even in a mixed meal also containing fat and protein,

the type of sweetener and the carbohydrate energy content can affect brain responses and might thus affect reward and satiety responses and feeding behavior.

In **Chapter 5 and 6** we study the effects of characteristics of food that are related to palatability rather than energy content as these factors could affect energy consumption trough their influence on the homeostatic and hedonic systems. In these chapters we determined the effects of consumption temperature and flavoring on the homeostatic and hedonic responses from the hypothalamus and VTA. Our results in **Chapter 5** show that after intake of both cold water and glucose containing drinks, the neuronal activity in the hypothalamus immediately decreases, whereas the activity increases after intake of water at room temperature. This indicates that consumption temperature can elicit or enhance the homeostatic response from the hypothalamus. In **Chapter 6** we determined the effect of added flavor by using ethyl butyrate (EB), a commonly used food flavoring with a fruity taste, to flavor both a glucose solution and plain water. The results of **Chapter 6** show that in the VTA the combination of glucose with EB flavoring led to a significant decrease in activity, which was threefold stronger than to glucose alone. The addition of EB flavoring to plain water (no caloric content) does not have any effect on the VTA. In the hypothalamus, only the ingestion of glucose, and not EB, was associated with changes in neuronal activity. Our results suggest that the hedonic response by the VTA is strongest to a combination of both flavor and energy and that the homeostatic satiety response of the hypothalamus is mainly driven by energy ingestion.

Deficits in central insulin action in diabetes type 2 are theorized to play a role in defective hypothalamic responses to glucose. Therefore, in **Chapter 7** we determined whether insulin, when centrally applied via the nose, influences the homeostatic response after glucose ingestion. Insulin signaling is an important factor in functional hypothalamic and whole brain response after glucose ingestion [1-4]. We found that centrally administrated insulin augmented the decrease in neuronal activity of the hypothalamus in response to glucose ingestion in normal weight and healthy subjects. This indicates that central insulin can affect the hypothalamic function to maintain glucose homeostasis and that intranasally applied insulin might have the potential to strengthen the defective response that was observed in patients with type 2 diabetes [2, 3].

In **Chapter 8** we determined whether the hypothalamic response to glucose ingestion was disrupted in patients with anorexia nervosa. Like in diabetes type 2 and obesity, energy homeostasis along with various other hypothalamic functions are severely disrupted in patients with anorexia nervosa. However, the results presented in **Chapter 8** shows that the hypothalamic response to glucose ingestion is not different between patients with anorexia nervosa and healthy controls. Despite the fact that the hypothalamic response to energy ingestion is not different these patients still manage

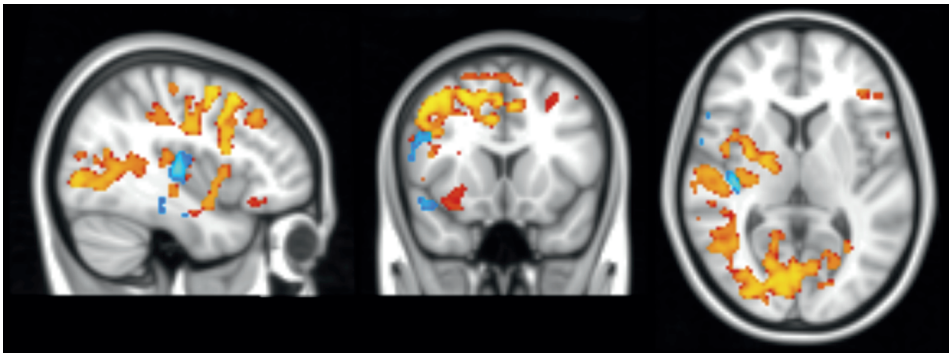

Figure 2. Brain areas showing changes in neuronal activity to both the ingestion of glucose and weight loss. Weight loss in obesity led to decreased activity in brain areas involved in feeding behavior, reward processing, and satiety after weight loss (indicated in blue, results from **Chapter 9**). These areas overlap with areas responding to the ingestion of glucose in normal weight subjects (indicated in orange, results from **Chapter 2**).

to refrain from eating, indicating that patients with anorexia nervosa might be overriding the hypothalamic signaling. Additionally we found that the volume of the cingulate cortex is decreased in anorexia nervosa. The cingulate cortex is a region known to be involved in emotion, attention, pain, and memory [5]. Together, these findings indicate that in anorexia nervosa direct hypothalamic nutrient sensing is not different but that reward and higher-order cognitive processes might be influencing and/or suppressing the hypothalamic signaling.

In **Chapter 9** we determined the effects of weight loss on the (altered) brain function in obesity by studying brain activity before and after a weight-loss intervention. Our data show that an 8-week weight loss intervention significantly decreased neural activity in parts of the insula, orbito-frontal cortex and in areas of several frontal gyri. These brain areas have previously been shown to have an increased activity in obesity [6-10] and are known to be involved in regulation of energy balance and feeding behavior [11]. We found that the neuronal activity in these areas was correlated with leptin, a marker of stored energy in the body. Interestingly, the areas that responded to weight loss, overlap with areas that showed decreased brain activity after glucose ingestion in **Chapter 2** (Figure 2). This suggest that maintaining the functional responses to glucose ingestion found in **Chapter 2** could be important for maintaining energy and glucose homeostasis. Taken together, these results indicate that alterations in neural activity in obesity are involved in glucose and energy homeostasis and that these alterations might be reversible by weight loss.

Overall conclusions, implications and future perspectives

In this thesis we found that different types of sugars and sweeteners elicit different functional brain responses in both the homeostatic and hedonic system. Additionally, we found that the flavoring and consumption temperature of the ingested substances can also influence these responses. Furthermore, we found that centrally administrated insulin can influence the brains response to glucose ingestion. In obese participants, with a disrupted energy balance, we found that weight loss appeared to normalizes the brain activity in various areas know to be affected by obesity. However, in patients with anorexia nervosa we found that the homeostatic response to glucose remained intact despite the disrupted energy balance.

When determining the functional responses to energy ingestion, we consistently found across the different chapters that glucose ingestion elicits the strongest and most widespread functional response from the brain in healthy normal weight subjects. We found that the response to other common sugars and non/low- nutritive sweeteners (both artificial and natural sugar derived sweeteners) is much less apparent or even absent. Furthermore, we demonstrated that the type of sweetener is an important determinant of functional brain responses, even in the context of other nutrients. This thesis shows that the use of glucose as a sweetener instead of other alternatives leads to the strongest satiety response and most immediate effect on feeding behavior. Therefore, when just regarding the brain, glucose appears the most preferable sweetener as the immediate homeostatic and hedonic effects might decrease continued consumption and overconsumption of energy.

When studying the brain function in obesity, where the maintenance of energy balance is disrupted, we found that similar areas respond to changes in body weight that respond to glucose ingestion. Furthermore, we show that brain areas that respond to glucose ingestion can be influenced by metabolic signals such as leptin and insulin, either endogenous or exogenous, that are often disrupted/altered in obesity and diabetes type 2. This, along with results from various other studies [2, 3, 12-14], and our finding that glucose elicits strong responses from the brain, suggests that the functional response to glucose and carbohydrates is important in maintaining energy and glucose homeostasis. Therefore further investigation into these responses should be an area of focus when aiming to find methods to achieve and/or restore normal energy intake and feeding behavior in obesity.

The combined results of the first chapters of this thesis show that energy content of food or drinks appears to be the most important determining factor in the homeostatic brain response and to a lesser extent in the hedonic response. However, we also demonstrated that consumption temperature has an effect on the homeostatic response, whereas flavoring without corresponding energy content does not elicit a lasting homeostatic response. This can be explained by the fact that while flavor in

itself does not have effects on homeostasis, drinking liquids at a low temperature is often deemed as thirst quenching can therefore have effects on the homeostatic regulation of thirst and fluid balance by the hypothalamus [15-17]. Our findings indicate that factors that influence homeostasis, like energy content and temperature, are important in determining hypothalamic responses but factors that do not directly affect homeostasis, like flavoring, might mainly affect the hedonic response.

An important limitation of this thesis is the fact that the functional brain responses have mostly been determined in a very homogenous group of healthy lean young men. Future research should focus on investigating functional brain responses in maintaining energy balance in a more heterogeneous study population with different body weights, fat percentages and feeding habits as these could all affect the brain function and responses [18-21]. Furthermore, investigation of these brain responses in females compared to males is important as females have a different (cerebral) glucose and lipid metabolism compared to males [22, 23]. Differences in eating behavior [24, 25] and taste perception [26] and different levels of inhibitory control over feelings of hunger have been also shown [27]. All of these factors will influence, or are influenced by, the central regulation of energy balance. To extrapolate the results of this thesis to the general population studies in female subjects are therefore necessary.

Overall, this thesis adds further insights into functional brain responses to energy ingestion and in the maintenance of energy balance. We found that glucose has a strong effect on these responses, especially on the homeostatic response, where other sugars and sweeteners in our modern day diet have lesser to very little effects on the brain. And while food characteristics such as temperature and flavor can influence the functional brain responses, both homeostatic and hedonic, the most important driving factor for these response is energy content. Additionally, in this thesis we show that hypothalamic and whole brain activity can be affected by central insulin signaling and weight loss. Still, we find that a disrupted energy balance does not necessarily correlate with an altered functional homeostatic response. Highlighting that even though the homeostatic response of the brain to maintain energy balance is important, the hedonic system and its capability to override the homeostatic system should not be overlooked when investigating feeding behavior. Therefore, future research should focus on what the responses found in this thesis mean in terms of feeding behavior and whether they can be predictive for energy consumption and (maintenance of) energy balance. This is especially important to investigate in subjects with a disrupted energy balance to determine if measurements of functional responses could be used to aid in treatment of this disrupted energy homeostasis. Furthermore, it is important that future investigations into central regulation of energy balance should be done in a more heterogenous group as it is expected that sex and body weight could lead to different responses and function of the brain in the maintenance of energy balance.

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CHAPTER 11

Nederlandse samenvatting

Een goede regulatie van energie balans en voedingsgedrag is cruciaal voor het behoud van de gezondheid. Verschillende veelvoorkomende gezondheidsproblemen in ons huidige samenleving gaan samen met, of worden veroorzaakt door, een combinatie van verstoord voedingsgedrag en een (daardoor) verstoorde energie balans. Naast de welbekende endocrinologische factoren die een rol spelen in de regulatie van voedingsgedrag en de energie balans wordt de rol van de hersenen meer en meer erkend. De hersenen reguleren de energie balans via twee systemen; het homeostatische en het hedonische systeem. Het homeostatische systeem reguleert de energie balans aan de hand van de actuele energie status en de behoefte naar energie van het lichaam en de hersenen. Het hedonische systeem beïnvloedt de energie balans doordat via dit systeem een beloningsreactie op de inname van energie, voedsel en drank ontstaat, wat een stimulans geeft om energie te nuttigen. Deze beloningsreactie kan het homeostatische systeem overstemmen, wat kan leiden tot een energie inname die niet overeenkomt met de actuele homeostatische behoefte naar energie van het lichaam.
Verandering en verstoring van de regulatie van de energie balans door de hersenen wordt gezien als een belangrijke factor in verschillende veel voorkomende ziektes zoals obesitas, diabetes type 2 en verscheidene eetstoornissen. Daarom is het van belang om meer inzicht te krijgen in de hersen functies die de energie balans reguleren en hoe deze functies zijn aangedaan in deze ziektes, maar ook hoe deze processen verlopen in gezonde deelnemers, om hiermee aanknopingspunten te vinden voor de behandeling van een verstoorde energie balans.
Om de hersen functies die verantwoordelijk zijn voor de regulatie van de energie balans te onderzoeken is functionele Magnetic Resonance Imaging (MRI) de aangewezen non-invasieve methode die gebruikt kan worden om deze functies te visualiseren en kwantificeren. Door gebruik te maken van functionele MRI is het mogelijk de neuronale activiteit en ook de neuronale connectiviteit te meten. Deze methodes zijn eerder succesvol gebruikt om hersengebieden te identificeren die een rol spelen in het behoud van de energie balans en om de reactie van deze gebieden op de inname van voedingstoffen te meten.

Het hoofddoel van dit proefschrift is het bestuderen en verder verduidelijken van functionele reacties van de hersenen op de inname van energie en voor het behoud van de energie balans met gebruik van functionele MRI. Dit alles in de hoop dat deze kennis bij kan dragen aan het vinden van neurofysiologische kenmerken en processen die mogelijk gebruikt kunnen worden als aanknooppunten voor de behandeling van obesitas, metabool syndroom en eetstoornissen.

In eerste instantie hebben we in **Hoofdstuk 2** naar de normale functionele reacties van de hersenen op de inname van glucose onderzocht. De resultaten van dit hoofdstuk laten zien dat na een nacht vasten, de inname van glucose leidt tot neuronale deactivatie en een daling in functionele netwerk connectiviteit. Deze resultaten kunnen geassocieerd worden met een verzadigings- en beloningsreactie van de hersenen, een vermindering in de behoefte naar energie en dus verminderde honger. Echter, deze beloningsreactie na de inname van glucose ook een reden zijn om glucose in te nemen. Om meer begrip te krijgen over het verschil in homeostatische en hedonische reacties op de inname van verschillende soorten suikers en zoetstoffen, hebben we in **Hoofdstuk 3** de reactie van de hypothalamus (homeostase) en het ventrale tegmentum (hedonisch) op andere suikers en zoetstoffen naast glucose onderzocht. De bevindingen van deze studie laten zien dat de hypothalamus en het ventrale tegmentum vooral reageren op de energetische waarde van de ingenomen suikers en dat enkel een zoete smaak van een zoetstof, zonder de bijbehorende energie, niet tot een langdurige reactie leidt in deze hersengebieden.

Met uitzondering van frisdrank en andere (met suiker) gezoete dranken worden suikers en zoetstoffen meestal genuttigd in combinatie met andere voedingsstoffen. Om deze reden hebben we in **Hoofdstuk 4** het effect op de hersenen van de inname van suikers en zoetstoffen, gecombineerd met vetten en eiwitten, bestudeerd. Hiervoor hebben we gebruik gemaakt van voeding shakes met vetten en eiwitten die gezoet werden met verschillende suikers en verschillende zoetstoffen zonder of met zeer weinig calorieën. In dit hoofdstuk vonden we opnieuw dat glucose, ook wanneer ingenomen in combinatie met vet en eiwit, het grootste effect heeft de op activiteit en connectiviteit van de hersenen. De shakes gezoet met de kunstmatige zoetstof sucralose, maar ook met de natuurlijke lage calorische zoetstof allulose, hadden zeer weinig tot geen effect op de hersenen. Dit betekent dat, ook wanneer suikers en zoetstoffen ingenomen worden in combinatie met andere voedingsstoffen, het type suiker/zoetstof bepalend lijkt voor de reactie van de hersenen en dus bepalend kan zijn voor de verzadigingsreactie en de regulering van voedingsgedrag.

Verschillende aspecten van voeding zoals smaak, geur, structuur en ook de temperatuur van de voeding/drank bij consumptie zijn belangrijk voor de aantrekkelijkheid van voeding en kunnen daarom het voedingsgedrag beïnvloeden. Om deze reden hebben we het effect van de temperatuur van voeding (**Hoofdstuk 5**) en de smaak (**Hoofdstuk 6**) op de homeostatische en hedonische reacties van de hersenen bestudeerd. De resultaten van **Hoofdstuk 5** laten zien dat, net als de inname van glucose, de inname van gekoeld water ook tot een directe afname van de neuronale activiteit van de hypothalamus. Hieruit blijkt dat ook de temperatuur van een drank/voeding een homeostatische reactie van de hypothalamus kan uitlokken en beïnvloeden. In **Hoofstuk 6** hebben we het effect van toegevoegde smaak bestudeerd. Hiervoor

hebben we de smaakstof ethyl butyraat, een veel gebruikte fruitige smaakstof, toegevoegd aan een glucose oplossing en aan gewoon water. In dit hoofdstuk vonden we dat de combinatie van ethyl butyraat en energie het sterkste effect heeft op de hedonische reactie van het ventrale tegmentum maar dat de homeostatische reactie van de hypothalamus vooral gedreven wordt door ingenomen energie.

De resultaten van **Hoofdstuk 2** tot en met **Hoofdstuk 6** laten zien dat verschillende factoren invloed kunnen hebben op de normale reacties van de hersenen in het behoud van de energie balans in gezonde deelnemers met een normaal gewicht. Zoals eerder genoemd is het bekend dat in verschillende ziektes de energie balans, en dus ook de regulering hiervan door de hersenen, verstoord is. Daarom hebben we in **Hoofdstuk 7, 8 en 9** gefocust op de hersen functie bij een verstoorde (signalering van) energie balans. Verschillende metabole signalen vanuit het lichaam hebben effect op de regulatie van de energie balans door de hypothalamus. Insuline is één van deze signalen die een belangrijke invloed heeft op de reactie van de hersenen op de inname van energie en specifiek op de inname van glucose. Verminderde gevoeligheid van de hersenen voor insuline bij diabetes type 2 wordt gezien als een reden voor de inadequate reactie van de hypothalamus op de inname van glucose bij patiënten met deze ziekte. In **Hoofdstuk 7** hebben we onderzocht of het mogelijk is de reactie van de hypothalamus op glucose te verbeteren door insuline toe te dienen via een neusspray, zodat deze direct in de hersenen kan worden opgenomen. De resultaten van dit hoofdstuk laten zien dat de reactie van de hypothalamus op de inname van glucose inderdaad sterker is als eerst insuline is toegediend. Dit betekent dat insuline toediening aan de hersenen via een neusspray mogelijk gebruikt zou kunnen worden om de inadequate homeostatische reactie van de hypothalamus bij diabetes type 2, en mogelijk ook bij obesitas, te verbeteren.

Bij patiënten met anorexia nervosa zijn de energie balans en het eetgedrag sterk verstoord en zijn tevens verscheidene andere functies van de hypothalamus aangetast. Daarom verwachten we dat de reactie van hypothalamus op energie inname mogelijk ook verstoord is bij deze patiënten. Om dit te bestuderen hebben we in **Hoofdstuk 8** de reactie van de hypothalamus van patiënten met anorexia nervosa op inname van glucose gemeten. Hoewel de hypothalamus niet normaal functioneert bij anorexia nervosa vonden wij geen verschil in de reactie van de hypothalamus op de inname van glucose in deze patiënten ten opzichte van gezonde deelnemers. Dit duidt erop dat, ondanks de sterk verstoorde energie balans, de reactie van de hypothalamus op de inname van energie normaal is. Dit zou er op kunnen wijzen dat hogere cognitieve functies en het hedonische systeem de normale signalering van de hypothalamus onderdrukken en/of negeren.

Obesitas wordt, net als anorexia nervosa, ook gekarakteriseerd door een sterk verstoorde energie balans en verstoord eetgedrag, echter leidt dit bij obesitas juist tot

een overtollige energie opslag. In de hersenen van personen met obesitas wordt een afwijkende functie van verschillende hersengebieden gevonden die betrokken zijn bij de regulering van energie balans en eetgedrag. De afwijkende functie manifesteert zich vaak als een verhoogde neuronale activiteit in deze hersengebieden. Het is echter onbekend of deze afwijkingen een oorzaak of gevolg zijn van de overtollige energie opslag en toegenomen lichaamsgewicht. In **Hoofdstuk 9** hebben we onderzocht of gewichtsverlies de hersen activiteit in deelnemers met obesitas kan veranderen en of deze veranderingen overeenkomen met veranderingen in (metabole) kenmerken van obesitas zoals BMI, vetpercentage en leptine niveaus. De resultaten van dit hoofdstuk laten zien dat na een 8 weken durend dieet verschillende hersengebieden betrokken bij het behoud van de energie balans een verlaging in activiteit vertonen. Tevens gaat deze daling in activiteit samen met een verlaging van de metabole kenmerken van obesitas. Dit duidt erop dat de veranderingen in activiteit van de hersenen die gevonden worden bij patiënten met obesitas mogelijk secundair zijn aan het overtollige gewicht. De gebieden waar wij een verandering in activiteit vonden na gewichtsverlies kwamen gedeeltelijk overeen met gebieden die een reactie vertonen op de inname van glucose in **Hoofdstuk 2**. Dit wijst erop dat de functionele reacties op de inname van glucose mogelijk ook belangrijk zijn in obesitas en dus waarschijnlijk belangrijk zijn voor het behoud van de energie balans.

Wanneer we alle resultaten van de hoofdstukken samen nemen heeft dit proefschrift bijgedragen aan een beter inzicht in de functionele hersen reacties die een rol spelen in het behoud van de energie balans. Het is duidelijk dat voornamelijk de inname van glucose een sterk effect heeft op de hersen functie, met name op de homeostatische reacties. Andere suikers en zoetstoffen die vaak genuttigd worden binnen ons moderne dieet hebben daarentegen veel kleinere tot geen effecten. Ook vonden we dat de temperatuur en de smaak van de ingenomen drank/voeding een effect kunnen hebben op de functionele reacties van de hersenen. Echter, de energetische waarde van de ingenomen stoffen is de grootste drijvende factor voor deze reacties. Naast deze bevindingen hebben we aangetoond dat de hersen activiteit kan worden beïnvloed door insuline signalering en door gewichtsverlies. Daarentegen vinden we ook dat een verstoorde energie balans niet per se samengaat met een verstoorde homeostatische reactie van de hersenen. Dit wijst erop dat, hoewel de homeostatische regulatie van de energie balans erg belangrijk is, de hedonische regulatie niet over het hoofd moet worden gezien in studies naar energie balans en eetgedrag, met name bij onderzoek in populaties met een verstoorde energie balans. Daarnaast is het belangrijk dat in toekomstige studies naar het behoud van energie balans gebruik wordt gemaakt van een heterogene studie populatie omdat verschillende populatie karakteristieken invloed zouden kunnen hebben op de in dit proefschrift bestudeerde functionele hersenen reacties.

APPENDIX

List of publications
Dankwoord
Curriculum vitae

LIST OF PUBLICATIONS

2012

1. CA Wijsman*, **AM van Opstal***, HE Kan , AB Maier, RG Westendorp, PE Slagboom, AG Webb, SP Mooijaart, D van Heemst. *Both authors contributed equally. Proton magnetic resonance spectroscopy shows lower intramyocellular lipid accumulation in middle-aged subjects predisposed to familial longevity. *Am J Physiol Endocrinol Metab. 2012 Feb;302(3):E344-8.*
2. M van Putten, D Kumar, M Hulsker, WM Hoogaars, JJ Plomp, **AM van Opstal**, M van Iterson, P Admiraal, GJ van Ommen, PA 't Hoen, A Aartsma-Rus. Comparison of skeletal muscle pathology and motor function of dystrophin and utrophin deficient mouse strains*. Neuromuscul Disord. 2012 May;22(5):406-17. doi: 10.1016/j.nmd.2011.10.011.*

2014

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2015

4. **AM van Opstal***, AM Westerink*, Teeuwisse WM, van der Geest MA, van Furth EF, van der Grond J. *Both authors contributed equally. Hypothalamic BOLD response to glucose intake and hypothalamic volume are similar in anorexia nervosa and healthy control subjects. *Front Neurosci. 2015 May 5;9:159. doi: 10.3389/fnins.2015.00159.*

2016

5. P Fotiadis*, S van Rooden*, J van der Grond, A Schultz, S Martinez-Ramirez, E Auriel, Y Reijmer, **AM van Opstal**, A Ayres, KM Schwab; Alzheimer's Disease Neuroimaging Initiative T, Hedden, J Rosand, A Viswanathan, M Wermer, G Terwindt, RA Sperling, JR Polimeni, KA Johnson, MA van Buchem, SM Greenberg, ME Gurol. *Both authors contributed equally. Cortical atrophy in patients with cerebral amyloid angiopathy: a case-control study. *Lancet Neurol. 2016 Jul;15(8):811-819. doi: 10.1016/S1474-4422(16)30030-8*.
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2017

7. **AM van Opstal**, S van Rooden, T van Harten, E Ghariq, G Labadie, P Fotiadis, ME Gurol, GM Terwindt, MJ Wermer, MA van Buchem, SM Greenberg, J van der Grond. Cerebrovascular function in presymptomatic and symptomatic individuals with hereditary cerebral amyloid angiopathy: a case-control study. *Lancet Neurol. 2017 Feb;16(2):115-122. doi: 10.1016/S1474-4422(16)30346-5.*

8. AA Akintola*, **AM van Opstal***, RG Westendorp, I Postmus, J van der Grond, D van Heemst. *Both authors contributed equally. Effect of intranasally administered insulin on cerebral blood flow and perfusion; a randomized experiment in young and older adults. *Aging. 2017 Mar 14;9(3):790-802. doi: 10.18632/aging.101192.*

9. ES van Etten, MM Verbeek, J van der Grond, R Zielman, S van Rooden, EW van Zwet, **AM van Opstal**, J Haan, SM Greenberg, MA van Buchem, MJ Wermer, GM Terwindt. β-Amyloid in CSF: Biomarker for preclinical cerebral amyloid angiopathy. *Neurology. 2017 Jan 10;88(2):169-176. doi: 10.1212/WNL.0000000000003486.*

10. **AM van Opstal***, AA Akintola*, M van der Elst, RG Westendorp, H Pijl, D van Heemst, J van der Grond. *Both authors contributed equally. Effects of intranasal insulin application on the hypothalamic BOLD response to glucose ingestion. *Scientific Reports 7, Article number: 13327 (2017) doi:10.1038/s41598-017-13818-x.*

2018

11. **AM van Opstal**, AA van den Berg-Huysmans, M Hoeksma, C Blonk, H Pijl, SARB Rombouts, J van der Grond. The effect of consumption temperature on the homeostatic and hedonic responses to glucose ingestion in the hypothalamus and the reward system. *Am J Clin Nutr. 2018 Jan 1;107(1):20-25. doi: 10.1093/ajcn/nqx023.*

12. **AM van Opstal**, A Hafkemeijer, AA van den Berg-Huysmans, M Hoeksma, C Blonk, H Pijl, SARB Rombouts, J van der Grond. Brain activity and connectivity changes in response to glucose ingestion. *Nutr Neurosci. 2018 May 27:1-8. doi: 10.1080/1028415X.2018.1477538.*

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17. **AM van Opstal**, AA van den Berg-Huysmans, M Hoeksma, C Blonk, H Pijl, SARB Rombouts , J. van der Grond. Effect of flavor on homeostatic and reward responses of the hypothalamus and ventral tegmental area. *Submitted.*

18. **AM van Opstal**, MA Wijngaarden, J van der Grond, H Pijl. Changes in brain activity after weight loss in obesity. *Submitted.*

19. **AM van Opstal**, A Hafkemeijer, AA van den Berg-Huysmans, M. Hoeksma, TPJ Mulder, H Pijl, SARB Rombouts, J van der Grond. Brain activity and connectivity changes in response to nutritive natural sugars, non-nutritive natural sugar replacements and artificial sweeteners. *Submitted.*

20. J van der Grond, MM Redelijkheid, **AM van Opstal**, G Labadie, J Barkey-Wolf, GM Terwindt, MJH Wermer, AA van den Berg-Huysmans, MA van Buchem, SM Greenberg, S van Rooden. Vascular reactivity as biomarker for hereditary cerebral amyloid angiopathy: reproducibility and progression. *Submitted.*

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CURRICULUM VITAE

Anna Maria "Annemarieke" van Opstal was born on the 15th of March 1988 and grew up in Zevenbergen, the Netherlands. After graduating from the Norbertus College in Roosendaal in 2006, she studied Bio-medical Sciences at the Leiden University Medical Center. After receiving her bachelor's degree she continued with a research master in Bio-medical Sciences. As part of her graduation, Annemarieke assisted in a neuroimaging study on amyloid deposition in hereditary cerebral amyloid angiopathy patients under the supervision of Dr. Sanneke van Rooden and Dr. Jeroen van der Grond.

After finishing her graduation in November 2012, Annemarieke started her PhD research at the Leiden University Medical Center at the department of Radiology under supervision of Dr. Jeroen van der Grond and Professor Serge Rombouts. In her PhD project, Annemarieke performed several industry initiated studies financed by Unilever Research & Development. These functional MRI projects focused on the effects of sugars and sweeteners and food characteristics on the brain. Alongside these industry initiated studies Annemarieke performed several other studies in collaboration with other departments of the Leiden University Medical Center including the EU funded Switchbox project with the department of Geriatrics. And additionally completed a study on functional brain responses in Anorexia Nervosa in collaboration with GGZ Rivierduinen.

Her findings resulted in more insights into functional brain responses that are important in the maintenance of energy balance as described in this thesis and give grounds for further investigations into these brain functions to determine neurophysiological markers that could aid in the management of obesity and eating disorders.

Currently, Annemarieke is continuing her work as a post-doc at the department of Radiology at the Leiden University Medical Center.