Central serotonin and dopamine transporters in overeating, obesity and insulin resistance

Koopman, K.E.M.

Publication date
2014

Document Version
Final published version

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Central Serotonin and Dopamine Transporters
in Overeating, Obesity and Insulin Resistance

Karin E.M. Koopman
CENTRAL SEROTONIN AND DOPAMINE TRANSPORTERS
IN OVEREATING, OBESITY AND INSULIN RESISTANCE

ACADEMISCH PROEFSCHRIFT

ter verkrijking van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college voor
promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op vrijdag 7 november 2014, te 14:00 uur

door
Karin Eva Maria Koopman
geboren te Oldenzaal

Copyright © 2014 K.E.M. Koopman, Amsterdam. All rights reserved. No part of this publication may be reproduced or transmitted in any form by any means, without written permission of the author.

Financial support for the printing of this thesis was provided by: Goodlife Healthcare, Chipsoft B.V., Hospira Benelux, Boehringer Ingelheim, Ipsen Farmaceutica B.V and the Academic Medical Center Amsterdam.
Chapter 1 General introduction.

PART I: Brain SERT and DAT in lean and obese humans.

Chapter 2 Assessing the optimal time point for the measurement of extrastriatal serotonin transporter binding with 123I-FP-CIT SPECT in healthy, male subjects.

Chapter 3 Decreased serotonin transporter immunoreactivity in the hypothalamic infundibular nucleus of overweight subjects.

Chapter 4 Diencephalic serotonin transporter binding, but not striatal dopamine transporter binding, is correlated to insulin sensitivity in obese women.

Chapter 5 Brain dopamine and serotonin transporter binding correlates with visual attention for food in lean men.

PART II: Neural and metabolic effects of hypercaloric diets in lean humans.

Chapter 6 Diet-induced changes in the lean brain: hypercaloric high-fat-high-sugar snacking reduces serotonin transporters in the hypothalamic region in lean men.

Chapter 7 Hypercaloric diets with increased meal frequency, but not meal size, increase intrahepatic triglycerides: a randomized controlled trial.

Chapter 8 A 6-week hypercaloric diet modulates sympathetic activity in proportion to a change in striatal DAT binding.

Chapter 9 General discussion.

Chapter 10 Appendix

Nederlandse samenvatting 162
Author Affiliations 166
Dankwoord 168
PhD Portfolio 172
About the author 174
General introduction
I. GENERAL INTRODUCTION

THE OBESITY EPIDEMIC

Obesity is defined by the World Health Organisation (WHO) as a BMI > 30 kg/m² and is a worldwide problem of pandemic proportions (www.who.int Fact sheet N°311, May 2014). In the USA one third of the population is obese [1,2] while in the Netherlands 31.5 % of the adult population is overweight (BMI ≥ 25 kg/m² and < 30 kg/m²) and 10.1% obese (statline.cbs.nl). Obesity increases the risk for the development of the metabolic syndrome [3] and is associated with co-morbidities, such as cardiovascular disease, type 2 diabetes mellitus (T2DM), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) and a number of malignancies (4-6). The health care costs related to obesity in the USA have recently been estimated at $147 billion per year, which is almost 10% of the National Health Care budget (7) while in the Netherlands estimated expenses on obesity-related health care approximated 505 million Euros yearly in 2002 (8) highlighting the societal relevance of obesity as a disease. Indeed, obesity has been recognized as a disease by the Obesity Society Council (9). Obesity-related costs are expected to rise with 10-20% in the next two decades, while a reduction in average BMI in the population could result in large financial benefits (7).

The question on ‘why do we become obese’ has engaged many researchers. Excess body fat is accumulated when energy intake (i.e. food) exceeds energy expenditure, and therefore excessive food intake and lack of physical exercise are considered major causes of obesity. Indeed, recent data from the WHO show that the prevalence of adults with insufficient physical activity is increasing in many countries around the world (www.who.int Fact sheet N°385, February 2014). The rise in the prevalence of obesity occurred simultaneously with the increase in availability and intake of high caloric palatable food rich in sugars and fat (10). Not only is excessive consumption of this type of food associated with obesity, but also with the development of fatty liver (11), abdominal obesity (12) and T2DM (13). Besides excessive fat and sugar consumption, meal pattern has changed over the last decades showing a trend for increasing meal frequency (i.e. snacking behaviour). American children consume up to 27% of their caloric intake from snacks (14) and snacking has been suggested to contribute to obesity as well as to its metabolic consequences (15-17). Thus, a reduction in physical activity and an increase in calorie dense food and snacking behaviour all result in weight gain. The key question is why individuals do not reduce caloric intake to match their caloric need.

Although educational level is inversely related to BMI (18;19), most adults are aware of the unhealthy aspects of foods rich in sugars and fat and of the fact that obesity increases the risk for diseases such as T2DM. Interestingly, in spite of this general awareness, the prevalence of obesity reaches epidemic proportions, thus showing that at least part of the population is not capable of regulating their own energy balance. Energy balance regulation is for a significant part under autonomic control and is not merely a conscious process. Although it is a semi-voluntary decision to eat or to exercise, feelings of hunger, satiety and motivation to eat are complex processes that cannot be influenced by decision-making. The regulation of hunger and satiety as well as ‘liking’ and ‘wanting’ of food is orchestrated by the brain and it is a generally accepted view that dysregulation of brain systems involved in regulation of food intake contributes to obesity (20). Homeostatic as well as rewarding components of feeding result in complex behavioural adaptations in response to actual caloric need. Apparently these adaptations seem to fail in at least one third of the adult population living in the Western world. This might be explained by either dysfunctional homeostatic control mainly regulated by the hypothalamus or by dysfunctional reward control mainly orchestrated in corticolimbic areas, or a combination of both. Because overall feeding behaviour results from a partially elucidated complex interplay between these major pathways, in depth studies are needed to disentangle the perturbations occurring in individuals that overeat. In humans, parts of these pathways can be studied by combining neuroimaging techniques and questionnaires on feeding behaviour before and after an intervention, either intra-individually or between groups. Subsequent unravelling of the molecular mechanisms is mainly based on data from studies in rodents. Interestingly, evidence exists for a role of the brain in the metabolic consequences of obesity, such as insulin resistance and dyslipidaemia (21). Brain areas involved in ho-
meostatic control of energy balance, and brain circuits involved in reward were recently shown involved in glucose and lipid metabolism [21-23]. Most data so far are from studies in rodents while translation to humans is urgently needed to understand the mechanisms with the ultimate aim to combat obesity and its metabolic consequences. This thesis describes results of studies in humans and focuses on serotonin transporters in the hypothalamic region and on dopamine transporters in the striatum, which are both known to play a central role in regulating energy balance.

NEUROBIOLOGICAL REGULATION OF FOOD INTAKE

To control food intake and energy balance, the brain receives and integrates peripheral signals from different organs (figure 1) involved in energy metabolism. This integration involves a complex network of nuclei within brainstem, hypothalamus and mesocorticolimbic areas. The hypothalamus serves as the homeostatic control centre regulating energy metabolism and it consists of many nuclei with different functions. Concerning the regulation of food intake the hypothalamic arcuate nucleus (ARC), also referred to as the infundibular nucleus (IFN) in humans, plays a prominent role. The blood-brain-barrier in the ARC is considered ‘leaky’ and therefore hormones can easily enter and activate ARC neurons to regulate energy metabolism (for review: [24]). Within the ARC, orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons when activated stimulate food intake, whereas anorexigenic proopiomelanocortin (POMC)/cocaine amphetamine related transcript (CART) neurons when activated inhibit food intake. ARC neurons project to several hypothalamic nuclei such as the ventromedial hypothalamic (VMH), dorsomedial hypothalamic (DMH) and lateral hypothalamic (LH) nuclei, all nuclei involved in maintaining energy homeostasis. In addition, the ARC also projects to the paraventricular nucleus (PVN), a nucleus centrally located within the hypothalamus and considered a control centre for integration of a variety of signals from within and outside the hypothalamus to regulate anterior pituitary function, energy expenditure and food intake [25]. The PVN additionally integrates information from gut signals via vagal afferents and the nucleus of the solitary tract (NTS) in the brainstem [26] [27], thereby contributing to eating behavior.

Hypothalamic areas such as the PVN and ARC receive dense projections from serotoninergic neurons originating in the dorsal raphe nuclei. The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) acts as a modulatory neurotransmitter to coordinate numerous cognitive, autonomic, and other functions to maintain homeostasis and therefore plays an important role in the regulation of energy balance [28]. Hypothalamic serotonin administration potently reduces food intake, and it has been shown that the integrity of the hypothalamus is required for normal responsiveness to serotonergic drugs [for review [29]]. Although the initial focus was on the role of the PVN in serotonin’s effects on feeding behavior [29], more recently serotonin’s involvement in the ARC has become evident (figure 2). 5HT fibers contact NPY neurons in the ARC [30] and a 5HT antagonist has been shown to increase the activity of NPY neurons in the ARC-PVN projection [31], however it is yet unknown whether this involves direct effects of serotonin on NPY neurons in the ARC. For anorexigenic neurons, however, it was shown that the serotonin reuptake inhibitor d-FEN increases the spontaneous firing rate of POMC neurons in the ARC which also express the 5HT2c receptors, while increasing the release of α-melanocyte stimulating hormone (MSH; cleavage product of POMC) thus providing a mechanism via which direct action of serotonin in the ARC inhibits food intake [32].

In human studies it has been shown that drugs that increase whole-body serotonergic signalling, such as the serotonin 2C receptor agonist lorcaserin and fenfluramine, exert hypophagic effects suggesting that, in line with the studies in rodents, serotonin plays a similar role in the regulation of food intake in humans [33-35]. In addition, subcortical serotonin transporter binding is negatively related to BMI in humans [36].

Taken together, the hypothalamus integrates incoming information on feeding status and energy stores via a complex network of orexigenic and anorexigenic cell populations and the neurotransmitter serotonin is involved in this complex regulatory system. Manipulating several intermediates of this network changes certain aspects of feeding behavior. It should be emphasized that most of the data on brain circuits involved in feeding behaviour are derived from studies in rodents, because the human brain is not easily accessible for in vivo studies. Interestingly, in contrast to the aforementioned neuropeptides in the ARC, the serotonin system can be visualized in vivo in the human brain using techniques like single photon emission computed tomography (SPECT), using a radioligand that binds to the serotonin transporter (SERT) providing a method to study the role of brain serotonin in human obesity.
Food intake is not only influenced by homeostatic control, but also by the rewarding properties of food (figure 3), either through the internal drive to eat, learned associations with food or the hedonic properties of food itself (37). Brain areas involved in reward range from the midbrain (ventral tegmental area; VTA) to limbic structures (ventral striatum, amygdala, hippocampus, and parts of the [prefrontal cortex] and are part of the mesocorticollimbic pathway or ‘reward pathway’ (38). Reward is often described to have two distinguishable components, namely wanting and liking, which refers to food-motivation and hedonic aspects, respectively. It has been postulated that separate neural substrates are involved in these two components, i.e. dopamine in wanting and opioids in liking (39). Within the striatal nucleus accumbens (NAc), parallel interconnecting circuits exist, involving these two substrates. Although dopamine was considered traditionally as the master regulator of hedonism, it is now believed that the ‘liking’ of food, or the hedonic sensation of pleasure when eating something, involves the striatal opioid system (e.g. enkephalins, β-endorphins) (40). In contrast, the ‘wanting’ of food, also called the motivation to obtain food or ‘incentive salience’ is attributed specifically to the striatal dopamine system (41;42). Food in itself and food-related cues (smell, sight) induce a striatal dopamine release which triggers the ‘wanting’ or pursuit of food (43). However, studies have also shown a relationship between striatal dopamine levels and meal pleasantness ratings in humans (44), suggesting that dopamine might play a role in liking as well, and that ‘liking’ and ‘wanting’ systems may not be completely separable.

Finally, the hypothalamic and mesocorticollimbic pathways are highly integrated to guarantee adequate feeding behavior in response to caloric needs. This is reflected by direct and indirect anatomical connections between the hypothalamus and the striatum, and between the hypothalamus and the VTA (38;40) (Figure 3). NPY infusion in the lateral hypothalamus, nucleus accumbens as well as in the VTA results in altered motivational behavior (45;46) and striatal opioid injections induce palatable feeding which can be blocked by inhibiting the activity of the lateral hypothalamus (47;48), demonstrating the involvement of all these brain areas and the functionality of connections between these structures to regulate feeding behavior.

**SEROTONERGIC – AND DOPAMINERGIC SIGNAL TRANSMISSION**

As stated, serotonin and dopamine are modulatory neurotransmitters in the integrated network of hypothalamic and mesocorticollimbic pathways that regulate energy balance. Serotonin is synthesized from tryptophan in the brainstem raphe nuclei and dopamine (DA) from phenylalanine in the midbrain VTA and substantia nigra. Ascending serotonergic fibers assemble in the medial forebrain bundle and project to a wide variety of brain regions: cortex, hippocampus, thalamus, hypothalamus, striatum, amygdala and nucleus accumbens (49). The VTA dopaminergic projections to the nucleus accumbens (NAc), medial prefrontal cortex, hippocampus and amygdala are mainly involved in the regulation of reward/motivation (50) whereas projections from the substantia nigra to the striatum, globus pallidus, and subthalamic nucleus play important roles in motor control (for review see: (51)).

From their production sites, the neurotransmitters are transported to presynaptic neurons where they are stored in vesicles and released in the synaptic cleft upon activation of the specific neuron with subsequent binding to the respective receptors. There are 5 dopamine receptor subtypes in mammals (D1R – D5R) (52) and 18 serotonin receptor subtypes of which several receptor subtypes have been implicated to play a role in the regulation of energy balance (28). Extracellular serotonin and dopamine concentrations are regulated by the serotonin (SERT) and dopamine (DAT) transporter respectively, that reside on the presynaptic cell surface (Figure 4). These transporters facilitate the re-uptake of unbound serotonin or dopamine into the presynaptic neuron for

---

The striatal dopamine system is inextricably linked to motivation and reward, although it should be emphasized that most work has been done in rodents. Obese subjects show increased motivation for food (68-70), which implies a different striatal response in obese subjects compared to lean controls. Two different theories on how the striatal dopamine system might lead to increased motivation for food and obesity have been developed. The ‘reward deficiency theory’ proposes that obese subjects are in a hypodopaminergic state, i.e. obese subjects experience less reward from food leading to a compensatory increase in food intake and eventually obesity (71). Indeed, when food is consumed or consumption is imagined, obese humans show less striatal activation compared to lean individuals (72,73), which is in line with decreased striatal dopaminergic D2/3 receptors (74-76) and a reduced amphetamine induced dopamine release in obese humans (76). In addition, individuals with a short Taq1a allele, which is associated with attenuated striatal dopamine signalling, are more susceptible to become obese (72,77) indicating that reduced dopamine signalling might contribute to developing obesity. Contrarily, the “reward-surfeit model” of obesity proposes hyperresponsiveness of the reward circuitry leading to overeating and subsequently obesity (78). Indeed, fMRI studies have shown that obese women show greater responses in gustatory and oral somatosensory regions as well as in the striatum compared to lean individuals when expecting and consuming food (79-81). Moreover, lean adolescents at risk for obesity by carrying the Taq 1 allele (i.e. compromised striatal dopamine signalling) showed increased caudate response to food (82). So far, most studies indicate that the assumed reward deficiency in obesity is a consequence of overeating or obesity rather than a cause. This is in line with rodent studies showing that eating high fat diets or diets with an increased fat/carbohydrate ratio decreases striatal D2/3 receptor availability in the absence of obesity (83,84). Knockdown of striatal D2 receptors rapidly accelerates the development of addiction-like reward deficits and the onset of compulsive-like food seeking in rats (86) indicating that decreased striatal D2/3 receptor levels might in turn contribute to overeating. As there are currently no studies available on the effect of overeating on the striatal dopamine system in humans, it remains speculative whether these rodent data can be translated to the human situation.

As previously stated, DATs are important regulators of the available amount of extracellular dopamine (53) and therefore potentially contribute to a hypo- or hyperdopaminergic striatum in obesity. At present it is unknown whether extracellular dopamine concentrations in food intake regulatory pathways are increased or decreased in humans. DAT levels could reflect available extracellular dopamine as long term systemic DA depletion results in reduced striatal DAT binding (87). However mice with a 90% reduction in DAT have have increased extracellular DA concentrations in the striatum and hypothalamus (88,89), indicating that downregulation of DATs could indeed contribute to high extracellular dopamine levels in the striatum. Interestingly, these mice show increased motivation to work for food (89). Similarly, diet-induced obese mice have lower DAT, lower D2/3 receptors and higher extracellular dopamine levels and show higher motivation for food (90). Since obese humans also have higher motivation for food (68-70) as well as re-

Figure 4
Neurotransmission.
duced D2/3 receptors [74-76], one would expect lower DATs in human obesity as well. Human studies indeed showed an inverse association between striatal DAT binding and BMI (90;91), although this finding was not reproduced by others (92;93).

Summarizing, most data indicate that reduced serotonergic signalling and low SERT availability are associated with hyperphagia and obesity. Furthermore, obese subjects have increased motivation for food and decreased D2/3 receptor binding and they might have lower DAT binding. It is unknown whether in humans, like in rodents, dopamine signalling is related to motivation for food, whether it is affected by food overconsumption and whether changes in the central dopamine system in human obesity are related to altered motivation for food.

Dietary Habits and Obesity

Obesity results from either a reduction in energy expenditure and/or an increase in caloric intake. Total caloric intake has increased over the last decades and surveys and studies on dietary habits report an increased intake of highly palatable fat and sugary food [10]. Caloric excess itself increases body weight and reduces insulin sensitivity even in healthy, non-obese individuals [94]. In addition, both increased fat and increased sugar consumption have been associated with weight gain and insulin resistance in humans [95-99]. Interestingly, rodent studies from our research group showed that a high fat high sugar diet, but not sugar or fat alone, resulted in an obese phenotype and insulin resistance even in healthy, non-obese individuals (94). In addition, both increased fat and increased sugar consumption have been associated with weight gains and insulin resistance in humans (95-99).

Obesity and hypercaloric feeding affect peripheral glucose metabolism by modulating insulin action also referred to as insulin sensitivity. This is a direct effect of the molecular consequences occurring in insulin sensitive tissues in the obese and hypercaloric state but might also be caused through changes in brain circuits known to regulate glucose metabolism. Glucose is a major fuel for many organs and in the fasting state, the majority of circulating glucose is taken up by the brain. Glucose concentrations are tightly regulated by peripheral hormones and by the autonomic nervous system. In short, blood glucose levels rise after a meal, which results in pancreatic insulin release that facilitates the uptake of glucose in insulin sensitive tissues via the glucose transporter subtype 4 (GLUT4). Besides the ingested glucose, gut hormones affect insulin secretion in a glucose dependent manner (106). After a meal, most of the ingested glucose is disposed in skeletal muscle, besides uptake in adipose tissue (107). After cellular uptake, glucose is either oxidized or stored. The amount of insulin-stimulated peripherally disposed glucose is a measure of peripheral insulin sensitivity. During hyperinsulinemia, glucose production by the liver is reduced and the ability of insulin to suppress endogenous glucose production by the liver is referred to as hepatic insulin sensitivity. In contrast, during periods of fasting, the plasma insulin concentration is low and glucagon, cortisol and growth hormone levels rise resulting in an increase in glycogenolysis and gluconeogenesis thereby preventing hypoglycemia. Besides the aforementioned hormones, the autonomic nervous system plays a role in glucose homeostasis. Already in 1855, Claude Bernard demonstrated a connection between the brain and peripheral glucose metabolism by showing a peak in plasma glucose levels after puncturing the fourth ventricle floor in a rabbit [108]. Since then, it has been discovered that the brain senses energy status through peripheral hormones and nutrients/substrates and modulates glucose production and uptake through the sympathetic and parasympathetic nervous systems [21;109]. Retrograde tracing studies have described anatomical connections projecting between the hypothalamus, the liver, adipose tissue and the pancreas [110;111], and different studies in several rodent models have shown that these connections are functional in terms of regulating glucose metabolism [112;113]. In fact, rodent studies have shown that part of the effect of insulin on glucose metabolism is through its signalling in the hypothalamus [114;115] although a recent study in dogs challenged this view [116]. Several nuclei within the hypothalamus contain glucose sensitive neurons and POMC and AgRP/NPY neurons of the arcuate nucleus have been shown to be involved in regulating glucose metabolism in rodents [21;117;118]. Currently, it is unknown how hypercaloric feeding and obesity affect the interaction between the brain and peripheral glucose metabolism in humans and whether this contributes to insulin resistance.
Insulin resistance is defined as a reduced biological effect of insulin on insulin sensitive metabolic pathways in a variety of tissues. In the setting of obesity, the underlying mechanisms of insulin resistance are complex and still not fully elucidated. Insulin resistance increases the risk for T2DM [119]. Besides genetic factors [120], low grade inflammation in adipose tissue [121], ER-stress, mitochondrial dysfunction and lipotoxicity/ectopic lipid accumulation all play a role in reducing insulin signalling and, thereby, insulin action [122]. In addition, specific macronutrients contribute to the development of insulin resistance independently of body weight gain. Thus, total intake of both dietary fat and sugars are involved in the development of insulin resistance [123-126], and in rodent studies it has been shown that consuming the combination of fat and sugar induces glucose intolerance within a week [105] in association with snacking feeding behaviour [104]. Whether in humans, specific macronutrients and eating patterns independently contribute to insulin resistance and nutrient handling is unclear. So far, it has been reported that obese women who frequently snack have a higher homeostatic model assessment for insulin resistance (HOMA-IR) compared to obese women with a non-snacking diet pattern [117]. As described above, insulin action and glucose metabolism are under control of the brain and therefore it seems likely that insulin resistance in obesity is partly mediated through obesity-induced changes in glucose regulating brain circuitries, such as the hypothalamus. Whether this is the consequence of the obese state or the ingested nutrients and eating patterns per se is difficult to dissect. Rats on a free choice high-fat and high sugar diet show changes in hypothalamic POMC and NPY expression [100], neurons known to be involved in glucose homeostasis, as well as glucose intolerance [101], although it is not certain whether these effects can be linked [127]. Hypothalamic POMC and NPY cells are insulin sensitive and antagonizing hypothalamic insulin action by locally blocking the insulin receptor, results in hyperinsulinemia and hepatic insulin resistance with increased glucose production [115;128]. To summarize, insulin resistance has a multifactorial etiology and rodent research indicates that the brain might contribute to its development.

**SEROTONIN AND DOPAMINE IN GLUCOSE METABOLISM**

Several lines of evidence exist showing a relationship between central serotonin and dopamine and peripheral glucose metabolism. In rodents, extracellular serotonin levels in the hypothalamic suprachiasmatic nucleus (SCN) are associated with glucose intolerance [129] and agonizing serotonin 2C receptors improves insulin sensitivity while knockout of serotonin 2C receptors results in hepatic insulin resistance [130;131]. Moreover, the latter could be reversed by re-expressing these receptors specifically in hypothalamic POMC neurons [132]. Besides, SERT deficient mice are hyperglycemic and hyperinsulinemic and have reduced insulin signalling in liver prior to the onset of obesity [60]. In humans, genetic studies have indicated a link between the whole body serotonin system and insulin resistance as relationships were shown in polymorphisms in the SERT promoter region [133] and in the serotonin 2C receptor promoter region [134]. Also, pharmacologically antagonizing serotonin 2C receptors in humans deteriorates insulin sensitivity [135] while pharmacologically agonizing the serotonin 4 receptor improves insulin sensitivity in T2DM patients [136], and long-term pharmacological inhibition of SERT with SSRIs causes hyperglycemia and a trend towards diabetes [66;137]. Whether these effects can be attributed to the hypothalamic serotonin system remains to be studied.

In conclusion, rodent studies point towards a strong link between hypothalamic serotonin and peripheral glucose metabolism and pharmacological and genetic studies seem to confirm this interaction to be functional in humans. In humans, it remains unclear whether hypothalamic serotonergic pathways independently contribute to insulin resistance, i.e. through a direct effect on glucose metabolism or indirect through its effect on body weight regulation.

Dopamine has also been linked to glucose metabolism [138]. Dopamine D2 receptor knockout mice are glucose intolerant [139] and conversely, agonizing dopamine D2 receptors with bromocriptine improves insulin sensitivity in rodents [140] and improves glycemic control and glucose tolerance in humans [141]. Moreover it was shown that drug-naive schizophrenic patients, characterized by central dopaminergic dysregulation display disturbed glucose metabolism [142], pointing to involvement of extra-hypothalamic dopamine signalling in glucose metabolism. Indeed, a recently published study from our group showed that deep brain stimulation in the NAc of rats resulted in an intensity-dependent increase in plasma glucose and glucagon that could not be attributed to stress [23]. Moreover unpublished data, also from our group, show improved peripheral insulin sensitivity during deep brain stimulation targeted at the NAc in humans with obsessive-compulsive disorder with insulin sensitivity being correlated with striatal dopamine release in those patients. Another recent unpublished observation from our group shows that depleting dopamine reduces insulin sensitivity in healthy lean men. These results suggest a link between striatal dopamine release in those patients, pointing to a link between striatal dopamine and insulin sensitivity. In addition, clinical studies have shown a positive relationship between Parkinson’s disease, a condition with degeneration of nigrostriatal dopamine neurons, and T2DM [143], although deep brain stimulation of the subthalamic nucleus did not result in changes in glucose metabolism in those patients [144]. Finally, we earlier reported a trend for a positive correlation between peripheral insulin sensitivity and striatal dopamine D2/3 receptor binding in obese women while another study showed a positive correlation between an insulin sensitivity index and availability of these receptors [145;146]. Also in rodents a negative correlation was found between striatal dopamine release and HOMA-IR [147].

In summary, dopamine signalling in different areas in the brain may be related to glucose metabolism and modulating extracellular dopamine affects insulin sensitivity, but more detailed studies are needed to elucidate which dopamine signalling pathways in which brain areas are involved in glucose homeostasis / insulin resistance in humans.
II. AIM AND OUTLINE OF THE THESIS

The general aims of this thesis were to study:

- Brain serotonin- and dopamine transporters in different metabolic conditions
- The effect of hypercaloric diets with different macronutrient compositions and consumed at different time points on brain serotonin and dopamine transporters and metabolism

PART I: BRAIN SERT AND DAT IN LEAN AND OBES HUMANS.

Part I contains four studies describing measures of central SERT and DAT in lean and obese humans. We measured both striatal DAT and diencephalic SERT binding availability in vivo using the radiotracer [18F]-FP-CIT and SPECT. Visualizing diencephalic SERT binding availability with this radiotracer is relatively new, and in chapter 2 we describe the optimal time point for this measurement after bolus injection of the radiotracer. In chapters 3 and 4 we study the differences in hypothalamic SERT content and diencephalic SERT binding between lean and obese humans, in post-mortem hypothalamic tissue and in vivo respectively. In addition, in chapter 4 we describe the relationship between in vivo diencephalic SERT and striatal DAT binding availability and insulin sensitivity in obese women. Chapter 5 describes the relationships between striatal DAT and diencephalic SERT binding availability, food related behavioural outcomes and food intake in lean men.

PART II: NEURAL AND METABOLIC EFFECTS OF HYPERCALORIC DIETS IN LEAN HUMANS.

Part II contains three studies that demonstrate the neural and metabolic effects of hypercaloric diets in healthy lean men. In chapter 6 we describe the effect of hypercaloric diets with different macronutrient compositions and consumed either with or in between the meals on diencephalic SERT availability. Chapter 7 describes the effect of these different hypercaloric diets on hepatic and abdominal fat and on insulin sensitivity. Finally, in chapter 8 we report the effects of a hypercaloric diet on striatal DAT availability and the autonomic nervous system activity.

REFERENCES


[21] Lam CK, Chari M, Lam TK. CNS regulation of glucose homeostasis. Physiology (Bethesda ) 2009 June;24:159-70.


[41] Berridge KC. The debate over dopamine’s role in reward: the case for incentive salience. Psychopharmacology (Berl) 2007 April; 191(3): 391-413.


Estellantes EH, Chabonneau E, Dietrich MS, Park S, Bradley BF, Magg K et al. Obese adults have visual attention bias for food cues: evidence for altered reward system function. Int J Obes (Lond) 2009 September; 33(9): 1063-73. 
Chapter 1 General Introduction


Chapter 1 General Introduction


Chapter 1 General Introduction


PART 1:

BRAIN SERT AND DAT IN LEAN AND OBESE HUMANS
Assessing the optimal time point for the measurement of extrastriatal serotonin transporter binding with 123I-FP-CIT SPECT in healthy male subjects

Karin E Koopman
Susanne E la Fleur
Eric Fliers
Mireille J Serlie
Jan Booij

Journal of Nuclear Medicine
2012; 53(7):1087-90
ABSTRACT

Background: 123I-N-v-fluoropropyl-2b-carboxymethoxy-3b-(4-iodophenyl) nortropane (123I-FP-CIT) is commonly used to assess the dopamine transporter (DAT) in the striatum. However, recent studies suggest that this tracer may be used also to assess binding to monoamine transporters in the midbrain of diencephalon, which may reflect predominantly serotonin transporter (SERT) binding. However, it is still unclear at what time point after injection, SPECT should be performed for optimal assessment of SERT with 123I-FP-CIT. Therefore, we examined the time-course of extrastriatal 123I-FP-CIT binding.

Methods: Nineteen healthy, male subjects were included and SPECT images were acquired up to 3 h after 123I-FP-CIT injection. Region of interest analysis was performed, and specific-to-nonspecific binding ratios were calculated.

Results: Specific-to-nonspecific 123I-FP-CIT binding ratios in the midbrain and diencephalon were significantly higher 2 h compared to 1 h after injection, and remained stable between 2 and 3 h after injection.

Conclusion: The optimal timeframe for assessing 123I-FP-CIT binding to extrastriatal SERT is between 2 and 3 h after injection of the tracer.

INTRODUCTION

In 2000, the radiotracer 123I-N-v-fluoropropyl-2b-carboxymethoxy-3b-(4-iodophenyl) nortropane (123I-FP-CIT) was registered in Europe to assess striatal dopamine transporter (DAT) binding and it is now commonly used to study the integrity of nigrostriatal dopaminergic neurons in-vivo (1). Recently, the same tracer was licensed in the United States. The optimal time-point for measuring striatal DAT is between 3 to 6 h after bolus injection of 123I-FP-CIT (2).

In-vitro studies showed a high affinity of 123I-FP-CIT for the DAT. However, this tracer also has a moderate affinity for the serotonin transporter (SERT) (3). Indeed, in healthy controls we recently showed that, after administration of the selective serotonin reuptake inhibitor paroxetine, 123I-FP-CIT binding was significantly blocked in the SERT-rich midbrain and diencephalon (4). Likewise, another recent study showed that thalamic binding of 123I-FP-CIT could be blocked by the selective serotonin reuptake inhibitor citalopram (5). The relative anatomical segregation between striatal DAT and extrastriatal SERT binding sets the condition for 123I-FP-CIT SPECT studies to examine also SERT in-vivo. Interestingly, recent clinical FP-CIT SPECT studies showed not only loss of striatal DAT but also loss of midbrain SERT binding in dementia with Lewy bodies and in Parkinson’s patients with depression (6,7). In these 2 studies, 123I-FP-CIT binding in SERT-rich areas was measured 3 h after injection, but it is not known whether this time-point represents the optimal time point is optimal for assessing FP-CIT binding to SERT.

Abi-Dargham et al. studied 4 healthy controls and showed that peak specific 123I-FP-CIT binding in the midbrain occurred at 72 ± 37 min after injection, followed by slow washout (3). In a previous study, we measured SERT binding in the midbrain and diencephalon only at 1 and 3 h after injection of 123I-FP-CIT (4). These preliminary studies gave us an idea of the fast kinetics of 123I-FP-CIT binding to extrastriatal SERT. However, since it is incompletely known what the optimal time point is for assessing SERT binding in extrastriatal areas using 123I-FP-CIT, the aim of the present study was to examine the time course of FP-CIT binding in the midbrain and diencephalon. Because previous 123I-FP-CIT studies showed that the optimal time point to assess striatal DAT is between 3 and 6 h after injection, and the binding potential is lower in the midbrain and diencephalon than in the striatum, we expected the optimal time point to assess extrastriatal SERT binding to occur earlier than 3 h after injection (1,2,4).

MATERIALS AND METHODS

SUBJECTS

Nineteen healthy male volunteers with a mean age of 23.5 y [range, 19-37 y] were included in this study. There were no known medical conditions or use of any medication, nor was a history of
any psychiatric disorder abuse of alcohol, drugs or nicotine. Written informed consent was obtained and the study was approved by the medical ethics committee.

**123FP-CIT SPECT**

Radiosynthesis of $^{123}$I-FP-CIT was performed as described earlier (8). Approximately 115 MBq $^{123}$I-FP-CIT (range 110-120 MBq) was injected intravenously as a bolus. All study subjects underwent SPECT imaging at 1, 2 and 3 h after injection of $^{123}$FP-CIT. The scan duration at each time point was approximately 35 min. Each study participant was pre-treated with potassium iodide to block thyroid uptake of free radioactive iodide. SPECT was performed using a 12-detector, single slice dedicated brain scanner (Neurofocus), using an acquisition protocol as described earlier with slight modifications (interslice distance, 5 mm; acquisition time 210 s per slice) (4,9).

**IMAGE RECONSTRUCTION AND ANALYSIS**

Images were corrected for attenuation and reconstructed in 3-dimensional mode, as described earlier. For quantification, a region-of-interest analysis was performed to determine specific binding activity in the midbrain and brain stem (herein called midbrain), diencephalon [Figure 1], and striatum as described earlier (4,9). Briefly, the 4 consecutive slices with the highest striatal, diencephalic, and midbrain binding were selected to assess binding to DAT and SERT, respectively. Activity in the cerebellum [Figure 1] was assumed to represent nondisplaceable activity (nonspecific binding and free radioactivity). Finally, a specific-to-nonspecific binding ratio was calculated as [(activity in region of interest minus nonspecific binding) divided by nonspecific binding] (10, 11).

**STATISTICAL ANALYSIS**

Data on SERT were not normally distributed, therefore, a non-parametric Wilcoxon signed ranks test was used for statistical analysis, to compare SERT binding ratios at 1 and 2 h, 2 and 3 h and 1 and 3 h after injection, and consequently data are expressed as median and range. Data on DAT were normally distributed. Therefore, a parametric paired t test was used to compare DAT binding ratios at 1 and 2 h, 2 and 3 h and 1 and 3 h after injection, and data are expressed as mean ± SEM.

**RESULTS**

**EXTRASTRIAL $^{123}$FP-CIT BINDING**

At almost all time points examined and in all subjects, $^{123}$FP-CIT binding was higher in the midbrain area and the diencephalon than in the cerebellum. The means and SEM for specific-to-nonspecific $^{123}$FP-CIT binding ratio 1, 2 and 3 h after injection are shown in Figure 2 for the midbrain and Figure 3 for the diencephalon. Two hours after injection, binding ratios were significantly [P<0.005] higher than at 1 h after injection in the midbrain [median, 0.25 [range 0.07 – 0.77] and 0.45 [range, 0.12 – 0.70]) A similar significant [P=0.001] increase between 1 and 2 h after injection was observed in the diencephalon [median, 0.35 [range, 0.24 – 0.84] and 0.58 [range, 0.36 – 1.19]). Three hours after injection, these ratios were slightly but not significantly [P=0.20] lower than 2 h after injection in the midbrain [median, 0.40 [range, 0.05 – 0.79]) and slightly but not significantly [P=0.93] higher than 2 h after injection in the diencephalon [median, 0.66 [range, 0.00 – 1.11]].

**STRIATAL $^{123}$FP-CIT BINDING**

As expected, in the DAT-rich striatum, the binding ratios were much higher than in the midbrain area [Figure 4]. These ratios increased over time, up to 3 h after injection. Mean binding ratios ± SEM were significantly higher 2 h [5.02 ± 0.18] than 1 hour [3.13 ± 0.21] after injection [P < 0.001] and 3 h [5.76 ± 0.31] versus 2 h after injection [P = 0.005; Figure 4].

**DISCUSSION**

In this study we found that the ratios of specific-to-nonspecific binding of $^{123}$FP-CIT in the midbrain and diencephalon peaked 2 h after injection of the radiotracer and remained stable up to 3 h after injection. The finding that the specific-to-nonspecific binding ratios of $^{123}$FP-CIT in the striatum increased up to 3 h after injection is in line with a previous study showing that these ratios peaked and remained stable 3-6 h post-injection (2).
123I-FP-CIT is still particularly known as a ligand for imaging DAT (1,2). However, recent research clearly shows that 123I-FP-CIT also binds to SERT, in the midbrain and diencephalon areas (4,5). DAT is distributed mainly in the striatum, whereas SERT is distributed mainly in the midbrain and diencephalon. Furthermore, specific extrastriatal 123I-FP-CIT uptake can be blocked by administration of a selective serotonin reuptake inhibitor that blocks SERT (4,5). 123I-FP-CIT has a lower affinity for SERT than DAT, and the expression of DAT in the striatum may be higher than that of SERT in the midbrain and diencephalon (4,5). Therefore we expected peak binding ratios of 123I-FP-CIT to SERT earlier than peak binding of 123I-FP-CIT to DAT. In the current study we indeed found a lower binding potential for SERT: mean peak binding potentials were 0.48 for SERT versus 5.02 for DAT. In line with this finding, peak binding for SERT in the midbrain and diencephalon was indeed earlier than peak binding for DAT in the striatum.

At the peak binding time point, association and dissociation to and from the transporter are equal. Therefore, this time point is optimal for measurement of the transporter (10,14). However, the binding peak time point varies between study subjects. A secular equilibrium is reached at a time when the ratio of specific to nonspecific binding is stable (15). This provides an equivalent that is related to the density of available transporters. In the current study, there was no true equilibrium for DAT and SERT binding, but there was a transient equilibrium. Therefore SERT and DAT densities may be overestimated (15,16). Nevertheless, in the current study, peak binding ratio was reached at 2 h after injection of the tracer. We therefore recommend measurement of SERT with 123I-FP-CIT between 2 and 3 h after injection of the tracer. Regarding count statistics, 2 h after injection is the most optimal time point for measurement. However, particularly in patients for whom a doubled in-scanner time to measure both SERT and DAT may be a problem, such as elderly or mentally ill patients, 3 h is a very reasonable alternative.

As discussed earlier, 123I-FP-CIT is not a selective tracer for the measurement of SERT. By contrast, 2-((2-((dimethylamino)methyl)phenyl)thio)-5-iodophenylamine (123I-ADAM) is a selective SERT SPECT tracer (17). However, because 123I-FP-CIT is widely used for measuring striatal DAT in, for example, Parkinson disease and dementia with Lewy bodies, it would be unfortunate not to let it serve its full purpose, particularly because recent clinical 123I-FP-CIT SPECT studies showed not only loss of striatal DAT but also loss of midbrain SERT binding in dementia with Lewy bodies, and in Parkinson patients with depression (4,5). This result suggested that information about extrastriatal SERT binding as measured with 123I-FP-CIT may be of interest for diagnostic as well as research purposes and can be obtained in the same scanning section in which striatal DAT binding is measured, without any additional radiation burden for the patients.

A relatively large interindividual variation in SERT binding potential was observed in our study.

---

**Figure 2.**
123I-FP-CIT specific-to-nonspecific binding ratios in the midbrain of young, healthy male volunteers 1, 2 and 3 hours after injection. Data expressed as individual data and median.

**Figure 3.**
123I-FP-CIT specific-to-nonspecific binding ratios in the diencephalon of young, healthy male volunteers 1, 2 and 3 hours after injection. Data expressed as individual data and median.

**Figure 4.**
123I-FP-CIT specific-to-nonspecific binding ratios in the striatum of young, healthy male volunteers 1, 2 and 3 hours after injection. Data expressed as mean and SEM.
sample. This observation is in accordance with the relatively large variation in SERT found in SPECT studies with the selective tracer \[^{123}\text{I}]\text{ADAM}\) [18,19], suggesting that the presently observed variation is not due to the nonspecificity of \[^{123}\text{I}]\text{FP-CIT}\). In the current study we measured specific-to-nonspecific binding ratios of \[^{123}\text{I}]\text{FP-CIT}\) in the midbrain and diencephalon only in healthy young, male subjects. There seems to be no large difference between healthy male and female subjects in brain SERT in several brain areas [20]. It is therefore likely that peak SERT binding ratios of \[^{123}\text{I}]\text{FP-CIT}\) in the midbrain and diencephalon are similar between the sexes. It is known that central SERT decreases with healthy aging [21]. Because we studied only healthy young controls, we cannot exclude the possibility that peak equilibrium is even earlier than 2 h after injection in the elderly, but further investigation is needed [15,16]. We performed measurements in healthy subjects. Because several medical conditions influence brain SERT (e.g. Parkinson disease and Lewy bodies dementia [5,6], panic disorder [19] and depression [22,23]), it is unknown at present whether peak extrastriatal SERT binding of \[^{123}\text{I}]\text{FP-CIT}\) is similar in subjects with any of these diseases. This also needs further investigation. Finally, we used a dedicated brain tomographic SPECT system. Because this system has a high in plane spatial resolution (~6.5 mm in full width at half maximum throughout the 20-cm field of view), we positioned the regions of interest on transversal slices.

CONCLUSION

When the radiotracer \[^{123}\text{I}]\text{FP-CIT}\) is used to examine SERT in the SERT-rich midbrain and diencephalon of young, healthy subjects, we recommend imaging at a single time point, between 2 and 3 h after injection.

REFERENCES


Decreased serotonin transporter immunoreactivity in the hypothalamic infundibular nucleus of overweight subjects

Karin E Koopman*
Anke J Borgers*
Peter H Bisschop
Mireille J Serlie
Dick F Swaab
Eric Fliers
Susanne E la Fleur
Anneke Alkemade
* Authors contributed equally

Frontiers in Neuroscience
2014 May 15;8:106
Chapter 3

ABSTRACT

Background: That serotonin plays a role in the regulation of feeding behavior and energy metabolism has been known for a long time. Serotonin transporter (SERT) play a crucial role in serotonin signalling by regulating its availability in the synaptic cleft. The neuroanatomy underlying serotonergic signalling in humans is largely unknown, and until now, SERT immunoreactivity in relation to body weight has not been investigated. We aimed to clarify the distribution of SERT immunoreactivity throughout the human hypothalamus and to compare SERT immunoreactivity in the infundibular nucleus (IFN), the human equivalent of the arcuate nucleus, in lean and overweight subjects.

Methods: First, we investigated the distribution of serotonin transporters (SERT) over the rostro-caudal axis of six postmortem hypothalami by means of immunohistochemistry. Second, we estimated SERT immunoreactivity in the IFN of lean and overweight subjects. Lastly, double-labelling of SERT with Neuropeptide Y (NPY) and melanocortin cell populations was performed to further identify cells showing basket-like SERT staining.

Results: SERT-immunoreactivity was ubiquitously expressed in fibers throughout the hypothalamus and was the strongest in the IFN. Immunoreactivity in the IFN was lower in overweight subjects (p<0.036). Basket-like staining in the IFN was highly suggestive of synaptic innervation. A very small minority of cells showed SERT double labelling with NPY, agouti-related protein and α-melanocyte stimulating hormone.

Conclusions: SERT is ubiquitously expressed in the human hypothalamus. Strong SERT immunoreactivity as observed in the IFN, a region important for appetite regulation, in combination with lower SERT immunoreactivity in the IFN of overweight and obese subjects, may point towards a role for hypothalamic SERT in human obesity.

INTRODUCTION

The brain serotonin (5-HT) system is known to be involved in the regulation of food intake and body weight. Several animal experimental studies manipulating endogenous serotonin synthesis, availability and metabolism, have made it clear that there is a negative relationship between the level of brain serotonin and food intake, in which increasing serotonin (by inhibiting reuptake or activating serotonin receptors postsynaptically) inhibits food intake [1]. In addition, over the last few years evidence has accumulated on the central serotonin system’s involvement in peripheral glucose metabolism [2]. A more recent study reports that serotonin transporter (SERT) knockout mice are obese and present pre diabetic symptoms such as glucose intolerance and insulin resistance [3].

A key brain area involved in the regulation of feeding behavior and in peripheral glucose metabolism is the hypothalamus. There are a number of anatomically distinct nuclei within the hypothalamus that are involved in these functions and that are closely interrelated [4]. A central role in this is played by the arcuate nucleus (ARC), or infundibular nucleus (IFN) as it is referred to in humans [5]. Several neuropeptides related to both feeding behavior and glucose metabolism have been localized in the ARC/IFN. Like has been shown for rodents, Neuropeptide Y (NPY), agouti-related protein (AGRP) and α-melanocyte stimulating hormone (αMSH) are localized in the human IFN [6,7]. When these neuropeptides are injected into the rodent brain, NPY and AGRP increase feeding, reduce insulin sensitivity and increase glucose production. On the other hand, αMSH decreases food intake and increases insulin action, thus reducing glucose production and enhancing glucose uptake [for review: (8)]. More recently we investigated the expression of these neuropeptides in the human IFN in relation to body mass index (BMI), and type 2 diabetes [6]. We found that AGRP expression showed a U-shaped correlation with BMI, and that NPY expression was lower in overweight and obese subjects, whereas αMSH revealed no relation to BMI.

Although it is clear that serotonin has an effect on feeding behavior and glucose metabolism in the rodent hypothalamus, data on human hypothalamus are scarce. What has been shown in humans is that polymorphisms related to serotonergic signalling are associated with BMI [9,10], however this does not provide evidence for hypothalamic serotonin as serotonin signals throughout the body and also widely throughout the brain. We did recently show that hypercaloric high fat high sugar snacking reduces diencephalic SERT [11]. Although diencephalon contains the hypothalamus, it remains to be determined whether SERT containing cells/fibers can be identified in the human IFN and other hypothalamic nuclei. If identified, the question arises which individual nuclei contain the densest SERT staining and how SERT staining in the IFN relates to neuropeptides known to be involved in energy metabolism.
In this paper we determined the distribution of SERT immunoreactivity in the human hypothalamus using a post-mortem approach. In addition, we compared SERT staining in the IFN of subjects with a BMI<25 to that of subjects with a BMI≥25 kg/m². Finally, we describe immunofluorescent double-labeling of SERT with the IFN neurotransmitters NPY, AgRP and αMSH to identify cell types showing basket-like staining of SERT-immunoreactive fibers, suggestive of synaptic innervation.

MATERIALS AND METHODS

SUBJECTS

For Experiment 1 we investigated the distribution of SERT using systematic sampling over the entire rostro-caudal axis of the hypothalamus of 6 subjects (3 male) without neurological or psychiatric disease ranging in age between 67 and 86 years. Clinicopathological and relevant medication data are presented in Table 1.

For Experiment 2 in which we related SERT staining in the IFN to BMI, we studied post-mortem hypothalamic tissue of 11 overweight and obese (6 male, median BMI 30.5 (range: 25.0-39.5) kg/m²; median age 76 (range: 65 - 100) years) with 12 non-obese (5 male, median BMI 20.1 (range 15.2-24.2) kg/m²; median age 64 (range: 50 - 92) years) subjects. Clinicopathological and relevant medication data have been published earlier (6), and are presented in Table 2.

In Experiment 3 we aimed to identify the immunocytochemical nature of the cells showing basket-like SERT immunoreactivity, using brain material of the subjects described in Experiment 1. All brain material was obtained from The Netherlands Brain Bank at The Netherlands Institute for Neuroscience [director Dr. I. Huitinga] in accordance with the formal permissions for brain autopsy and for the use of human brain material and clinical information for research purposes.

<table>
<thead>
<tr>
<th>Table 1. Brain material Experiment 1.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject</td>
</tr>
<tr>
<td>94017</td>
</tr>
<tr>
<td>50004</td>
</tr>
<tr>
<td>60354</td>
</tr>
<tr>
<td>98556</td>
</tr>
<tr>
<td>95016</td>
</tr>
<tr>
<td>01005</td>
</tr>
</tbody>
</table>

Table 2. Brain material Experiment 2.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age</th>
<th>PD</th>
<th>Fix</th>
<th>Cause of death, clinical diagnoses</th>
</tr>
</thead>
<tbody>
<tr>
<td>95072</td>
<td>F</td>
<td>62</td>
<td>7</td>
<td>51</td>
<td>Cardiovascular disease, old CVA</td>
</tr>
<tr>
<td>98072</td>
<td>M</td>
<td>79</td>
<td>17</td>
<td>31</td>
<td>Pancreatitis, COPD, CVA, renal insufficiency</td>
</tr>
<tr>
<td>98091</td>
<td>F</td>
<td>30</td>
<td>41</td>
<td>72</td>
<td>Respiratory insufficiency, pansy, hypertension</td>
</tr>
<tr>
<td>98077</td>
<td>M</td>
<td>54</td>
<td>8</td>
<td>59</td>
<td>Respiratory insufficiency, COPD, cardiac insufficiency, PICA, adenocarcinoma</td>
</tr>
<tr>
<td>98073</td>
<td>M</td>
<td>88</td>
<td>7</td>
<td>44</td>
<td>Respiratory insufficiency, COPD, cardiac insufficiency, PICA, adenocarcinoma</td>
</tr>
<tr>
<td>91005</td>
<td>F</td>
<td>65</td>
<td>09</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>10016</td>
<td>M</td>
<td>81</td>
<td>04</td>
<td>50</td>
<td>72</td>
</tr>
<tr>
<td>98552</td>
<td>F</td>
<td>62</td>
<td>8</td>
<td>73</td>
<td>21</td>
</tr>
<tr>
<td>98161</td>
<td>F</td>
<td>60</td>
<td>8</td>
<td>67</td>
<td>22</td>
</tr>
<tr>
<td>98070</td>
<td>M</td>
<td>60</td>
<td>02</td>
<td>30</td>
<td>108</td>
</tr>
<tr>
<td>98095</td>
<td>F</td>
<td>75</td>
<td>20</td>
<td>63</td>
<td>64</td>
</tr>
<tr>
<td>98099</td>
<td>M</td>
<td>60</td>
<td>7</td>
<td>88</td>
<td>24</td>
</tr>
<tr>
<td>95070</td>
<td>M</td>
<td>76</td>
<td>19</td>
<td>133</td>
<td>25</td>
</tr>
<tr>
<td>98013</td>
<td>M</td>
<td>70</td>
<td>6</td>
<td>68</td>
<td>26</td>
</tr>
<tr>
<td>98054</td>
<td>F</td>
<td>92</td>
<td>7</td>
<td>67</td>
<td>27</td>
</tr>
<tr>
<td>98058</td>
<td>M</td>
<td>82</td>
<td>13</td>
<td>38</td>
<td>27</td>
</tr>
<tr>
<td>91021</td>
<td>F</td>
<td>56</td>
<td>05</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>98171</td>
<td>F</td>
<td>70</td>
<td>24</td>
<td>62</td>
<td>30</td>
</tr>
<tr>
<td>98100</td>
<td>F</td>
<td>76</td>
<td>10</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td>98065</td>
<td>F</td>
<td>76</td>
<td>14</td>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td>98105</td>
<td>F</td>
<td>71</td>
<td>7</td>
<td>53</td>
<td>32</td>
</tr>
<tr>
<td>98060</td>
<td>M</td>
<td>65</td>
<td>11</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td>98072</td>
<td>M</td>
<td>78</td>
<td>18</td>
<td>42</td>
<td>39</td>
</tr>
</tbody>
</table>

HISTOLOGY

Brains were dissected at autopsy and the hypothalamus was fixed in 10% phosphate-buffered formalin at room temperature (RT) for 1-2 months. After dehydration in graded ethanol series, tissues were cleared in toluene and embedded in paraffin. Coronal serial sections (6 μm) were cut over the entire rostro-caudal axis of the hypothalamus. For anatomical orientation, every 100th section was collected and mounted on chrome alum-gelatine coated glass slides and subsequently dried for two days at 37°C, followed by Nissl staining.

ANTIBODY CHARACTERIZATION

Mouse monoclonal anti-human SERT antibody was purchased from Millipore, MAb Technologies Inc. (Stone Mountain, GA; catalogue no. Mab5618). Antibody specificity has been reported before and was supported using Western blotting (12-15). Rabbit polyclonal anti-human AgRP antibody was obtained from Phoenix Pharmaceuticals (Belmont, CA; catalogue no. H-003-53). AgRP staining disappeared after pre-adsorption with AgRP, and was not affected by cross adsorption using the NPY peptide (7). The αMSH antibody was raised against the αMSH C-terminal, which is modified in αMSH free acid, and absent in ACTH, minimizing cross-reaction with other POMC products. Staining was abolished after pre-adsorption with the αMSH peptide (16).

IMMUNOHISTOCHEMISTRY

Histological and immunocytochemical processing was performed as described previously, with some minor modifications (6). For SERT-immunohistochemistry, a series of coronal sections at 100-
section intervals over the entire rostro-caudal axis of the hypothalamus was mounted on Superfrost plus slides (Menzel Glaser, Germany) and dried for at least 2 days at 37°C. This resulted in 6-12 sections per subject. Next, antigen retrieval was performed using microwave treatment [17] and sections were stained using the avidin biotinylated complex method [18], according to the following protocol. Sections were deparaffinised in xylene and rehydrated through graded ethanol series. After rinsing in distilled water, the sections were washed in TBS (pH 7.6) and antigen retrieval was performed using microwave treatment (10 min, 700W) in TBS (pH 7.6). After adjustment to RT, sections were incubated in the primary antibody diluted 1:5000 in SUMI (supermix, 0.05M Tris, 0.15M NaCl, 0.5% Triton X-100 (Sigma, Zwijndrecht, The Netherlands), and 0.25% gelatine (Merck, Darmstadt, Germany) (pH 7.6) overnight at 4°C. The slides were rinsed in TBS (pH 7.6, 3x5min) and incubated for 1h at RT in the second antibody (biotinylated horse antirabbit 1:4000 in SUMI; Vector Laboratories, Burlingame, CA). After rinsing in TBS (pH 7.6, 3x5min), the sections were incubated 1h at RT in avidine biotinylated complex (1:800 in SUMI; Vector Laboratories, Burlingame, CA) and subsequently rinsed in TBS (pH 7.6, 3x5 min). Finally, sections were incubated in 0.5 mg/ml 3,3′-diaminobenzidine (Sigma) in TBS containing 0.2% ammonium nickel sulphate (BDH; Brunschwig, Amsterdam, The Netherlands) and 0.01% H2O2 (Merck) for approximately 1.5 min. The reaction was stopped in distilled water. The sections were dehydrated in ascending series of ethanol, cleared in xylene, and covered slipped using Entellan (Merck, Darmstadt, Germany).

**DOUBLE LABELING BY IMMUNOHISTOCHEMISTRY**

To identify cell types showing basket-like staining of SERT-immunoreactive fibers in the IFN, we performed immunofluorescent double staining of respectively SERT with α-MSH (1:20000), NPY (1:10000) and AgRP (1:3000) in hypothalamic sections containing the IFN of all 6 subjects.

After overnight primary antibody incubation at 4°C the slides were rinsed in TBS (pH 7.6, 3x5min) and incubated in the secondary antibodies (biotinylated horse antimouse 1:4000 in SUMI; Vector laboratories, Burlingame, CA) for 1h at RT. Following rinsing in TBS (pH 7.6, 3x5min), the sections were incubated 1h at RT in avidine biotinylated complex (1:800 in SUMI; Vector Laboratories, Burlingame, CA), subsequently rinsed in TBS (pH 7.6, 3x5min) and incubated in biotinylated tyramide (1:750 in SUMI, 0.01% H2O2 (Merck, Darmstadt, Germany)) for 15 min at RT followed by rinsing in TBS (pH 7.6, 3x5 min). SERT was detected in green by streptavidin-Alexa488 (1:1000; Invitrogen, Eugene, OR). The other peptides were visualized in red by respectively anti-rabbit Alexa594 (1:1000; Invitrogen, Eugene, OR) for NPY or AgRP and anti-sheep Alexa594 (1:1000; Invitrogen, Eugene, OR) for α-MSH. This fluorochrome-conjugated antibody incubation was performed for 1h at RT, followed by overnight incubation at 4°C. Vectashield with DAPI (Vector laboratories, Inc, Burlingame, CA) was used for nuclear staining and cover slipping. The sections were stored under dark conditions at 4°C until further analysis. Colocalization was assessed by visual inspection.

**QUANTITATIVE ANALYSIS**

SERT immunoreactivity was quantified using an unbiased masking procedure of the 3,3′-diaminobenzidine-Ni precipitate [6]. For quantification of the immunoreactive signal, gray values of the DAB-Ni precipitate in the IFN were analyzed by computer-assisted densitometry using Image pro (Media Cybernetics, Silver Spring) and software developed at the Netherlands Institute for Neuroscience. Every 10th section containing the IFN was analyzed and estimates of the immunoreactivity were made by averaging the signal density of the 3 sections that showed the highest signal, resulting in arbitrary units (a.u.) [19].

**STATISTICAL ANALYSIS**

The data of the second experiment were analyzed with SPSS for Windows, version 19.0 (SPSS Inc. Chicago, Illinois, USA). Group differences in numerical variables were evaluated using the Mann-Whitney U test for not normally distributed parameters. A stepwise linear regression analysis was performed to investigate the effects of possible confounding factors. The statistical significance level for all analyses was set at p<0.05 (two-sided).

**RESULTS**

Many SERT-immunoreactive fibers and few scattered SERT-positive basket cells were found throughout the entire hypothalamus, except in the white matter tracts (Figure 1). Basket-like staining was defined as aggregation of staining surrounding neuronal cell bodies, creating a basket-like appearance, suggestive of synaptic innervation. The general distribution of SERT was comparable in all subjects studied (Figure 1A). A strong inter-individual variation in staining intensity was observed. SERT positive fibers were present throughout the entire hypothalamus, with a denser network of SERT-immunoreactive fibers in the perifornical area and in close proximity to the anterior commissure. A plexus along the ependyma of the third ventricle wall also showed strong SERT-immunoreactivity (Figures 1B). In addition, the highest fiber density was observed in the IFN, and the supraoptic nucleus (SCN), which is the central biological clock of the human hypothalamus (Figures 1C and D). In these areas, cell bodies and capillaries were directly surrounded by clusters of SERT-immunoreactive fibers, highly suggestive of SERT-positive nerve endings in contact with SERT-negative perikarya and capillaries (high power inserts of Figures 1C and D). The supraoptic nucleus, paraventricular nucleus, lateral tuberal nucleus and tuberomamillary nucleus contained relatively small numbers of SERT-immunoreactive fibers. No differences were observed between males and females.
SERT-staining was observed in the IFN of all subjects. IFN SERT-immunoreactivity was lower in overweight subjects than in non-overweight subjects (p=0.036) (Figure 2A). An example of the difference between overweight and non-overweight subjects is illustrated in Figures 2B-C. A stepwise linear regression analysis performed for the factors age, sex, fixation duration, and postmortem delay did not reveal any significant results.

To further characterize the cell types showing basket-like staining of SERT-immunoreactive fibers in the IFN, we performed immunofluorescent double staining of SERT with NPY, AGRP or αMSH on sections containing the IFN. SERT-immunoreactive fibers were present at all levels in all studied subjects. Many single-labelled cells expressing NPY, AGRP and αMSH-immunoreactive cells were found in the IFN in all subjects. A very small minority of neurons immunoreactive for αMSH or AGRP showed basket-like SERT staining. As SERT showed no clear colocalization with NPY, neurons showing basket-like SERT staining remained largely unidentified (Figure 3 and 4). Of note, the labelling of SERT in the IFN was predominantly localized within the IFN, somewhat lateral to the NPY, AGRP- and αMSH-immunoreactive cells. A number of these cells appeared to be strongly activated, as indicated by the presence of two nucleoli. Together, our results indicate that SERT axons project to a minority of αMSH and AGRP neurons, as well as to currently unidentified subgroups of neurons in the IFN.

We showed that SERT protein is extensively expressed in the human hypothalamus. Moreover, one of the nuclei most heavily innervated was the SCN, which is in agreement with the distribution of SERT immunoreactivity reported in rodents [20] as well as in human and nonhuman primates [21,22]. Interestingly, when analysing SERT protein in post mortem sections of the IFN of overweight and obese subjects and comparing them to lean individuals, the amount of SERT protein was clearly reduced in obesity. This is in line with a large number of knockout and transgenic mouse studies, which, collectively, show an inverse relationship between brain serotonin signalling and food intake, as well as with pharmacological studies targeting serotonin signalling in rodents [for review: (1)]. We cannot exclude that BMI in these subjects was influenced by severe illness, including malignancies such as adenocarcinomas. In addition, some subjects received antidiabetic medication. Immunoreactivity for serotonin has been reported before in the rodent ARC, where stained fibers were found in the immediate vicinity of capillaries and neurons [23]. Surprisingly, our double-labelling study indicates that these SERT immunoreactive fibers in the IFN only partly

**DISCUSSION**

We showed that SERT protein is extensively expressed in the human hypothalamus. Moreover, one of the nuclei most heavily innervated was the SCN, which is in agreement with the distribution of SERT immunoreactivity reported in rodents [20] as well as in human and nonhuman primates [21,22]. Interestingly, when analysing SERT protein in post mortem sections of the IFN of overweight and obese subjects and comparing them to lean individuals, the amount of SERT protein was clearly reduced in obesity. This is in line with a large number of knockout and transgenic mouse studies, which, collectively, show an inverse relationship between brain serotonin signalling and food intake, as well as with pharmacological studies targeting serotonin signalling in rodents [for review: (1)]. We cannot exclude that BMI in these subjects was influenced by severe illness, including malignancies such as adenocarcinomas. In addition, some subjects received antidiabetic medication. Immunoreactivity for serotonin has been reported before in the rodent ARC, where stained fibers were found in the immediate vicinity of capillaries and neurons [23]. Surprisingly, our double-labelling study indicates that these SERT immunoreactive fibers in the IFN only partly...
A series of immunofluorescent photomicrographs showing serotonin transporter (SERT) immunostaining (shown in green) combined with markers for major cell types (shown in red) of suprachiasmatic nucleus (SCN) and infundibular nucleus (IFN). Cell nuclei are shown in blue. SERT immunofluorescent staining (green) combined with A) arginine vasopressin (AVP, red) in the SCN, high power insert shows SERT staining in close proximity of AVP-positive neurons which was observed in a very small minority of cells; B) Vasointestinal peptide (VIP, red), high power insert illustrates the absence of SERT immunoreactivity surrounding VIP-positive neurons; C) Neuropeptide Y (NPY, red), and D) α-Melanocyte stimulating hormone (α-MSH, red), note the SERT staining surrounding the α-MSH positive neurons.

Interestingly, another area with dense innervation of SERT fibers was the suprachiasmatic nucleus (SCN), the area in the brain were the biological clock resides. This finding on dense innervation is in agreement with literature showing serotonin as well as SERT containing fibers in the SCN in other species (for review: [28]) and is in line with the important role of serotonin as regulator of the circadian phase. This innervation is also likely to affect feeding behavior and glucose metabolism, which are clearly under the influence of the SCN.

Pharmacological targeting of the serotonin system for the treatment of obesity has received a lot of attention, but the use of pharmacological compounds affecting serotonin signalling has been
complicated by unwanted side-effects, such as increased heart rate, hypertension, headaches and nausea (2). The use of specific serotonin reuptake inhibitors for treatment of an array of psychiatric and neurological disorders also shows the importance of serotonin in relation to body weight. Side-effects of specific serotonin reuptake inhibitors include increased appetite and increased body weight (29;30). Our results on SERT distribution in the human hypothalamus provides an anatomical framework for future investigations regarding the role of the serotonergic system in the human hypothalamus in terms of body weight regulation.

CONCLUSION

We show in post-mortem hypothalamic tissue, SERT is ubiquitously expressed with the highest density in the IFN and SCN. Moreover, SERT immunoreactivity was lower in the IFN of overweight and obese subjects than lean subjects, which may point towards a role for hypothalamic SERT in human obesity.

ACKNOWLEDGMENTS

Brain material was obtained from the Netherlands Brain Bank (director I. Huttiga). We wish to acknowledge Bart Fisser for his excellent technical assistance.

REFERENCES


Diencephalic serotonin transporter binding, but not striatal dopamine transporter binding, is correlated to insulin sensitivity in obese women

Karin E Koopman
Ruth I Versteeg
Jan Booij
Mariëtte T Ackerman
Eric Fliers
Susanne E la Fleur
Mireille J Serlie

Manuscript in preparation
ABSTRACT

Background: Striatal dopamine receptors and cerebral serotonin transporters are reduced in obese humans. Obesity is associated with insulin resistance, but it is unknown whether brain serotonin and dopamine relate to insulin sensitivity independently of obesity.

Methods: To examine this relationship, we measured serotonin transporter (SERT) binding in the diencephalon and striatal dopamine transporter (DAT) binding using 123I-FP-CIT SPECT in insulin sensitive obese (ISO), insulin resistant obese (IRO) (total n=10) and matched lean women (n=8). We assessed insulin sensitivity using an oral glucose tolerance test.

Results: Body mass index (BMI) was similar in ISO and IRO women. Striatal DAT binding was similar in lean, ISO, and IRO women, and did not correlate with BMI or insulin sensitivity. In contrast, SERT binding in the diencephalon was significantly lower in IRO women compared to lean and ISO women, and correlated positively with insulin sensitivity. SERT binding did not correlate with BMI.

Conclusions: We conclude that available serotonin transporters in the diencephalon, but not striatal dopamine transporters are positively correlated with insulin sensitivity in obese women independently of BMI. Our results demonstrate an independent effect of hypothalamic SERT on glucose metabolism and indicate that SERT might be a potential target in the treatment of diabetes.

INTRODUCTION

Serotonin is a monoamine neurotransmitter involved in the regulation of food intake and body weight (1). In rodent studies, brain serotonin has been linked to obesity. Neurochemical serotonin depletion in the rodent brain results in obesity and hyperphagia (2-4). Inversely, infusion of serotonin into the hypothalamus decreases body weight in Zucker rats (5). In humans, pharmacological challenge of the serotonin system induces weight loss (6;7). Recently, the serotonin-2C receptor agonist lorcanerin was approved by the Food and Drug Administration (FDA) as a weight loss treatment (8). Furthermore, short-term use of selective serotonin reuptake inhibitors (SSRIs), which inhibit reuptake of serotonin via the serotonin transporter (SERT), reduces body weight (7). This suggests that in humans an increase in serotoninergic signalling, via a serotonin-2C receptor agonist or inhibition of SERT, may be favourable for body weight management. Furthermore, in humans, SERT binding in subcortical regions (caudate nucleus-putamen-thalamus) is negatively associated with BMI (9), suggesting that serotoninergic signalling is diminished in obesity.

Aside from the established association between body weight and the serotonin system, many studies point towards a regulatory role for serotoninergic signalling in glucose metabolism. Mice lacking the serotonin-2C receptor in proopiomelanocortin (POMC) neurons have normal body weight but exhibit hyperglycemia, hyperinsulinemia, and insulin resistance (10). SERT deficient mice are hyperglycemic and hyperinsulimemic and have reduced insulin signalling in liver prior to the occurrence of obesity (11). In overweight non-diabetic humans, levels of plasma serotonin metabolites decrease after oral glucose (12). Short-term treatment with SSRIs decreases fasting blood glucose levels in nondiabetic patients (13) and SERT polymorphisms are linked to the occurrence of diabetes (14). Most studies in humans address systemic serotonin, while body weight regulation and glucose metabolism are mainly orchestrated by the brain, and more specifically within the hypothalamus. Therefore, we studied hypothalamic SERT binding in obese humans. In addition, to further explore the relationship between body weight, insulin resistance, and hypothalamic SERT, we studied obese insulin sensitive and obese insulin resistant women and compared them to matched lean controls.

In addition to the hypothalamic serotonergic system, the striatal dopaminergic system has been associated with body weight and glucose metabolism. Obese subjects have lower striatal dopamine D2/3 receptor (D2/3R) binding availability (15;16) and striatal D2/3R binding is associated with insulin sensitivity in humans (17). Additionally, D2R knockout mice have a blunted insulin response and are glucose intolerant (18). Increasing dopaminergic signalling using the dopamine receptor agonist bromocriptine in rodents and humans improves glucose metabolism (19;20), and in obese rodents this was accompanied by an increase in striatal dopamine transporter [DAT] expression (21). The latter finding suggests that DAT is a representative reflection of dopaminergic
signalling. To study whether striatal DAT is different in lean versus obese subjects and affected by the metabolic state, we also measured DAT binding in the same group of obese insulin sensitive and obese insulin resistant subjects, as well as in the lean controls. Finally, we measured SERT binding in the diencephalon and striatal DAT binding with \[^{123}\text{I} \text{FP-CIT}\] SPECT and assessed insulin sensitivity via an oral glucose tolerance test using the Matsuda Index.

**MATERIALS AND METHODS**

**SUBJECTS**

Study subjects included ten obese premenopausal women (mean age: 31.3±9.7 years; mean BMI: 37.2±4.8 kg/m²) and 8 matched lean controls (mean age: 30.9±10.5 years; mean BMI: 21.3±1.3 kg/m²). Participants were in self-reported good health, had normal liver, renal, and thyroid function, did not smoke and did not use any medication. Those with a history of psychiatric or eating disorders (e.g. binge eating, restraint eating) were excluded. All subjects had a self-reported stable weight during the 3 months prior to inclusion. We divided the obese group in ISO and IRO subgroups, based on the Matsuda Index cut-off point for insulin resistance of 5.0 [23]. The study protocol was approved by the institutional review board/medical ethics committee of the Academic Medical Center Amsterdam. Written informed consent was received from all participants after explanation of the nature of the study.

**IMAGING OF THE SEROTONIN TRANSPORTER (SERT) AND DOPAMINE TRANSPORTER (DAT)**

Each participant underwent single photon emission computed tomography (SPECT) imaging with the radioligand \[^{123}\text{I} \text{FP-CIT}\]. A total dose of 115 MBq (range: 110-120 MBq; specific activity > 750 MBq/mmol; radiochemical purity > 98%, produced according to GMP criteria at GE Healthcare, Eindhoven, The Netherlands) was given intravenously. This tracer can be used to visualize and quantified adequately in the diencephalon ((hypo)thalamic region) and striatum at 2 and 3 hours after bolus injection as described previously [24]. Participants were scanned at 10:30 AM after an overnight fast. Each participant was pre-treated with potassium iodide to block thyroid uptake of free radioactive iodide. SPECT imaging was performed using a 12-detector, single slice brain-dedicated scanner (Neurofocus, 810, Strichman Medical Equipment, Cleveland, OH, USA) using an acquisition protocol as described previously with slight modifications (interslice distance: 5 mm; acquisition time: 210 sec/slice) [24]. All scans were reconstructed in 3D mode and corrected for attenuation. For quantification, a region-of-interest (ROI) analysis to determine specific binding activity in the striatum and diencephalon was performed by a well-trained researcher as described previously [25]. Briefly, the 4 consecutive slices with the highest binding were selected to assess binding to DAT and SERT. Activity in the cerebellum (3 consecutive slices) was assumed to represent non-displaceable activity (nonspecific binding and free radioactivity). A specific-to-nonspecific binding ratio (SNS-BR) was calculated as [ROI-binding minus cerebellar-binding / cerebellar-binding]. The outcome measure was SNS-BR.

**ORAL GLUCOSE TOLERANCE TEST**

Within one week of the SPECT scan and following an overnight fast, the oral glucose tolerance test (OGGT) was performed using 75 grams of glucose dissolved in 300 ml of water. An intravenous catheter was inserted in the right forearm and the subjects remained seated during the entire OGGT. Venous blood samples were drawn after ingestion of the glucose load, at T=0, 30, 60, 90, and 120 minutes.

**LABORATORY ANALYSIS**

Plasma glucose concentrations were measured with the glucose oxidase method using a Biosen C-line plus glucose analyzer (EKF Diagnostics, Barleben/Magedebug, Germany). Insulin and cortisol were both measured on an IMMULITE 2000 system (Siemens Healthcare Diagnostics B.V., Breda, The Netherlands). Cortisol was measured with a chemiluminescent immunometric assay (intra-assay variation: 7-8%; total-assay variation: 7-8%; detection limit: 50 nM). Insulin was measured with a chemiluminescent immunometric assay (intra-assay variation: 3-6%; total-assay variation: 4%; detection limit: 15 pmol/l). C-peptide levels were measured with a \[^{125}\text{I} \text{radioimmunoassay}\] (Merck Millipore, St. Charles, MO, USA) (intra-assay variation: 6-9%; total-assay variation: 7-11%; detection limit: 50 pmol/l). Glucagon was measured with the \[^{125}\text{I} \text{radioimmunoassay}\] (Merck Millipore) (intra-assay variation: 9-10%; total-assay variation: 5-7%; detection limit: 15 ng/l).

**INSULIN SENSITIVITY**

We used the Matsuda Index (MI) to assess insulin sensitivity. MI is calculated from plasma insulin and glucose response during a 2-hour OGGT and is well validated with clamp derived peripheral glucose disposal rates [26,27].

\[ \text{MI} = \frac{10,000}{\sqrt{\text{[FPG} \times \text{FPI}] \times \text{[mean glucose (mmol/L) \times mean insulin (pmol/L)]}}.} \]

**STATISTICS**

All data were analysed using SPSS for Windows, version 20.0 (SPSS Inc. Chicago, Illinois, USA). Data were tested for normality. To compare data between the lean, ISO, and IRO groups we used 1-way ANOVA with a posthoc Bonferroni. To compare data between the lean and obese group, we used the Student’s T-test. We used Pearson correlations for correlation analysis in the whole cohort and the obese and lean subgroups. For all analyses, a P < 0.05 was considered statistically significant and P < 0.1 was considered a trend. Sample size calculation was based on
a previous study that showed that diencephalic SERT binding in lean subjects was 0.51±0.17 (24). To detect a difference with a significance level α=0.05, power=80%, variance of means = 0.029 and common standard deviation=0.17, we needed n=5 subjects per group (calculated with nQuery Advisor 7.0 software).

RESULTS

Baseline characteristics are presented in Table 1. Based on the Matsuda Index (MI), the obese subjects were divided into ISO and IRO subgroups (Table 2). Subjects with MI ≥ 5.0 were considered ISO and subjects with MI < 5.0 were considered IRO (23). We could not calculate the MI from one obese subject, due to haemolytic samples that precluded adequate performance of the insulin assay. Based on the curve for C-peptide and a calculated HOMA-IR of 0.47 (HOMA-IR=[FPG*(FPI*6.945)]/22.5) with a suggested cut-off value for insulin resistance >2.2 (28), this obese participant was considered insulin sensitive.

LEAN VERSUS OBSESE SUBJECTS

As expected, the obese subjects had higher fasting glucose concentrations, C-peptide concentrations, and a trend towards higher fasting insulin concentrations, as well as reduced insulin sensitivity compared to the lean subjects (Table 1).

INSULIN SENSITIVE VERSUS INSULIN RESISTANT OBSESE SUBJECTS

ISO subjects were comparable to lean subjects for all measured parameters except BMI, while BMI did not differ between the ISO and IRO groups (Table 2 and Figure 1A). Plasma concentrations of cortisol and glucagon did not differ between the three groups (Table 2).

SERT BINDING, BUT NOT STRIATAL DAT BINDING, IS LOWER IN INSULIN RESISTANT WOMEN

Overall, SERT binding in the diencephalon was not different between the lean and obese subjects [Table 1]. However, IRO women had significantly lower SERT binding compared to the ISO and lean women, while there was no difference between the lean and ISO subjects (Table 2 and Figure 1C). Striatal DAT binding did not differ between the lean and obese subjects (Table 1). In addition, no differences in striatal DAT binding were found between lean, ISO, and IRO subjects (Table 2).

SERT binding, but not striatal DAT binding, correlates with insulin sensitivity

SERT binding did not correlate with BMI in either the lean or obese subjects (p=0.105 [lean + obese], p=0.709 [lean only] and p=0.249 [obese only] respectively), neither did striatal DAT binding (p=0.891 [lean + obese], p=0.562 [lean only] and p=0.350 [obese only] respectively). In the lean subjects, no correlation was found between SERT availability and insulin sensitivity (p=0.891 (lean + obese); p=0.709 (lean only) and p=0.249 (obese only) respectively); neither did striatal DAT binding (p=0.865). In the obese subgroup, SERT binding positively and significantly correlated with insulin sensitivity (p=0.025, R=0.73, r^2=0.5332, Figure 2). DAT availability did not correlate with insulin sensitivity in lean or obese subjects.

DISCUSSION

Our data reveal a robust correlation (r > 0.50) between SERT binding in the diencephalon and insulin sensitivity, independent of BMI in obese women. In contrast, striatal DAT, an important regulator of dopamine signalling, did not correlate with insulin sensitivity. Neither SERT nor DAT binding were associated with BMI.

Table 1. Lean vs obese subjects

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>Obese</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Age [yr]</td>
<td>30.9±10.5</td>
<td>31.3±9.7</td>
<td>0.93</td>
</tr>
<tr>
<td>BMI [kg/m²]</td>
<td>21.3±1.3</td>
<td>37.2±4.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Glucose [mmol/L]</td>
<td>4.38±0.39</td>
<td>4.87±0.38</td>
<td>0.016</td>
</tr>
<tr>
<td>Insulin [pmol/L]</td>
<td>23.3±12.8</td>
<td>106.2±1734</td>
<td>0.009</td>
</tr>
<tr>
<td>Cortisol [nmol/L]</td>
<td>467±241</td>
<td>3293±1709</td>
<td>0.142</td>
</tr>
<tr>
<td>Glucagon [ng/L]</td>
<td>72.6±11</td>
<td>94.3±347</td>
<td>0.174</td>
</tr>
<tr>
<td>Matsuda Index</td>
<td>13.7±7.9</td>
<td>3.8±3.7</td>
<td>0.028</td>
</tr>
<tr>
<td>Diencephalic SERT binding [SN-SBR]</td>
<td>0.89±0.32</td>
<td>0.69±0.34</td>
<td>0.036</td>
</tr>
<tr>
<td>Striatal DAT binding [SN-SBR]</td>
<td>4.59±1.06</td>
<td>4.78±1.19</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. | N=9 obese subjects.

Table 2. Insulin sensitive vs insulin resistant obese subjects

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>ISO</th>
<th>IRO</th>
<th>ANOVA</th>
<th>P</th>
<th>F</th>
<th>Iso vs ISO</th>
<th>Iso vs IRO</th>
<th>Iso vs Lean</th>
<th>Iso vs IRO</th>
<th>Iso vs Lean</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age [yr]</td>
<td>30.9±10.5</td>
<td>35.4±10.9</td>
<td>27.2±7.1</td>
<td>0.44</td>
<td>0.08</td>
<td>0.018</td>
<td>1.000</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI [kg/m²]</td>
<td>21.3±1.3</td>
<td>35.5±4.0</td>
<td>38.9±5.3</td>
<td>40.01</td>
<td>40.21</td>
<td>0.413</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose [mmol/L]</td>
<td>4.38±0.39</td>
<td>4.78±0.29</td>
<td>4.76±0.67</td>
<td>0.05</td>
<td>0.70</td>
<td>1.000</td>
<td>0.092</td>
<td>0.372</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin [pmol/L]</td>
<td>23.3±12.8</td>
<td>272±11.2</td>
<td>185.2±163.6</td>
<td>0.01</td>
<td>6.42</td>
<td>0.001</td>
<td>0.014</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol [nmol/L]</td>
<td>481.4±1092</td>
<td>680.0±232</td>
<td>1972±338.3</td>
<td>0.02</td>
<td>5.76</td>
<td>0.100</td>
<td>0.213</td>
<td>0.824</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucagon [ng/L]</td>
<td>72.6±11</td>
<td>151.5±90.3</td>
<td>190.3±29.4</td>
<td>0.03</td>
<td>1.20</td>
<td>1.000</td>
<td>1.000</td>
<td>0.485</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matsuda Index</td>
<td>13.7±7.9</td>
<td>9.3±1.0</td>
<td>3.0±1.8</td>
<td>0.01</td>
<td>10.27</td>
<td>0.002</td>
<td>0.016</td>
<td>0.686</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diencephalic SERT binding</td>
<td>0.89±0.32</td>
<td>0.95±0.35</td>
<td>0.41±0.29</td>
<td>0.02</td>
<td>5.63</td>
<td>0.027</td>
<td>0.032</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Striatal DAT binding</td>
<td>4.59±1.06</td>
<td>4.89±1.62</td>
<td>4.57±0.67</td>
<td>0.09</td>
<td>0.022</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ISO: insulin sensitive obese subjects; IRO: insulin resistant obese subjects. Data are presented as mean ± SD. | N=9 obese subjects.
SERT is an essential part of the serotonergic signal transmission cascade. Although the interaction between SERT, extracellular serotonin, and serotonin signal transmission is dynamic and complex, it is clear that SERT is the driving force of rapid reuptake of extracellular serotonin and thereby an essential regulator of serotonergic neurotransmission (29). However, although acute inhibition of SERT function results in increased levels of extracellular serotonin (30), chronic inhibition of SERT might result in decreased serotonergic signalling, given the detrimental long-term effects of SSRI use on food intake and body weight (31;32). In addition, it has been shown that SERT deficiency in mice results in decreased expression of brain serotonin-2A/C receptors and SERT, while over-expression in mice results in increased brain serotonin-2A/C receptors (33), suggesting that prolonged reduction of SERT function corresponds with lower serotonergic signalling. Since obesity and insulin resistance represent a chronic state of metabolic perturbation, we interpret our finding of lower hypothalamic levels of SERT in the insulin resistant obese subjects as an indication of reduced serotonergic signalling.

To the best of our knowledge, this is the first study examining SERT binding in the human diencephalon in relation to metabolic health, i.e. insulin sensitivity in obesity. Although one rodent study showed that hypothalamic infusion of serotonin results in insulin resistance (34), our results are consistent with a larger number of rodent studies demonstrating that long-term inhibition of SERT induces insulin resistance (11;35), and with human studies indicating an increased risk of type-2 diabetes (T2DM) in subjects on SSRIs (36;37) and an association between a polymorphism in the SERT promoter region and the occurrence of diabetes (14). An association between the use of SSRIs and the occurrence of T2DM might be biased by the relationship between obesity and depression (38). However, Brown, et al showed that SSRIs, but not tricyclic antidepressants, are associated with T2DM, which indicates that modulating brain serotonin activity independently affects glucose metabolism (37).

Our results are consistent with earlier studies demonstrating that modulation of serotonergic signalling by targeting the serotonin-2C receptor or the SERT itself affects glucose metabolism (6-8). While most studies in humans pharmacologically manipulate systemic serotonergic signalling, we focused specifically on SERT in the diencephalon in a stable weight and stable metabolic state condition. Therefore, our results may indicate an independent effect of hypothalamic SERT on glucose metabolism.

The underlying pathways explaining the relationship between insulin resistance and SERT binding remain speculative. The absence of a relationship with BMI precludes a straightforward fat mass-induced reduction in SERT activity. However, the development of insulin resistance in obese humans is a complex interplay of many tissue-specific and circulating factors, including cytokines, adipokines, gut peptides, and hormones, and which of these components contributes to diminished...
SERT activity is unknown. In addition, lower SERT activity itself might induce insulin resistance, as studies in rodents with SSRIs have found that inhibiting SERT activity precedes insulin resistance (14, 34). Subjects with genetically determined lower SERT levels might be more vulnerable to develop insulin resistance during weight gain; however, longitudinal studies in humans are needed to prove this. Surprisingly, the correlation between SERT binding and measures of insulin sensitivity was only present in the obese subjects, suggesting that the interplay between caloric intake, metabolic health, and SERT binding in the diencephalon is altered in obesity. Furthermore, diet composition and diet pattern might contribute to changes in SERT binding in obese insulin resistant individuals, as we recently showed that in lean individuals a Western style diet, i.e. a hypercaloric high-fat-high-sugar snacking diet (39) reduces SERT binding in the diencephalon.

A negative relationship between BMI and cerebral SERT binding has been demonstrated in humans. However, in that study, insulin sensitivity was not measured and it is therefore unknown whether the correlation between SERT and BMI is primary or an epiphenomenon of a correlation between SERT and insulin sensitivity (9). Moreover, in that study SERT availability was assessed in the cortex and subcortex and not specifically in the area of the hypothalamus.

In contrast to the findings for SERT, we did not find a correlation between BMI and striatal DAT binding or insulin sensitivity and striatal DAT binding. Although a recent rodent study reported that both striatal DAT and D2R density are decreased in an animal model of diet-induced obesity (40), our data are consistent with two recently published European multi-centre studies in humans finding no association between BMI and striatal DAT binding in lean and overweight humans (40-42). Whereas DAT binding may not be affected by BMI in humans, we and others have previously reported on lower striatal D2/ D3 dopamine receptors in obese compared to lean individuals (15,16). Moreover, based on previous publications, a relationship between insulin resistance and the dopamine system is evident. Stimulating the dopaminergic system with bromocriptine (a D2R agonist) results in improvements in insulin sensitivity in T2DM patients and is approved by the FDA as an antidiabetic therapy (19;43). Inversely, blocking the D2R in mice that are inherently resistant to the harmful effects of high-fat food induces an unfavourable metabolic profile (44). Dopaminergic signalling, like serotonergic signalling, is complex and highly dynamic. Extracellular dopamine levels are mainly regulated by DAT, but how this relates to D2/3R activity or dopamine release is not completely understood (45). It is unknown whether lower striatal D2/3R activity in human obesity is caused by lower available extracellular dopamine, regulated by DAT, or active down-regulation of the receptors by chronically higher extracellular dopamine. However, the current results make the first mechanism less likely. It might be that the effects of the dopamine system on body weight and insulin sensitivity are postsynaptic rather than presynaptic and that this is not reflected by altered striatal DAT. In addition, it is important to remember that interactions between the serotonergic and dopaminergic signalling pathways might play a significant role in metabolic function (46).

Our study has some limitations: first, it was conducted exclusively in small groups of premenopausal women. Although in our sample, age and SERT binding did not correlate, a negative correlation between SERT binding and age has previously been described (47). Therefore the correlation between SERT and insulin sensitivity might be different for the elderly or the very young. Second, we used mathematic models to calculate insulin sensitivity instead of the hyperinsulenic euglycemic clamp, which is considered the gold standard for measuring insulin sensitivity. However, the Matsuda Index correlates well with clamp-derived insulin sensitivity ($r=0.73$) and therefore provides a reasonable estimation (27). Third, because of the limited spatial resolution of SPECT, we cannot identify the hypothalamic nuclei involved in the observed correlation at this stage. However, we previously measured SERT immunoreactivity in post-mortem human hypothalamic tissue and found that SERT is mainly located in the hypothalamic infundibular nucleus (IFN) and is lower in the IFN of obese compared to lean subjects (48).

CONCLUSION

In obese women, SERT binding in the region of the hypothalamus is positively correlated to insulin sensitivity independently of BMI. It remains to be studied whether lower SERT binding in the presence of obesity induces insulin resistance or whether factors determining metabolic health in human obesity also affect SERT expression in the diencephalon.
ACKNOWLEDGEMENTS

We wish to acknowledge our research assistant Martine van Vessem for her help with the oral glucose tolerance tests and we thank Dr JM Chou-Green of CG Scientific Editing for English editing.

REFERENCES


[27] Matsuda M, Defronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetology Care 1999 September; 22(9):1462-70.
[45] Schmitt KC, Reith ME. Regulation of the dopamine transporter: aspects relevant to SERT, but not DAT, is related to insulin sensitivity.


SERT, but not DAT, is related to insulin sensitivity.
Brain serotonin- and dopamine transporter binding correlates with visual attention bias for food in lean men

Karin E Koopman
Anne Roefs
Danne CE Elbers
Eric Fliers
Jan Booij
Mireille J Serlie
Susanne E la Fleur

Submitted
ABSTRACT

Background: In rodent studies the striatal dopamine (DA) system and the (hypo)thalamic serotonin (5-HT) system are involved in the regulation of feeding behavior. Disturbed feeding behavior is a hallmark of obesity and given the current obesity epidemic it is of importance to understand how the 5-HT and DA systems relate to motivation to eat in humans since this might lead to novel treatment modalities in the battle against obesity.

Methods: We included 36 lean male subjects and measured striatal DA transporter (DAT) and diencephalic SHT transporter (SERT) binding with 11-FP-CIT SPECT. Visual attention bias for high- and low caloric food, a measure of food-related motivation, and degree of impulsivity were measured using response-latency based computer tasks. Craving and emotional eating were assessed with questionnaires and ratings of hunger by means of VAS scores. Ad libitum food intake was assessed through a self-reported online dietary intake list.

Results: Striatal DAT and diencephalic SERT binding positively correlated with visual attention bias for food (p=0.004; r=0.54 and p=0.001; r=0.60, respectively), but not with ratings of hunger, craving or impulsivity. Striatal DAT and diencephalic SERT binding did not correlate with ad libitum food intake while visual attention bias for food positively correlated with total caloric intake (p=0.001; r=0.63), protein intake (p=0.005; r=0.49), carbohydrate intake (p=0.03; r=0.38) and fat intake (p=0.01, r=0.44) indicating that a faster response to a visual food stimulus correlates with higher food intake.

Conclusion: These results confirm a role for the 5-HT and DA system in the regulation of motivational feeding behavior in non-obese, healthy humans. In addition, this study confirms that attention bias for food measured with the latency-based computer task positively correlates with total food and macronutrient intake.

INTRODUCTION

The brain plays a critical role in regulating food intake and the amount of consumed food is determined by hunger and satiety signals as well as by the accessibility and the rewarding properties of food and a person’s motivation to obtain food. One of the brain neurotransmitters involved in the latter aspect is striatal dopamine (DA). Although in literature striatal DA is often used as a surrogate for hedonism, the striatal DA system is in fact related to the motivation to seek a reward and not so much to the hedonic value of the reward (1;2). Rodent studies have shown a relationship between food-related motivational behavior and the striatal DA system. Thus, stimulating DA release with amphetamine in rats specifically amplified incentive salience for a food reward but not hedonic ‘liking’ (3) and motivation to obtain a food reward is reduced when DA antagonists are administered in the striatum of rats (4). Moreover, food itself as well as the sight or smell of food serve as stimuli for striatal DA release (5;6). The relationship between food motivated behavior and the striatal DA system in humans however has not been investigated in depth.

A method to assess motivational impact of rewarding stimuli in humans is by measuring visual attention for the specific stimulus (7), and recently computerized tests to assess visual attention bias for food stimuli became available. Since then, it has repeatedly been shown that visual attention bias for food-related stimuli is reduced in obesity, indicating that obese subjects respond faster to food related cues (8-10). It has however consistently been shown that obese subjects have decreased striatal DA D2/3 receptor binding (11-13), but antagonizing the DA D3 receptor did not affect visual attention bias for food in overweight and obese humans (14). Another study showed that striatal DA D2 receptor availability measured with positron emission tomography (PET), which is indicative for extracellular dopamine, increased after non-hedonic food stimulation (display of food without consumption), indicating that DA in the human striatum is indeed involved in motivation to obtain food (15). Direct evidence for a relationship between visual attention bias for food stimuli as a measure of motivational impact of a food stimulus and the striatal DA system in healthy, lean humans is lacking.

Whereas the striatal DA circuit is only involved in motivational behavior to obtain food, the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) in the hypothalamus has been related to homeostatic regulation of hunger and satiety signalling (16). The hypothalamic 5-HT system has extensively been linked to overfeeding and excess weight in animals and humans. For instance depleting hypothalamic 5-HT in rodents causes uncontrollable hyperphagia and obesity (17-19) and a hypercaloric high fat high sugar-snacking diet reduces diencephalic SHT-transporters (SERT) in humans (20). Brain SERT binding may be decreased in obese humans (21;22) and mutations in the 5-HT 2A and 2C receptor promoter gene are associated with obesity (23-25). In contrast to the extensive data on 5-HT in overfeeding or obesity, limited data are available on its potential
regulatory role in a non-obese condition. Serotonergic drugs such as the 5-HT 2C receptor agonist lorcaserin, currently in use as an anti-obesity drug, suppress hunger and increase satiety in obese but also in lean humans (26) resulting in significant weight loss (27). It has not been investigated whether in lean humans there is an association between the hypothalamic 5-HT system, ad libitum food intake and food-intake-related behavioural outcomes.

We hypothesized that striatal DA transporter (DAT) binding relates to visual attention for food as a measure of food-related motivation, and that SERT binding in the diencephalon, a brain region including thalamus and hypothalamus, relates to hunger. Moreover, we hypothesized that both striatal DAT and hypothalamic SERT binding would be related to total food intake. To test these hypotheses we measured striatal DAT and diencephalic SERT binding with 123I-FP-CIT SPECT and correlated these outcomes with ad libitum food intake, visual attention bias for food, hunger feelings, impulsivity and craving.

MATERIALS AND METHODS

The subjects described in this study were included in a hypercaloric diet intervention trial. The effects of the hypercaloric diet intervention on metabolism, liver fat and diencephalic SERT binding were published previously (20;28).

STUDY POPULATION

A total of 36, healthy, male subjects with a mean age of 22.2±2.5 years and a mean body mass index (BMI) of 22.3±1.4 kg/m² were included in the current analyses. Exclusion criteria were a history of psychological- or psychiatric disorders, eating disorders assessed with the Eating Disorders Examination Questionnaire [EDE-Q] (29;30), substance and alcohol abuse, unstable weight, any medical condition, performance of excessive sports, shift work and use of any medication. The study was approved by the institutional review board of the AMC, Amsterdam. Written informed consent was received from all participants prior to the start of study participation.

IMAGING OF DAT AND SERT

After an overnight fast subjects underwent a single photon emission computed tomography (SPECT) scan after intravenous administration of 115 MBq 123I-FP-CIT (range 110-120 MBq; specific activity > 750 MBq/mmol; radiochemical purity > 98%, produced according to GMP criteria at GE Healthcare, Eindhoven, The Netherlands) measuring SERT binding 2 hours and DAT binding 3 hours after injection, as these are the optimal time points for visualizing SERTs and DATs with 123I-FP-CIT SPECT (31;32). Each participant was pre-treated with potassium iodide to block thyroid uptake of free radioactive iodide. SPECT imaging was performed using a 12-detector, single slice brain-dedicated scanner (Neurofocus Inc, Medfield, MA, USA), using an acquisition protocol as described earlier with slight modifications (interslice distance 5 mm, acquisition time 210 sec per slice) (31). All scans were reconstructed in 3D and corrected for attenuation. For quantification, a region-of-interest (ROI) analysis to determine specific binding activity in the diencephalon and striatum was performed by a well-trained researcher and confirmed by a second analyst as described earlier (31). Briefly, the 4 consecutive slices with the highest binding were selected in the diencephalon (including hypothalamus/thalamus) to assess binding to SERT and in the striatum to assess binding to DAT. Activity in the cerebellum (3 consecutive slices) was assumed to represent non-displaceable activity (nonspecific binding and free radioactivity). A specific-to-nonspecific binding ratio [SNS-BR] was calculated as [ROI-binding minus cerebellar-binding / cerebellar-binding]. The SNS-BR was used as the outcome measure. Due to a technical problem with the SPECT scanner scans of 6 subjects had to be excluded from the analyses, therefore data on DAT and SERT binding were available in 30 subjects.

NEUROPSYCHOLOGICAL TASKS AND QUESTIONNAIRES

The tasks and questionnaires were selected to assess functions associated with the brain 5-HT- and DA-ergic systems and were performed and filled in after an overnight fast of approximately 12 hours, at the same day on which the SPECT scan was performed.

1. Delay Discounting Task: a computerized task for assessment of impulsive decision making. Subjects choose between a smaller immediate reward (money) or a larger future reward. The future reward ranged from two weeks to 10 years from now. When subjects are inclined to choose the smaller, immediate amount of money over large, delayed money, they are characterized by greater levels of impulsivity. A detailed description of this task was previously published (33). Behavioural performance was analysed by plotting the indifference points (the points where immediate and delayed reward were equally valued) at different time points and the area under the curve was used as a behavioural outcome (34).

2. Food related Visual Search Task: a computerized task that measures visual attention or distraction for a particular object (target) among other objects (distractors). In the current study we measured visual attention with high caloric- or low caloric food as target and the distractors were bags or balls. To measure distraction by food the situation was reversed: the targets were balls or bags and the distractors were low caloric- or high caloric food. The visual search task was programmed using E-prime software (Eprime 1.0; Psychology Software Tools Inc, Pittsburg; PA, USA). Participants initially completed 12 practice trials (with pictures of tools and clocks) followed by 154 experiment trials. The task lasted approximately 15 minutes and was split into two blocks of trials, separated by a brief break. Each trial started with a brief tone, followed by a fixation cross (500 ms). Then a 5 × 4 matrix containing 20 visual stimuli was presented. The participant was instructed to respond as quickly and as accurately as possible, and to press the right key in case of an odd-one-out (one picture in a category dif-
bird from the other 19 pictures), and the left key in case of no odd-one-out (20 pictures in the same category). The matrix remained on screen until the participant responded or for a maximum of 20 s. Then a new trial started. We assessed both reaction time (RT) and accuracy. Trials with an inaccurate response were not included in the analyses. RT outliers were excluded from the analyses if less than 200 ms, greater than 2000 ms or greater than 3 standard deviations above each participant’s mean. RTs for high caloric- and low caloric tar-gets were corrected for RT for a neutral target by subtraction and likewise, RTs for distraction by high caloric- and low caloric objects were corrected for RT for neutral objects by subtraction. The RTs for attention and distraction corrected for the RT for neutral targets are called the visual attention or distraction bias. A negative attention bias means that subjects responded faster to a food target than to a neutral target.

3. Dutch Eating Behaviour Questionnaire (DEBQ): a 33-item self-report for assessment of re-stained, emotional and external eating behaviour [35].

4. The Dutch 31-item version of the Barratt Impulsiveness Scale (BIS-11), scored on a four-point scale, was used for assessment of self-reported impulsiveness [36].

5. General Food Craving Questionnaire-Trait (G-FCQ-T): a four-factor, 21-item questionnaire to assess general trait food cravings [37].

6. Hunger and Appetite: assessed with 100 mm visual analogue scales (VAS) anchored at each end with descriptive extremes (e.g. extremely hungry – not hungry at all). Subjects were required to mark a point on the scale within the anchored points, which related to how hungry they felt.

**FOOD INTAKE**

We assessed food intake with an online diet journal designed by the Dutch Nutrition Centre (eet-meeter.voedingscentrum.nl). Participants reported their ad libitum food intake at this website during 7 days prior to the measurements. Reported intake was compared to the calculated eucaloric need (1.4x measured energy expenditure using indirect calorimetry) to exclude underreporting. In addition we measured body weight before- and after this week, which had to be stable, to validate that the reported intake was eucaloric and weight-maintaining. We suspected underreporting of ad libitum food intake in 2 subjects (reported intake about 1000 kcal lower than expected based on energy expenditure measurements) Therefore food intake data of these 2 subjects were excluded and analyses on food intake were performed on data of 34 subjects.

**STATISTICS**

Statistical analyses were performed using SPSS version 20.0 (IBM SPSS, Chicago, Illinois, USA). Data were tested for normality. To evaluate correlations between the studied parameters, we first performed univariate linear regression analysis. We subsequently performed forward multivariate analysis with the rule that the maximum amount of variables was no more than 10% of the ana-lysed amount of observations. In most cases this meant that the maximum amount of simultaneously entered variables was 2 or 3. We tested for multicollinearity in all the multivariate analyses. There was multicollinearity between different visual attention biases (high caloric, low caloric and total) and between total caloric intake and specific macronutrient intake and therefore these parameters could not be analysed in the same model. We performed multivariate analysis to detect whether outcomes had independent associations with (1) striatal DAT and diencephalic SERT binding and (2) visual attention bias for high caloric food and low caloric food. Correlations were considered as significant when was p<0.05 and as a trend when p<0.1.

**RESULTS**

Baseline characteristics of the study participants are presented in Table 1.

**UNIVARIATE CORRELATIONS WITH STRIATAL DAT AND DIENCEPHALIC SERT BINDING**

Striatal DAT binding was significantly associated with visual attention bias for food in general (high caloric + low caloric food, figure 1A), with visual attention bias for low caloric food and with REE (kCal/kg) (Table 2). Subjects with lower striatal DAT binding responded faster to a visual food stimulus and vice versa. Visual attention bias for high caloric food and BMI tended to be associat-ed with striatal DAT binding (Table 2). In addition, VAS hunger score positively correlated with

<table>
<thead>
<tr>
<th>Table 1. Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
</tr>
<tr>
<td>Age (yrs)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>Weight (kg)</td>
</tr>
<tr>
<td>REE (kCal/kg)</td>
</tr>
<tr>
<td>striatal DAT availability (SNS-BR)</td>
</tr>
<tr>
<td>diencephalic SERT availability (SNS-BR)</td>
</tr>
<tr>
<td>Total caloric intake (kCal)</td>
</tr>
<tr>
<td>Carbohydrate intake (gram)</td>
</tr>
<tr>
<td>Carbohydrate intake (% of total caloric intake)</td>
</tr>
<tr>
<td>Fat intake (gram)</td>
</tr>
<tr>
<td>Fat intake (% of total caloric intake)</td>
</tr>
<tr>
<td>Protein intake (gram)</td>
</tr>
<tr>
<td>Protein intake (% of total caloric intake)</td>
</tr>
</tbody>
</table>

Data presented as mean ± SD. Measures were performed in fasting condition. Caloric and macronutrient intake presented as the average daily intake based on the weekly intake. BMI = body mass index; REE = resting energy expenditure; DAT = dopamine transporter; SERT = serotonin transporter; SNS-BR = specific to nonspecific binding ratio.
striatal DAT binding (Table 2). The delay discounting task, a measure for impulsivity and questionnaires on impulsivity, craving and appetite did not correlate with striatal DAT binding (Table 2). Diencephalic SERT binding was positively correlated with REE (Table 2) and attention bias for food in general (high caloric + low caloric food, figure 1B), as well as with attention bias for high and low caloric food separately (i.e. lower SERT binding was associated with a faster response to a visual food stimulus). Impulsive decision making as measured with the delay discounting task tended to be negatively correlated with diencephalic SERT binding (Table 2). BMI and the other tasks and questionnaires did not correlate with diencephalic SERT binding (Table 2). Moreover, striatal DAT binding correlated with diencephalic SERT binding (p=0.008, r=-0.48, r²=0.23).

**Table 2. Associations with striatal DAT and diencephalic SERT availability using univariate linear regression analysis**

<table>
<thead>
<tr>
<th>Striatal DAT binding</th>
<th></th>
<th></th>
<th>Diencephalic SERT binding</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>0.34</td>
<td>0.17</td>
<td>0.26</td>
<td>0.20</td>
<td>0.05</td>
</tr>
<tr>
<td>REE (kCal/kg)</td>
<td>0.17</td>
<td>0.02</td>
<td>0.17</td>
<td>0.20</td>
<td>0.05</td>
</tr>
<tr>
<td>Total caloric intake (kCal)</td>
<td>0.12</td>
<td>0.01</td>
<td>0.20</td>
<td>0.20</td>
<td>0.04</td>
</tr>
<tr>
<td>Carbohydrate intake (grams)</td>
<td>0.20</td>
<td>0.07</td>
<td>0.17</td>
<td>0.15</td>
<td>0.02</td>
</tr>
<tr>
<td>Protein intake (grams)</td>
<td>0.11</td>
<td>0.01</td>
<td>0.56</td>
<td>0.22</td>
<td>0.05</td>
</tr>
<tr>
<td>Attention bias food in general (ms)</td>
<td>0.54</td>
<td>0.29</td>
<td>0.004</td>
<td>0.60</td>
<td>0.36</td>
</tr>
<tr>
<td>Attention bias high caloric food (ms)</td>
<td>0.37</td>
<td>0.13</td>
<td>0.06</td>
<td>0.55</td>
<td>0.30</td>
</tr>
<tr>
<td>Attention bias low caloric food (ms)</td>
<td>0.60</td>
<td>0.36</td>
<td>0.001</td>
<td>0.54</td>
<td>0.29</td>
</tr>
<tr>
<td>Distraction bias food in general (ms)</td>
<td>0.09</td>
<td>0.00</td>
<td>0.004</td>
<td>0.05</td>
<td>0.00</td>
</tr>
<tr>
<td>Delay Discounting</td>
<td>-0.09</td>
<td>0.01</td>
<td>0.71</td>
<td>-0.36</td>
<td>0.13</td>
</tr>
<tr>
<td>BIS</td>
<td>0.06</td>
<td>0.00</td>
<td>0.26</td>
<td>0.17</td>
<td>0.03</td>
</tr>
<tr>
<td>craving (GFCQ-I)</td>
<td>0.14</td>
<td>0.02</td>
<td>0.48</td>
<td>0.13</td>
<td>0.02</td>
</tr>
<tr>
<td>VAS Hunger score</td>
<td>0.36</td>
<td>0.13</td>
<td>0.05</td>
<td>0.25</td>
<td>0.06</td>
</tr>
<tr>
<td>VAS appetite score</td>
<td>0.22</td>
<td>0.09</td>
<td>0.12</td>
<td>0.31</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**MULTIVARIABLE PREDICTION MODEL FOR STRIATAL DAT AND DIENCEPHALIC SERT BINDING**

The multivariable model for striatal DAT binding, including all variables with p<0.1 in the univariate analysis, predicted almost 50% of the variation in striatal DAT binding (p=0.01, r²=0.47), and visual attention bias for food was the only significant independent predictor of striatal DAT binding (p=0.02). A similar multivariable model for diencephalic SERT binding, including all variables with p<0.1 in the univariate analysis, predicted 45% of the variability in diencephalic SERT binding (p=0.005; r²=0.45) and visual attention bias for food was the only significant independent predictor (p=0.006).

**MULTIVARIABLE PREDICTION MODEL FOR VISUAL ATTENTION BIAS**

To detect independent associations with visual attention bias we performed multivariable analysis with diencephalic SERT binding, striatal DAT binding and BMI as independent variables. Visual attention bias for food in general was for 40% predicted by this model (p=0.009, r²=0.40) however none of the variables showed independent predictive value (SERT p=0.06, DAT p=0.20, BMI p=0.69). Subsequently we tested predictive value of the above-mentioned variables with p<0.1 in the univariate analysis, predicted almost 50% of the variation in striatal DAT binding (p=0.01; r²=0.47), and visual attention bias for food was the only significant independent predictor of striatal DAT binding (p=0.02). A similar multivariable model for diencephalic SERT binding, including all variables with p<0.1 in the univariate analysis, predicted 45% of the variability in diencephalic SERT binding (p=0.005; r²=0.45) and visual attention bias for food was the only significant independent predictor (p=0.006).
variables on visual attention bias for low caloric and high caloric food pictures. Visual attention bias for low caloric food was for 41% predicted by this model (p=0.007; r=0.64; r²=0.41) and only striatal DAT binding was independently correlated with visual attention bias for low caloric food (p=0.045) corrected for diencephalic SERT binding (p=0.22) and BMI (p=0.48). Visual attention bias for high caloric food was for 30% predicted by this model (p=0.038 r=0.55; r²=0.30) and only diencephalic SERT binding was independently correlated with visual attention bias for high caloric food (p=0.030) corrected for striatal DAT binding (p=0.83) and BMI (p=0.72).

**DISCUSSION**

We show that striatal DAT binding and diencephalic SERT binding significantly correlated with attention bias for visual food stimuli in lean, healthy men on a eucaloric ad libitum diet. Lower striatal DAT and diencephalic SERT binding corresponded with a faster response to a visual food stimulus. In addition, attention bias for visual food stimuli correlated with total food and individual macronutrient intake while striatal DAT and diencephalic SERT binding did not independently correlate with food intake. Taken together, our findings point to a physiological role for DA and 5-HT signaling in attention for food, whereas factors in addition to DA- and 5-HT signaling are involved in the actual food intake.

The positive correlation between striatal DAT binding and visual attention bias for food, indicates that lower striatal DAT binding is associated with a high visual attention bias. DATs are located presynaptically in dopaminergic neurons and facilitate reuptake of DA from the synaptic cleft (38) and thus lower DAT binding could reflect higher available extracellular concentrations of DA suggesting that more available dopamine induces a higher visual attention bias for food in the fasting condition. In line, mice in which DAT expression is genetically downregulated to 10% of expression levels in wild type mice have increased extracellular DA concentrations in the striatum and hypothalamus (39;40). Interestingly the mice with lower DAT (and thus higher extracellular DA) show higher intake when the cost for food was increased reflecting higher motivation and willingness to work (39). In addition, upon striatal DA depletion animals do not feed at all demonstrating that DA is essential for motivation for food in general (41). Translating these data from rodents to the findings in our lean subjects suggest that the increased visual attention for pictures of food could reflect higher motivation for food through higher striatal dopamine. In contrast to lower DAT binding reflecting higher extracellular DA, striatal DAT binding is reduced after long term systemic DA depletion (42,43) and therefore we cannot draw firm conclusions on striatal extracellular dopamine concentrations in relation to our measured outcomes. Moreover, our results may only apply to a non-obese condition and non-pathological feeding behavior since in obesity striatal DA...
release is reduced in response to a food-related stimulus, resulting in a higher motivation to eat while DAT binding was not shown to be altered in the obese state (12,44).

Interestingly, when analysing visual attention bias for food in more detail, striatal DAT binding was related to visual attention bias for low caloric and not high caloric food. Given the role of DA in reward pathways and the direct effects of eating fat on striatal DA receptor binding in male Wistar rats (45), this finding is unexpected and suggests that the striatal DA system relates more to preferred foods and not to palatability of food.

Visual attention bias for food also correlated with diencephalic SERT binding, showing that subjects with the lowest SERT binding responded fastest to a visual food stimulus. Similar to striatal DAT binding, lower SERT binding could reflect higher or lower extracellular 5-HT levels. It has been shown that SERT knockout mice have increased striatal extracellular 5-HT and greater depolarization-induced increases in striatal 5-HT (46), as well as reduced food intake (47). However, these mice also have reduced serotonin 2A/C receptors, suggesting that there may be reduced 5-HT signaling (48). In humans short term inhibition of SERT with selective serotonin reuptake inhibitors (SSRIs) and thus increased extracellular serotonin levels, results in reduced food intake (26). However, long term SSRI use results in body weight (re)gain (49,50) and in obese subjects, representing a chronic state of excessive food intake and a high visual attention bias for food (8-10), SERT is reduced (21,22) suggesting that chronically low SERT availability is associated with decreased extracellular 5-HT. This is in line with our finding showing that in weight stable healthy, lean humans visual attention bias for food positively correlates with food intake and inversely with diencephalic SERT binding and our previous findings on lowering of SERT binding after a hypercaloric high fat high sugar snacking diet (20). In summary, although most data point towards lower SERT binding was measured in the diencephalon which contains both the hypothalamus and thalamus, and while the hypothalamus exerts homeostatic control on hunger and satiety, the (paraventricular nucleus of the) thalamus is considered a relay between the hypothalamus and limbic structures involved in reward behavior and receives dense 5-HT projections (51,52). We thus speculate that the correlation between SERT binding and visual attention bias for high caloric food relates to 5-HT’s involvement in reward behavior. Due to limited spatial resolution of current available neuroimaging techniques, it is not possible to disentangle SERT binding in the paraventricular nucleus of the thalamus from SERT binding in the surrounding structures.

Further, SERT binding was specifically correlated with visual attention bias for high but not low caloric food pointing to relation with rewarding rather than homeostatic aspects of food. Indeed, SERT binding was measured in the diencephalon which contains both the hypothalamus and thalamus, and while the hypothalamus exerts homeostatic control on hunger and satiety, the (paraventricular nucleus of the) thalamus is considered a relay between the hypothalamus and limbic structures involved in reward behavior and receives dense 5-HT projections (51,52). We thus speculate that the correlation between SERT binding and visual attention bias for high caloric food relates to 5-HT’s involvement in reward behavior. Due to limited spatial resolution of current available neuroimaging techniques, it is not possible to disentangle SERT binding in the paraventricular nucleus of the thalamus from SERT binding in the surrounding structures.

Finally, we show that visual attention bias for food (both high caloric and low caloric) correlates with actual food intake. The correlation is the strongest for total caloric intake, but the three main macronutrients (fat, carbohydrates and protein) also correlate with visual attention bias for high caloric food, whereas only fat intake correlates with visual attention bias for low caloric food. Werthmann et al previously showed that a high visual attention bias for chocolate leads to higher chocolate intake (53) but to our knowledge we are the first to show a more general relationship between visual attention bias for food and recorded food intake. Our study was conducted in healthy, young, Caucasian men and therefore the results might be different for older subjects, female subjects or subjects of other ethnicities.

CONCLUSION

We show that striatal DAT binding and diencephalic SERT binding correlate with measures of motivational feeding behavior in non-obese, healthy conditions, in line with a key role for dopamine and serotonin in the motivation to eat. At present it is unknown how SERT and DAT binding in imaging studies under fasting conditions relate to extracellular concentrations of 5-HT and dopamine, respectively. Therefore further experimental studies are needed to elucidate their respective roles in feeding behaviour in humans. In addition, changes in SERT and striatal DAT might predispose to disturbed feeding behaviour and might be a target for eating disorders, obesity included.

ACKNOWLEDGEMENTS

We wish to acknowledge Lynne S. Wolbert and Elsmarieke van de Giessen for their assistance with the computer tasks and questionnaires.
Castellanos EH, Charboneau E, Dietrich MS, Park S, Bradley BP, Mogk K et al. Obese adults have visual attention bias for food cue images: evidence for altered reward system function. Int J Obes (Lond) 2009 September;33(9):1063-73.


Luquet S, Magnan C. The central nervous system at the core of the regulation of energy homeostasis. Front Biosci (Schol Ed) 2009;4:1486-65.

Breisch ST, Zemlan FP, Hoebel BG. Hyperphagia and obesity following serotonin depletions by intraventricular p-chlorophenylalanine. Science 1976 April 1;192(4237):382-5.


Schmitt KC, Reith ME. Regulation of the dopamine transporter: aspects relevant to...


(45) van de Giessen E, de Fleur SE, de Beker, van den Brink W, Bossuyt JS, Free-choice and no-choice high-fat diets affect striatal dopamine D2/3 receptor availability, caloric intake, and adiposity. Obesity (Silver Spring) 2012 August 20;20(8):1738-40.


(52) Parsons MP, Li S, Kirovic GJ. The paraventricular nucleus of the thalamus as an interface between the orexin and CART peptides and the shell of the nucleus accumbens. Synapse 2006 June 15;59(8):480-90.

PART 2:

NEURAL AND METABOLIC EFFECTS OF HYPERCALORIC DIETS IN LEAN HUMANS
Diet-induced changes in the lean brain: hypercaloric high-fat-high-sugar snacking reduces serotonin transporters in the hypothalamic region in lean men

Karin E Koopman
Jan Booij
Eric Fliers
Mireille J Serlie
Susanne E la Fleur

Molecular Metabolism
2013 Aug 7;2(4):417-22
ABSTRACT

**Background:** It is evident that there is a relationship between the brain’s serotonin system and obesity. Although it is clear that drugs affecting the serotonin system regulate appetite and food intake, it is unclear whether changes in the serotonin system are cause or consequence of obesity.

**Methods:** To determine whether obesogenic eating habits result in reduced serotonin transporter (SERT) binding in the human hypothalamic region, we included 25 lean, male subjects who followed a 6-weeks-hypercaloric diet, which were high-fat-high-sugar (HFHS) or high-sugar (HS) with increased meal size or -frequency (=snacking pattern). We measured SERT binding in the hypothalamic region with SPECT.

**Results:** All hypercaloric diets significantly increased body weight by 3-3.5%. Although there were no differences in total calories consumed between the diets, only a hypercaloric HFHS-snacking diet decreased SERT binding significantly by 30%.

**Conclusion:** We here show for the first time in humans that snacking may change the serotonergic system increasing the risk to develop obesity.

---

INTRODUCTION

Obesity is a worldwide health problem of epidemic proportions and is a consequence of an ongoing dysbalance in energy intake and energy expenditure. Why so many people are not capable to balance their energy metabolism is still poorly understood. To target the obesity epidemic, it is important to understand the mechanisms underlying the chronic intake of energy surplus. The hypothalamus is viewed as the centre of feeding regulation [1] and it has been shown that brain circuits involved in food intake and appetite control are disturbed in rodent models of obesity. Besides the hypothalamic control of food intake, nuclei in the brainstem and the mesolimbic cortex are also involved in this complex regulatory system [2].

The neurotransmitter serotonin innervates most of these brain structures and is therefore a likely candidate to coordinate hunger and satiety signalling to and from the periphery. Indeed, serotonin acts as an anorexigenic signal: food intake increases both extracellular serotonin levels and serotonin turnover in the brain while serotonin inhibits food intake and promotes satiety [3,4]. In addition, neurochemical depletion of brain serotonin, as well as genetic and pharmacological manipulations of several parts of the serotonin system in rodents, results in obesity and hyperphagia [5-7].

Serotonin transporters (SERTs) are determinants of serotonin bioavailability in the synaptic cleft and are thereby important regulators of serotonergic transmission by facilitating reuptake of serotonin from the synaptic cleft into the presynaptic neuron. After reuptake, serotonin can be released into the cleft again and stimulate signal transmission [8]. Lower cerebral SERT binding was reported in an obese rat model [9] while in humans cerebral SERT binding measured with PET was inversely related to body mass index (BMI) [10]. In line with these findings, a SERT promoter polymorphism has been identified as risk factor for obesity [11]. Finally, methylation of the SERT promoter correlates with obesity measures in monozygotic twins [12]. Interestingly, drugs that increase extracellular serotonin via inhibition of SERT reduce food intake and result in a decrease in body weight in both animals [13] and in humans [4]. However, long-term use of selective serotonin reuptake inhibitors (SSRIs) is associated with obesity [14,15]. The mechanisms underlying this difference remain unclear.

Thus, lower SERT is clearly associated with obesity; however whether this is cause or consequence, and whether this is explained by BMI per se or excessive food intake remains to be determined. As obesity may result from either change in meal pattern and/or meal composition we here studied the relationship between cerebral SERT and different patterns of excessive food intake. We therefore measured cerebral SERT with SPECT before and after a 6 weeks hypercaloric diet intervention in lean men.
**MATERIALS AND METHODS**

**SUBJECTS**
We included 39 healthy lean men (age 22.6 ± 3.5 years; BMI 22.4 ± 1.5 kg/m²). All subjects had normal insulin sensitivity defined as HOMA-IR <2.5. Subjects had no history of neuropsychiatric or eating disorders and did not use any medication. They were not known with substance abuse and did not perform shift work. All subjects had healthy eating behaviour assessed via an online diet journal (eetmeter.voedingscentrum.nl) defined as a eucaloric diet containing ~45-50% carbohydrates, ~30-35% fat and ~15-20% protein. The study was approved by the medical ethics committee of the AMC Amsterdam. Written informed consent was received from all participants prior to the start of study participation.

**DIETS:**
Subjects were randomised into one of 4 hypercaloric diet groups or a control group. Subjects in the control group (N=5) underwent all the measurements but did not follow a diet. All hypercaloric diets were based on a 40% caloric surplus on top of the ad libitum diet. The diet was followed for 6 consecutive weeks. Subjects were contacted weekly. Randomisation is displayed in Figure 1. The hypercaloric diet groups were:

1. High-fat high-sugar (HFHS) diet using a liquid meal (Nutridrink Compact, Nutricia® Advanced Medical Nutrition; Zoetermeer, The Netherlands) 3 times a day to be consumed together with the 3 daily main meals. This group represents the HFHS-increased meal size (HFHS-S) group.
2. High-fat high-sugar (HFHS) diet using a liquid meal (Nutridrink Compact, Nutricia®) 3 times a day in between the 3 daily main meals (2 to 3 hours after each meal). This group represents the HFHS-increased meal frequency (HFHS-F) group.
3. High-sugar (HS) diet using commercially available sugar-sweetened beverages 3 times a day to be consumed together with the 3 daily main meals. This group represents the HS-increased meal size (HS-S) group.
4. High-sugar (HS) diet using commercially available sugar-sweetened beverages 3 times a day in between the 3 daily main meals (2 to 3 hours after each meal). This group represents the HS-increased meal frequency (HS-F) group.

The nutritive value of Nutridrink Compact® was as follows: 240 kcal/100 ml; 16 En% protein (mainly casein), 47 En% carbohydrates (mainly maltose and polysaccharides) and 35 En% fat (mainly mono- and polyunsaturated fat). As high-sugar liquids, the subjects consumed commercially available soft drinks. A list of drinks with comparable nutritive value of which subjects could choose their favourite beverage was used. The soft drinks contained no fat or protein and were mainly sweetened with sucrose. The ad libitum diet was monitored online. Subjects registered their daily food consumption on a website (eetmeter.voedingscentrum.nl). When caloric intake was lower than caloric need, assessed on measured resting energy expenditure (REE), subjects were instructed to increase their healthy ad libitum diet.
**RESTING ENERGY EXPENDITURE (REE)**

REE was measured by use of indirect calorimetry. VO\textsubscript{2} and VCO\textsubscript{2} were measured in the supine position during 30 min using a ventilated hood system (Sensor Medics, Vmax Encore 29N, Anaheim, CA). REE and respiratory exchange ratio (RER) were calculated as described previously [16]. The abbreviated Weir equation was used to calculate the 24-hour energy expenditure.

**SPECT IMAGING**

After an overnight fast subjects underwent a single photon emission computed tomography (SPECT) scan 2 h after intravenous administration of 115 MBq \textsuperscript{123}I-FP-CIT (range 110-120 MBq; specific activity > 750 MBq/mmol; radiochemical purity > 98%, produced according to GMP criteria at GE Healthcare, Eindhoven, The Netherlands). In a previous study, we showed that this is the optimal time-point to measure SERT with \textsuperscript{123}I-FP-CIT SPECT [17]. Each participant was pretreated with potassium iodide to block thyroid uptake of free radioactive iodide. SPECT imaging was performed using a 12-detector, single slice brain-dedicated scanner (Neurofocus), using an acquisition protocol as described earlier with slight modifications (interslice distance 5 mm, acquisition time 210 sec per slice) [18]. All scans were reconstructed in 3D and corrected for attenuation.

**IMAGE ANALYSIS**

For quantification, a ROI analysis was performed to determine specific binding activity in the diencephalon, which includes the hypothalamus and thalamus, as described previously [18]. Briefly, the 4 consecutive slices with the highest diencephalic binding were selected to assess binding to SERT. Activity in the cerebellum (3 consecutive slices) was assumed to represent non-displaceable activity (nonspecific binding and free radioactivity). Finally, a specific-to-nonspecific binding ratio was calculated as activity in ROI minus cerebellar binding/cerebellar binding [19]. This ratio was used as the outcome measure.

**STATISTICS**

For body weight and food intake data we used a 1-way ANOVA to determine overall effects. If a significant result was found, posthoc analysis (Students t-test) was performed with Bonferroni correction to compare data between the different groups. For analysis of SERT binding changes within the hypercaloric diet groups, we used 2 way ANOVA’s to determine overall effects. If significant effects were determined, we used paired student t-tests to compare the data before and after the diet intervention within the different hypercaloric diet group.

**Figure 2**

Body weight gain and food intake. HFHS = high-fat-high-sugar; HS = high-sugar. S = increased meal size; F = increased meal frequency.

2A) All hypercaloric diet groups showed significantly increased body weight compared to the control group. There were no differences in body weight gain between the hypercaloric diet groups (data are depicted in mean ±SEM; *p<0.01).

2B) Caloric intake in kCal/day during the intervention period: Ad libitum intake is depicted in light gray and added HFHS or HS calories are depicted in dark gray. Total intake, and also ad lib and diet intake were similar in all hypercaloric diet groups, whereas intake for all hypercaloric diet groups were increased compared to the control group (data depicted as mean ± SEM; ***p<0.0001).

2C) Carbohydrate- and fat intake in kCal/day displayed as mean and SEM. Subjects on HFHS consumed more fat and carbohydrate calories, but equal fat calories, compared to controls. No significant differences were detected between HFHSS and HFHSS, and between HSS and HS-F (data depicted as mean ± SEM; ***p<0.0001).
RESULTS

RANDOMIZATION AND BODY WEIGHT
39 subjects were randomised and completed the 6-week diet period. Two subjects were excluded because compliance with the diet was uncertain and one subject was excluded because of excessive alcohol consumption during the days prior to the second study day. In 6 subjects the SPECT images could not be used because of technical failure. Randomisation and drop-out rate is displayed in Figure 1. The final data analyses were performed on 30 subjects with a mean age of 22.1±2.5 years and a mean BMI of 22.5±1.3 kg/m². Baseline characteristics are described in Table 1. Subjects in all 4 diet groups significantly gained body weight compared to the control group (p<0.0001). Control subjects consumed a eucaloric diet of ~2400 kcal/day consisting for ~45-50% of carbohydrates, ~30-35% of fat and for ~15% of protein. As expected, the subjects in the HS groups consumed excessive carbohydrates (~60% of total caloric intake; p<0.0001 compared to baseline diet), which was significantly higher compared to the control and HFHS group (p<0.0001). Absolute intake of protein (~103 gram per day at baseline versus ~112 gram per day during the diet, p<0.02), and fat (~98 gram per day at baseline versus 112 gram per day during the diet, p<0.0001), were higher during the HS diet. However, due to excessive carbohydrate intake, relative intake of protein (~15% at baseline versus ~12% during the diet, p<0.0001) and fat (~35% at baseline versus ~27% during the diet p<0.0001) were decreased. There was no difference in carbohydrate intake between both HS groups (p=0.33).

Subjects in the HFHS groups consumed both excessive fat (~94 gram per day at baseline versus ~147 gram per day during the diet, p<0.0001) and carbohydrates (~297 gram per day at baseline versus ~441 gram per day during the diet, p<0.0001) with no difference between the HFHS groups (p=0.35). Both HFHS groups had a higher absolute intake of protein (~98 gram per day before versus ~146 gram per day during the diet; p<0.0001). Relative diet composition remained stable at ~45% carbohydrate, ~35% fat and ~15% protein. Subjects in both HFHS-groups consumed similar amounts of Nutridrink®.

CALORIC INTAKE
Ad libitum nutrient intake was similar between the diet groups. Diet intervention data are displayed in table 2 and figures 2B and 2C. The subjects in the 4 diet groups all consumed a hypercaloric diet compared to the control group (p<0.0001). Control subjects consumed a eucaloric diet of 106 kcal/day consisting for ~45-50% of carbohydrates, ~30-35% of fat and for ~15% of protein. As expected, the subjects in the HS groups consumed excessive carbohydrates (~60% of total caloric intake; p<0.0001 compared to baseline diet), which was significantly higher compared to the control and HFHS group (p<0.0001). Absolute intake of protein (~103 gram per day at baseline versus ~112 gram per day during the diet, p<0.02), and fat (~98 gram per day at baseline versus 112 gram per day during the diet, p<0.0001), were higher during the HS diet. However, due to excessive carbohydrate intake, relative intake of protein (~15% at baseline versus ~12% during the diet, p<0.0001) and fat (~35% at baseline versus ~27% during the diet p<0.0001) were decreased. There was no difference in carbohydrate intake between both HS groups (p=0.33).

Subjects in the HFHS groups consumed both excessive fat (~94 gram per day at baseline versus ~147 gram per day during the diet, p<0.0001) and carbohydrates (~297 gram per day at baseline versus ~441 gram per day during the diet, p<0.0001) with no difference between the HFHS groups (p=0.35). Both HFHS groups had a higher absolute intake of protein (~98 gram per day before versus ~146 gram per day during the diet; p<0.0001). Relative diet composition remained stable at ~45% carbohydrate, ~35% fat and ~15% protein. Subjects in both HFHS-groups consumed similar amounts of Nutridrink®.

SERT in the hypothalamic region
A 2 way ANOVA detected an overall effect of size versus frequency on SERT specific-to-nonspecific binding ratio (SNS-BR) in the hypothalamic region (p=0.01), with lower SERT binding when surplus calories were consumed in between the meals (frequency) compared to surplus calories consumed with the meals (size) (Figure 3A). When analysing the changes in SERT binding within the specific hypercaloric diet groups and within the control group, SERT binding activity in the HFHS-F group was 30% reduced after the diet (0.65±0.15 vs 0.46±0.13; p=0.004) (Figure 3B). In the other 3 hypercaloric diet groups and in the control group, there was no significant change in SERT binding over the 6 weeks of treatment (data not shown).

We here show for the first time that in lean healthy male subjects SERT binding in the diencephalon, which includes the hypothalamus, decreases after a hypercaloric HFHS diet. This decrease is only observed when the caloric surplus is consumed in between meals but not when caloric surplus is consumed together with the meals. This suggests a selective effect of hypercaloric snacking on diencephalic SERT that is absent following increased meal size. Our data are in line with, and extend, previous findings showing a negative correlation between SERT and BMI [10;12], and the

Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>HFHS</th>
<th>HFHSF</th>
<th>HS</th>
<th>HSF</th>
<th>CP</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Age [yr]</td>
<td>20.0±1.4</td>
<td>21.3±1.1</td>
<td>20.0±1.6</td>
<td>20.7±2.9</td>
<td>22.0±3.1</td>
<td>ns</td>
</tr>
<tr>
<td>BMI [kg/m²]</td>
<td>22.3±0.9</td>
<td>22.6±0.7</td>
<td>22.3±0.8</td>
<td>23.0±1.3</td>
<td>22.3±2.1</td>
<td>ns</td>
</tr>
<tr>
<td>Body weight [kg]</td>
<td>78.0±5.6</td>
<td>80.6±5.5</td>
<td>79.5±7.8</td>
<td>80.5±8.4</td>
<td>76.6±7.7</td>
<td>ns</td>
</tr>
<tr>
<td>Resting Energy Expenditure (REE)</td>
<td>1026±101</td>
<td>1095±179</td>
<td>1095±190</td>
<td>1095±179</td>
<td>1095±179</td>
<td>ns</td>
</tr>
<tr>
<td>Calorie intake (kcal)</td>
<td>2566±317</td>
<td>2692±341</td>
<td>2601±165</td>
<td>2426±301</td>
<td>2490±262</td>
<td>ns</td>
</tr>
</tbody>
</table>

Data are displayed as mean ± SD or ns = not significant.

Table 2. BMI and food intake before and after a hypercaloric diet.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HFHS</th>
<th>Baseline</th>
<th>T=6 weeks</th>
<th>Hypocaloric</th>
<th>Test</th>
<th>Baseline</th>
<th>Hypocaloric</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI [kg/m²]</td>
<td>22.3±1.1</td>
<td>22.2±2.2</td>
<td>22.4±1.2</td>
<td>23.1±1.2</td>
<td>22.7±1.1</td>
<td>23.4±1.2</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Caloric intake (kcal/24h)</td>
<td>2490±262</td>
<td>2572±271</td>
<td>2610±219</td>
<td>3060±231</td>
<td>2512±206</td>
<td>3934±257</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Carbohydrate (gram)</td>
<td>322±68</td>
<td>299±51</td>
<td>297±42</td>
<td>441±37</td>
<td>272±46</td>
<td>340±61</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Carbohydrate %</td>
<td>41±10</td>
<td>40±10</td>
<td>40±10</td>
<td>40±13</td>
<td>40±10</td>
<td>40±13</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Fat (gram)</td>
<td>19±3</td>
<td>16±1</td>
<td>18±4</td>
<td>16±4</td>
<td>14±4</td>
<td>14±4</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>19±3</td>
<td>16±1</td>
<td>18±4</td>
<td>16±4</td>
<td>14±4</td>
<td>14±4</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Protein (gram)</td>
<td>92±17</td>
<td>89±14</td>
<td>94±12</td>
<td>140±11</td>
<td>103±46</td>
<td>112±30</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Protein (%</td>
<td>15±3</td>
<td>15±3</td>
<td>15±3</td>
<td>15±3</td>
<td>15±3</td>
<td>15±3</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>

Data are displayed as mean ± SD. HS = hypercaloric high-fat-high-sugar diet; HFHS = hypercaloric high-fat-high-sugar diet. Within the HFHS and the HFHS-F group there were no significant differences (see kg/m²) and for legibility the groups are combined in this table. Treated meal is = not significant; * p<0.05; ** p<0.01; *** p<0.001.

DISCUSSION

We here show for the first time that in lean healthy male subjects SERT binding in the diencephalon, which includes the hypothalamus, decreases after a hypercaloric HFHS diet. This decrease is only observed when the caloric surplus is consumed in between meals but not when caloric surplus is consumed together with the meals. This suggests a selective effect of hypercaloric snacking on diencephalic SERT that is absent following increased meal size. Our data are in line with, and extend, previous findings showing a negative correlation between SERT and BMI [10;12], and the...
The HFHS groups consumed more protein, which was inevitable since the liquid meal (Nutridrink®) contained protein. Dietary proteins are involved in brain satiation mechanisms: they induce gut peptide secretion (e.g. GLP-1, PPY) and production of hormones such as insulin and ghrelin. These neuropeptides and hormones can signal to brain regions involved in hunger and satiety including the hypothalamus (23). There is currently no evidence for the presence of a relationship between SERT and dietary proteins. A few rodent studies have investigated the possibility of a relationship between dietary protein and serotonin in the brain but did not report a consistent association (24;25). One study showed activation of the hypothalamic serotonin system after ingestion of animal proteins in rats (26). However, in the present study the protein surplus is casein and not animal proteins. Moreover, the reduction in SERT was found only in the HFHS-F group while protein intake did not significantly differ from the HFHS-S group making it unlikely that the effect on the brain is explained by higher protein intake.

In the present study we investigated SERT binding in the diencephalon. The diencephalon includes the hypothalamus, which is a key regulatory brain centre for energy balance (1). Future studies will need to establish in which specific nucleus or nuclei SERT is decreased after similar diet interventions.

Understanding the role of SERT in the hypothalamus in the regulation of food intake and body weight is difficult. The interaction between SERT, extracellular serotonin and serotonin signal transmission is dynamic and complex (20). Moreover, serotonin is produced not only in the brain but also in the enteric nerve system. Total SERT depleted animals have increased extracellular serotonin and display an obese insulin resistant phenotype without an increase in food intake (21) while the short-term use of SERT inhibitors is associated with lower food intake and body weight (4). On the other hand, chronic use of SSRIs might increase the risk for obesity in humans (14;22). It could well be that there is a difference between acute and chronic SERT inhibition on extracellular serotonin levels and thereby on food intake and body weight control. The change in SERT in our subjects might be a physiological response to reduce food intake through increasing extracellular serotonin. However, one would then expect a decrease in all hypercaloric diet groups, but we found a decrease only in the HFHS-F group. Therefore it seems more likely that a decrease in SERT is accompanied by a decrease in extracellular serotonin, also explaining the failure of SSRIs for long-term weight loss maintenance. A decrease in extracellular serotonin might result in less inhibition of food intake predisposing especially snacking individuals to obesity. However, this remains to be investigated.

CONCLUSION

A hypercaloric 6-week HFHS snacking diet decreases SERT within the diencephalon in lean men while increasing glucose intake or increasing meal size did not affect SERT. Interestingly, in today’s society there is increasing snacking behavior and it has been shown that snacking is associated with obesity (27-29). We propose that a decrease in diencephalic SERT caused by high-fat-high-sugar snacking might be considered as a mechanism for disturbed appetite control resulting in hyperphagia and obesity.
REFERENCES


Hypercaloric diets with increased meal frequency, but not meal size, increase intrahepatic triglycerides: a randomized controlled trial

Karin E Koopman
Matthan WA Caan
Aart J Nederveen
Anouk Pels
Mariëtte T Ackermans
Eric Fliers
Susanne E la Fleur
Mireille J Serlie

Hepatology
2014 Aug; 60(2): 545-53
ABSTRACT

Background: American children consume 27% of calories from high-fat and high-sugar snacks. Both sugar and fat consumption have been implicated as a cause of hepatic steatosis and obesity but the effect of meal pattern is largely understudied. We hypothesized that a high meal frequency, compared to consuming large meals, is detrimental in the accumulation of intrahepatic and abdominal fat.

Methods: To test this hypothesis, we randomized 36 lean, healthy men to a 40% hypercaloric diet for 6 weeks or a eucaloric control diet and measured intrahepatic triglyceride content (IHTG) using proton magnetic resonance spectroscopy (H-MRS), abdominal fat using magnetic resonance imaging (MRI) and insulin sensitivity using hyperinsulinemic euglycemic clamp with a glucose isotope tracer before and after the diet intervention. The caloric surplus consisted of fat and sugar (high-fat-high-sugar; HFHS) or sugar only (high-sugar; HS) and was consumed together with, or between, the 3 main meals, thereby increasing meal size or meal frequency.

Results: All hypercaloric diets similarly increased body mass index (BMI). Increasing meal frequency significantly increased IHTG (HFHS mean relative increase of 45%; p=0.016 and HS mean relative increase 110%; p=0.047) whereas increasing meal size did not (2-way analysis of variance [ANOVA] size vs frequency p=0.03). Abdominal fat increased in the HFHS-frequency group (+63.3 ± 42.8 ml; p=0.004) and tended to increase in the HS-frequency group (+46.5 ± 50.7 ml; p=0.08). Hepatic insulin sensitivity tended to decrease in the HFHS-frequency group only. Peripheral insulin sensitivity was not affected.

Conclusion: A hypercaloric diet with high meal frequency increased IHTG and abdominal fat independent of caloric content and body weight, whereas increasing meal size did not. This study suggests that snacking, a common feature in the Western diet, independently contributes to hepatic steatosis and obesity.

INTRODUCTION

Obesity is a worldwide health problem and associated with hepatic steatosis and intra-abdominal fat accumulation. Although obesity and hepatic steatosis often coincide, hepatic steatosis can be present in lean subjects and is not present in all obese humans (1), suggesting that factors besides obesity contribute to fat accumulation in the liver. An obvious candidate to be involved is the diet.

Caloric content (2) and individual macronutrients are associated with hepatic steatosis. Short-term high-fat diets increase IHTG in lean and obese humans (3,4) and induce robust hepatic steatosis in rodents (5) and dietary glucose and fructose stimulate de novo lipogenesis (DNL) (6) and increase IHTG, even in lean subjects (7,8). Moreover, cross-sectional studies have identified the consumption of sugar-sweetened softdrinks as a dietary factor predicting hepatic steatosis (9). Recent human studies however showed that overfeeding resulted in accumulation of IHTG without a differential effect of fructose, glucose or fat (10,11). This suggests that macronutrient composition is not the only determining dietary factor in IHTG accumulation. A factor less often considered is the frequency and timing of food intake. This is remarkable, since 27 % of US children’s daily calories come from snacks (12) and also in obese women excessive caloric intake mainly comes from snacks in between meals (13). Interestingly, when provided with the choice to consume saturated fat and liquid sugar separate from their balanced chow pellets, rats increase their meal frequency, show persistent hyperphagia and become obese (14). Whether snacking specifically affects IHTG is unknown.

Hepatic steatosis increases the risk for non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis and is associated with insulin resistance (15,16). How hepatic steatosis interferes with insulin sensitivity in humans is only in part elucidated. We recently showed in patients with familial hypobetalipoproteinemia, which is characterized by massive IHTG accumulation, that hepatic steatosis, per se is not associated with insulin resistance (17). Interference of lipid metabolites with insulin signaling is a general concept in obesity-associated insulin resistance, and macronutrients themselves are able to modulate glucose production and insulin sensitivity independent of obesity (18,19). Rats snacking fat and sugar develop insulin resistance within 1 week (20), and female adolescents who reported consumption of frequent snacks throughout the day have a higher homeostatic model assessment of insulin resistance (HOMA-IR) compared to non-snacking controls (21). Randomized controlled studies on the effect of increasing meal frequency or meal size with different macronutrient combinations on insulin sensitivity and IHTG are currently unavailable and was the aim of this study. We hypothesized that increasing meal frequency, representing a snacking eating pattern, negatively affects IHTG and insulin sensitivity.
MATERIALS AND METHODS

STUDY PARTICIPANTS:
We recruited 37 Caucasian, lean men [age 22 (19-27) years, BMI 22.5 (19.5-24.5) kg/m²] via local advertisements. Participants were healthy, had no family history of type 2 diabetes (T2DM) and a normal oral glucose tolerance test (22). Other exclusion criteria were use of medication, substance abuse (nicotine or drugs, alcohol > 2 units/day), history of eating or psychiatric disorders, exercise > 3 hours/week, and an unhealthy ad libitum diet. A healthy diet contained balanced macronutrient composition following the Dutch guidelines (23). Self-reported body weight was stable in the 6 months before study participation, thereby excluding a hypocaloric or hypercaloric state. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Medical Ethics Committee of the AMC Amsterdam. Written informed consent was obtained from all study participants before the start of study participation.

STUDY DESIGN:
A schematic overview of the study design is presented in Figure 1. After inclusion subjects started the 1-week run-in phase: they reported their ad libitum intake on an online diet journal (eetmeter.voedingscentrum.nl). Body weight before- and after this week had to be similar; the consumed amount of calories was then considered adequate for weight maintenance, i.e. eucaloric. Subjects were then randomised into 1 of 4 hypercaloric diet groups (N=8/group) or a control group (N=5). The control group underwent all measurements but continued the weight maintaining ad libitum diet. The diet was followed for 6 consecutive weeks. Subjects visited the research unit weekly for measurement of body weight and resting energy expenditure (REE) and diet monitoring, subjects daily reported their ad libitum intake online. When ad libitum caloric intake was lower than caloric need (1.4 x REE), subjects were instructed to increase their ad libitum intake. After the intervention subjects were monitored until they returned to their baseline body weight. The baseline characteristics, study design and changes in body weight have previously been reported (24).

HYPERCALORIC DIETS:
All diets consisted of a 40% caloric surplus on top of the ad libitum weight maintaining diet (calculated as 1.4 x REE). The hypercaloric diet groups were:

1. HFHS-size group: high-fat-high-sugar (HFHS) diet using Nutridrink Compact ® 3 times a day, consumed together with the 3 daily main meals.
2. HFHS-frequency group: high-fat-high-sugar (HFHS) diet using Nutridrink Compact ® 3 times a day, consumed 2 to 3 hours after each meal.
3. HS-size group: high-sugar (HS) diet using commercially available sucrose-sweetened beverages 3 times a day, consumed together with the 3 daily main meals.
4. HS-frequency group: high-sugar (HS) diet using commercially available sucrose-sweetened beverages 3 times a day, consumed 2 to 3 hours after each meal.

Nutridrink Compact ® (Nutricia® Advanced Medical Nutrition; Zoetermeer; The Netherlands) is a liquid meal with nutritive value of 240 kcal/100 ml; 16 energy% protein (mainly casein), 49 energy% carbohydrates (mainly maltose and polysaccharides) and 35 energy% fat (mainly unsaturated fat). As high-sugar liquids subjects consumed commercially available sucrose-sweetened (~50% glucose/50% fructose) softdrinks. The softdrinks contained no fat or protein. Participants chose their beverage from a list of softdrinks with comparable nutritive value. Soft-drinks contained 43.3 (range 36-49) kcal/100 ml and 10.3 (range 9-12) gram/100 ml of sucrose. Participants consumed on average 1000 ml 3 times per day.

RESTING ENERGY EXPENDITURE (REE):
REE was measured using indirect calorimetry. VO2 and VCO2 were measured in the supine position for 20 minutes using a ventilated hood system (Vmax Encore 29; SensorMedics, Anaheim, CA). REE was calculated as described previously (25). The abbreviated Weir equation was used to calculate 24-hour energy expenditure.
IHTG MEASUREMENT USING PROTON MAGNETIC RESONANCE SPECTROSCOPY (1H-MRS)

1H-MRS measurements were performed on a clinical 3.0T Philips Intera scanner (Philips Healthcare, Best, The Netherlands). After performing T1-weighted coronal and axial localizer images of the abdomen, a voxel of 20 x 20 x 20 mm was positioned in the right hepatic lobe, avoiding inclusion of the diaphragm and edges of the liver, as well as vascular and biliary structures. Voxel size and acquisition time were standardized for all subjects. Spectra were acquired avoiding inclusion of the diaphragm and edges of the liver, as well as vascular and biliary structures. Voxel size and acquisition time were standardized for all subjects. Spectra were acquired using first-order iterative shimming, a PRESS sequence with relaxation time/echo time (TR/TE) 2000/35 ms and 64 signal acquisitions during free breathing [26]. 1H-MRS data were processed using jMRUI software. The water non-suppressed spectra were used to quantify the lipid signal resonances. Relative fat content was expressed as a ratio of the fat peak area over the cumulative water and fat peak areas (1.3 ppm / (1.3 ppm + 4.65 ppm)). Calculated peak areas of water and fat were corrected for T2 relaxation. Percentage IHTG was calculated as previously described [26].

ABDOMINAL FAT QUANTIFICATION USING MR

We performed abdominal fat measurements in abdominal MRIs acquired on a clinical 3.0T Philips Intera scanner (Philips Healthcare) at baseline and after the diet period. The abdominal MRIs were bias field corrected [27] and then automatically segmented with in-house developed software, written in MATLAB (The Mathworks, Matwick, MA). In short, subcutaneous fat was segmented using snakes, after which visceral fat was segmented by intensity thresholding. The data were then manually corrected by one well-trained researcher blinded for the randomisation with ITK-SNAP 2.2 software. We analysed abdominal fat in 10 consecutive slices at the level of lumbar vertebrae L3/L4, which has been shown to be representative for total abdominal fat [28].

TWO-STEP HYPERINSULINEMIC EUGLYCEMIC CLAMP

Insulin sensitivity was measured with a 2-step hyperinsulinemic euglycemic clamp after an overnight fast in supine position as described previously [29]. In short, subjects were admitted to the Metabolic Clinical Research Unit of the AMC after an overnight fast and studied in the supine position. Subjects were randomly allocated to one of the four hypercaloric diet groups or the control group. Randomisation was not blinded. We performed simple, non-stratified randomisation by drawing lots.

Calculations and Statistics

Endogenous glucose production (EGP) and peripheral glucose uptake (rate of disappearance, Rd) were calculated using modified versions of the Steele equations for the non-steady state and expressed as micromoles/kilograms/minute as described previously [33;34]. We calculated caloric intake/day as the mean of the complete diet period of 6 weeks. When normality tests showed normal distribution, data before and after the diet within the groups were compared using paired Student t-test. Otherwise, the Wilcoxon matched pairs test was used. Between-group dif-
ferences were analysed using a 2-way analysis of variance (ANOVA) with a post-hoc Bonferroni for multiple comparisons.

RESULTS

RECRUITMENT AND BASELINE CHARACTERISTICS

After 37 subjects completed the study protocol, we excluded 2 subjects from the analyses because of uncertain diet compliance. We furthermore excluded 1 subject because of excessive alcohol consumption during the last hypercaloric intervention week. Two subjects were replaced by newly recruited participants. Baseline characteristics of the subjects are presented in Table 1. Subjects were lean and had normal insulin sensitivity and IHTG. Control subjects consumed more carbohydrate and less fat at baseline compared to the 4 hypercaloric groups, but intake was similar between the 4 hypercaloric groups (Table 1).

CONTROL SUBJECTS

In the control group, BMI remained stable between the T=0 weeks and T=6 weeks measurements (22.3±2.1 vs. 22.2±2.2 kg/m²; p=0.37). Colotic intake and intake of specific macronutrients were stable during the observational period (data not shown). IHTG (1.34±0.54 vs. 1.15±0.26%; p=0.50), abdominal fat (0.57±0.25 vs. 0.56±0.11 liter; p=0.92), insulin-mediated suppression of EGP (75.0±7.1 vs. 73.0±14.7%; p=0.81) and peripheral rate of disappearance of glucose (64.8±8.7 vs. 68.3±5.1 umol/kg/min; p=1.00) did not change after the observational period. Control subjects were included to show reproducibility of the measurements only and are therefore not further analysed.

CALORIC INTAKE

Food intake and macronutrient composition during the hypercaloric interventions are presented in Table 2 and Figure 2A. In summary, ad libitum nutrient intake was similar between the four diet groups. There was no difference in carbohydrate and fat intake between both HS groups and between both HFHS groups respectively. There were no side effects or adverse events reported by subjects on any of the 4 diets.

BMI AND REE

Subjects gained 2.5 ± 1.7 kg within the 6 weeks. All hypercaloric diet interventions resulted in an increase in BMI (Table 3) with no differences between the diet groups (Figure 2B). REE did not change in any of the diet groups (Table 3).

IHTG significantly increased in the HFHS-frequency (0.98±0.91% vs. 1.38±1.26% [mean relative increase 45%]; p=0.018) and the HS-frequency (1.49±0.95% vs. 3.10±2.16% [mean relative increase 110%]; p=0.043) groups (Figure 3). The increase in IHTG tended to be higher in the HS-frequency group (p=0.07). In the 2 groups with increased meal size, IHTG did not change (HFHS-size 0.85±0.32% vs. 1.05±1.19%; p=0.208; HS-size 0.80±0.45% vs. 0.93±2.37%, p=0.917) [Figure 3A].

2-way ANOVA analysis of the 4 hypercaloric diet groups showed an overall effect of size versus frequency (p=0.03, F=5.435) but not of HFHS vs. HS (p=0.13, F=2.418).

Table 1: Baseline characteristics of study participants.

<table>
<thead>
<tr>
<th>Group</th>
<th>Controls</th>
<th>HFHS5</th>
<th>HFHS10</th>
<th>HS5</th>
<th>HS10</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>23.0±3.1</td>
<td>22.2±2.9</td>
<td>23.5±1.9</td>
<td>22.0±2.5</td>
<td>21.9±3.8</td>
<td>0.84</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.6±3.3</td>
<td>23.3±3.6</td>
<td>22.5±1.5</td>
<td>21.7±1.3</td>
<td>22.6±3.8</td>
<td>0.90</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78.6±37</td>
<td>78.5±36</td>
<td>68.3±61</td>
<td>74.4±79</td>
<td>81.0±88</td>
<td>0.70</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>79.0±5.4</td>
<td>80.7±3.1</td>
<td>81.3±13</td>
<td>79.0±2.9</td>
<td>81.4±2.2</td>
<td>0.25</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>2.8±1.7</td>
<td>3.0±1.3</td>
<td>3.3±1.4</td>
<td>3.0±1.2</td>
<td>3.7±1.4</td>
<td>0.14</td>
</tr>
<tr>
<td>Resting Energy Expenditure (kcal/kg)</td>
<td>2.2±1.8</td>
<td>2.3±2.3</td>
<td>2.4±2.7</td>
<td>2.4±2.7</td>
<td>2.5±2.5</td>
<td>0.02</td>
</tr>
<tr>
<td>Caloric intake (kCal/kg)</td>
<td>249.0±382</td>
<td>2566±317</td>
<td>2616±334</td>
<td>3333±303</td>
<td>2456±292</td>
<td>0.64</td>
</tr>
<tr>
<td>Carbohydrate intake (% of total kcal)</td>
<td>45.4±6.7</td>
<td>45.2±6.4</td>
<td>45.2±6.4</td>
<td>44.5±6.6</td>
<td>44.5±6.6</td>
<td>0.03</td>
</tr>
<tr>
<td>Fat intake (% of total kcal)</td>
<td>30.4±3.8</td>
<td>31.7±3.0</td>
<td>32.5±3.0</td>
<td>32.6±3.0</td>
<td>32.6±3.0</td>
<td>0.006</td>
</tr>
<tr>
<td>Alcohol intake (% of total kcal)</td>
<td>0.1±0.1</td>
<td>0.2±0.2</td>
<td>0.2±0.2</td>
<td>0.2±0.2</td>
<td>0.1±0.1</td>
<td>0.16</td>
</tr>
<tr>
<td>Insulin sensitivity</td>
<td>45.3±13.4</td>
<td>46.1±13.6</td>
<td>57.2±21.0</td>
<td>70.0±11.4</td>
<td>57.2±28.7</td>
<td>0.21</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.4±0.6</td>
<td>1.2±0.2</td>
<td>1.7±0.8</td>
<td>1.7±0.3</td>
<td>1.7±0.2</td>
<td>0.19</td>
</tr>
<tr>
<td>Plasma TC (mmol/L)</td>
<td>0.72±0.09</td>
<td>0.70±0.09</td>
<td>0.69±0.09</td>
<td>0.68±0.09</td>
<td>0.68±0.09</td>
<td>0.16</td>
</tr>
<tr>
<td>Free fatty Acids (mM/L)</td>
<td>0.46±0.21</td>
<td>0.23±0.09</td>
<td>0.24±0.28</td>
<td>0.48±0.20</td>
<td>0.48±0.20</td>
<td>0.09</td>
</tr>
<tr>
<td>Hepatic triglyceride content (%)</td>
<td>1.34±0.15</td>
<td>0.86±0.34</td>
<td>0.90±0.86</td>
<td>0.80±0.89</td>
<td>0.80±0.89</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. Glucose, insulin, triglycerides (TG) and free fatty acids were determined in the fasting state.

Table 2: Food intake per randomisation group during the hypercaloric diet.

<table>
<thead>
<tr>
<th>Group</th>
<th>HFHS5</th>
<th>HFHS10</th>
<th>HS5</th>
<th>HS10</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total caloric intake (kCal)</td>
<td>3474±137</td>
<td>3957±198</td>
<td>3474±404</td>
<td>3614±381</td>
<td>0.11</td>
</tr>
<tr>
<td>Ad libitum caloric intake (kCal)</td>
<td>2640±141</td>
<td>2880±171</td>
<td>2550±469</td>
<td>2633±216</td>
<td>0.13</td>
</tr>
<tr>
<td>Carbohydrate intake (g)</td>
<td>437±33</td>
<td>444±38</td>
<td>500±118</td>
<td>511±81</td>
<td>0.10</td>
</tr>
<tr>
<td>Ad libitum carbohydrate intake (g)</td>
<td>303±33</td>
<td>306±37</td>
<td>278±68</td>
<td>263±27</td>
<td>0.16</td>
</tr>
<tr>
<td>Fat intake (g)</td>
<td>137±16</td>
<td>136±17</td>
<td>220±60</td>
<td>248±65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ad libitum fat intake (g)</td>
<td>100±17</td>
<td>112±4</td>
<td>102±19</td>
<td>115±11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Carbohydrate intake (% of total kCal)</td>
<td>43±5</td>
<td>43±6</td>
<td>43±5</td>
<td>43±6</td>
<td>0.00</td>
</tr>
<tr>
<td>Fat intake (% of total kCal)</td>
<td>14±13</td>
<td>15±5</td>
<td>10±24</td>
<td>10±24</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ad libitum protein intake (g)</td>
<td>98±11</td>
<td>100±7</td>
<td>100±24</td>
<td>104±8</td>
<td>0.48</td>
</tr>
<tr>
<td>Protein intake (g)</td>
<td>44±7</td>
<td>44±6</td>
<td>44±6</td>
<td>44±6</td>
<td>0.00</td>
</tr>
<tr>
<td>Relative caloric intake (% of total kCal)</td>
<td>47±4</td>
<td>46±2</td>
<td>58±3</td>
<td>56±2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Relative fat intake (% of total kCal)</td>
<td>35±4</td>
<td>35±4</td>
<td>27±4</td>
<td>29±3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD; intake per day (mean over the complete 6 week diet period).

Table 3: Intrahepatic triglyceride content (IHTG).

<table>
<thead>
<tr>
<th>Group</th>
<th>HFHS5</th>
<th>HFHS10</th>
<th>HS5</th>
<th>HS10</th>
</tr>
</thead>
<tbody>
<tr>
<td>T=0 weeks</td>
<td>0.98±0.91%</td>
<td>1.38±1.26%</td>
<td>1.49±0.95%</td>
<td>3.10±2.16%</td>
</tr>
<tr>
<td>T=6 weeks</td>
<td>1.49±0.95%</td>
<td>3.10±2.16%</td>
<td>1.49±0.95%</td>
<td>3.10±2.16%</td>
</tr>
</tbody>
</table>

IHTG: intrahepatic triglyceride content; HS: high fat-high sugar diet; HFHS: high fat-high sugar-frequency and high fat-high sugar-size diet.
Total abdominal fat significantly increased in the HFHS-frequency group and tended to increase in the HS-frequency group. In the HFHS-size and HF-size group abdominal fat did not change (Table 3). The increase in abdominal fat was not different between the 2 frequency groups (p=0.50). The increase in total abdominal fat was mainly caused by an increase in subcutaneous fat in both frequency groups (Table 3). Fat in the visceral compartment tended to increase in the HFHS-frequency group and was unchanged in all other groups (Table 3).
Hypercaloric snacking increases intrahepatic triglycerides

Table 4. Increased meal size vs increased meal frequency

<table>
<thead>
<tr>
<th>Increased Meal Size</th>
<th>Increased Meal Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td><strong>End diet</strong></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.0 ± 0.9</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>101 ± 6</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.6</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>38.7 ± 6.4</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>217 ± 16.5</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>68.0 ± 16.6</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>80.3 ± 0.36</td>
</tr>
<tr>
<td>Free fatty acids (mM)</td>
<td>0.41 ± 0.17</td>
</tr>
<tr>
<td>FFA (mg/dl)</td>
<td>11.9 ± 1.0</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>75.3 ± 1.7</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>64.2 ± 2.9</td>
</tr>
<tr>
<td>Step 1 FFA suppression (%)</td>
<td>92.4 ± 6.2</td>
</tr>
<tr>
<td>Step 2 FFA suppression (%)</td>
<td>92.9 ± 3.8</td>
</tr>
<tr>
<td>Intravenous adipose tissue (%)</td>
<td>0.42 ± 0.12</td>
</tr>
<tr>
<td>Subcutaneous adipose tissue (%)</td>
<td>0.16 ± 0.06</td>
</tr>
<tr>
<td>Intravenous triglycerides (%)</td>
<td>0.83 ± 0.38</td>
</tr>
</tbody>
</table>

**GLUCOSE METABOLISM**

Fasting glucose and EGP did not change upon the diet interventions. Fasting insulin levels slightly but significantly increased in the HS-S group only (Table 3). Hepatic insulin sensitivity expressed as percent insulin-mediated suppression of baseline EGP tended to decrease in the HHFS-frequency group (Table 3) but not in the other groups. Peripheral insulin sensitivity did not change in any of the hypercaloric diet groups (Table 3). In the HHFS-frequency group insulin-mediated suppression of FFA significantly decreased (Table 3).

**GLUCOREGULATORY HORMONES, leptin and plasma lipids**

Plasma leptin concentrations increased in all diet intervention groups [Table 3]. Glucoregulatory hormones did not change. Fasting plasma concentrations of triglycerides (TG) increased upon the HHFS-frequency diet only.

**OVERALL EFFECTS OF INCREASING MEAL SIZE VERSUS MEAL FREQUENCY**

In Table 4 the differences between pooled data from the meal size (HS-S and HHFS-S) and meal frequency (HS-F and HHFS-F) hypercaloric diet groups are shown. While BMI significantly increases in both groups, only increasing meal frequency significantly increases IHTG and abdominal (subcutaneous and visceral) fat and reduces insulin-mediated suppression of circulating fatty acids.

**DISCUSSION**

We show that a 6-week hypercaloric snacking diet increases IHTG and abdominal fat in lean men while increasing meal size does not. Moreover, we show that this was irrespective of the macronutrients in the diet, as both snacking sugar and snacking fat and sugar resulted in IHTG and abdominal fat accumulation. However, the increase in IHTG tended to be higher in the HS-frequency group, indicating that the frequent snacking of sugar leads to the most profound accumulation of IHTG. Although frequent consumption of snacks has been linked to obesity (13,14), we are the first to provide evidence that overeating by consuming frequent meals, and not large meals, contributes to fat accumulation in liver independent of body weight gain. It has been shown that consumption of excessive carbohydrates above caloric need substantially increases fractional DNL [35] and glycogen synthesis [11]. The trend we demonstrate for a higher increase in IHTG in the HS-frequency group compared to the HHFS-frequency group (which consumed fewer carbohydrates) is in line with this hypothesis, although subjects in the size groups also consumed excessive carbohydrates but spread over 3 meals. Snacking of mono- and polysaccharides seems to exert the same effect on IHTG as our HS diets contained monosaccharides whereas our HHFS diets contained polysaccharides.

The underlying molecular mechanisms remain to be elucidated. Continuous delivery of nutrients through the portal vein might yield a different metabolic response compared to a pattern of fasting and feeding cycles. Our data suggest that a continuous flow of nutrients to hepatocytes stimulates DNL either through induction of carbohydrate responsive transcription factors like CHREBP or insulin-mediated induction of SREBP1c, PPARγ or IRS [36,37]. Besides the nutrients, increased flux of portal FFA might stimulate DNL [38] since it has been reported that the plasma FFA pool accounts for 60% of IHTG in humans with non-alcoholic fatty liver disease (NAFLD) [6]. Lipolysis rates from abdominal adipose tissue were not directly measured in our study, but insulin-mediated suppression of plasma FFA, a marker of insulin sensitivity of adipose tissue, was reduced in the HHFS-, but not HS-, frequency group. A reduction in β-oxidation is another possible mechanism since in obesity-related hepatic steatosis both increased DNL and decreased β-oxidation have been shown in rodents [39]. The mechanisms of excessive storage of liver TGs might be different when subjects are exposed to high-sugar versus high-fat-high-sugar diets. Increased IHTG and abdominal fat are risk factors for insulin resistance and T2DM and our data imply that a long-term hypercaloric snacking diet increases the risk for perturbed glucose metabolism independently of obesity. A reduction in hepatic insulin sensitivity was observed in the HHFS-frequency group compared to the HHFS-size group, suggesting that fat and sugar when consumed in excess and as between-meal snacks independently affect hepatic glucose metabolism. However, the effect was relatively modest. Although some studies show an association between dietary sugar consumption and insulin resistance and the prevalence of diabetes [10,19,40], it is difficult to dissect whether this is a direct effect of carbohydrates overconsumption or secondary to the induction of obesity. Moreover, the eating pattern was not always monitored in those studies. We did not observe changes in peripheral insulin sensitivity in any of the diet groups studied. We previously showed that a period of 4-7 weeks of a hypercaloric diet significantly decreased insulin sensitivity [31]. However, in that study subjects were older, had a higher baseline BMI and gained more body weight.
The study was conducted under free-living conditions with a risk of non-compliance. However, weekly visits and intensive phone and email contact with the participants have ensured good compliance with the diets, confirmed by a steady weekly increase in body weight. Furthermore, this study is conducted in healthy, young, Caucasian, male volunteers. Therefore, the results might be different in older subjects, female subjects and subjects from different ethnicities. Therefore, the results of this study cannot be extrapolated to the general population. Because our intervention was a short-term diet, results might be different during longterm exposure to hypercaloric diets. We did not include a high-fat only group and therefore the specific effect of a high-fat high-frequency diet remains unknown. The total increase in IHTG in our subjects was relatively modest and might be different in other populations.

Finally, the lack of an effect of the short-term hypercaloric diet intervention on insulin sensitivity might become apparent in other populations since we showed previously that short-term hypercaloric diets in a somewhat older population affected whole body insulin sensitivity [31].

Reports estimate that American children consume 27% of calories from snacks [12] and snacking is common in obese women [13]. Our findings are therefore an actual reflection of eating habits in today’s society and might in part be an explanation for the increased number of children and adults with hepatic steatosis and T2DM [41,42]. Our data indicate that attention should be paid to diet patterns besides caloric intake in general in the treatment of subjects with hepatic steatosis and abdominal obesity. In obese subjects who presumably consume a hypercaloric diet, snacking should be strongly discouraged. In addition, one might hypothesize that consuming fewer meals might be beneficial in reducing hepatic and abdominal fat accumulation.

CONCLUSIONS

Hypercaloric diets with increased meal frequency, representing snacking, increase IHTG and abdominal fat in lean men, whereas similar diets with increased meal size do not. This suggests that food intake pattern independently of caloric excess and weight gain contributes to the occurrence of hepatic steatosis and abdominal obesity. Besides, hypercaloric snacking of fat and sugar tended to reduce hepatic insulin sensitivity. Therefore, reducing snacking behaviour and encouraging consuming 3 meals per day might have favourable metabolic consequences in the long term and might reduce the prevalence of non-alcoholic fatty liver disease.

REFERENCES


A 6-week hypercaloric diet modulates sympathetic activity in proportion to a change in striatal DAT binding

Karin E Koopman
John M Karemaker
Eric Fliers
Jan Booij
Mireille J Serlie
Susanne E la Fleur

Submitted
ABSTRACT

Background: The autonomic nervous system (ANS) and striatal dopamine are related to food intake. In conditions of overfeeding such as in obesity, the striatal dopamine D₁/D₃ receptors are reduced and increased sympathetic activity has been reported. We hypothesized that increased food intake itself is related to changes in ANS activity and the striatal dopaminergic system.

Methods: We studied ANS activity and striatal dopamine transporter (DAT) binding in 31 healthy, lean men before and after a 6-week hypercaloric diet. We measured striatal DAT binding with 123I-FP-CIT SPECT, heart rate variability (HRV) in the supine and upright position as a derivative of ANS activity and plasma ghrelin, leptin, insulin, and glucose in the fasted condition.

Results: Supine low frequency (LF) power, mainly reflecting sympathetic activity, increased (p=0.04) and striatal DAT binding tended to decrease (p=0.08) after the hypercaloric diet. In addition, LF response to a sympathetic stimulation (supine to upright position) was blunted after the hypercaloric diet (p=0.02). Leptin increased while plasma insulin tended to increase and plasma concentrations of glucose and ghrelin remained stable. ∆ supine LF power was inversely associated with ∆ striatal DAT binding (p=0.02).

Conclusion: A short-term hypercaloric diet in non-obese humans causes obesogenic alterations in ANS activity. The inverse relationship between ∆ LF and ∆ DAT binding indicates a link between hypercaloric feeding induced changes in striatal dopaminergic signalling and sympathetic activity.

INTRODUCTION

In rodents, feeding status clearly affects the autonomic balance between the parasympathetic and sympathetic nervous system with fasting suppressing and overfeeding increasing sympathetic activity [1-3]. Also in humans, small amounts of weight gain result in changes in autonomic balance reflecting a relative sympathetic predominance although the methodology to assess autonomic balance differed between studies (5,6). It is unclear whether metabolic or other factors associated with weight gain or increased caloric intake itself perturb the autonomic nervous system (ANS) balance. Indeed several studies, using heart rate variability (HRV) as a measure of autonomic balance, show that a meal reduces the HF power, which reflects parasympathetic activity, to a greater extent than the LF power, reflecting sympathetic activity, thereby resulting in a predominant sympathetic nervous system (SNS) activity postprandially and pointing to a role for feeding itself in the autonomic dysbalance observed in weight gain conditions [4-6].

Thus so far, available studies conclude on SNS predominance after weight gain, however the underlying mechanism is not completely known. Feeding and overfeeding evoke several hormonal responses that could be involved in changes in ANS. Higher insulin levels might play a role as in conditions of hyperinsulinemia, such as insulin resistance, muscle sympathetic nerve activity [MSNA], a reliable method to assess SNS activity, increases [7]. Moreover, intravenous insulin infusion in healthy subjects increases MSNA, decreases parasympathetic activity [8] and increases relative sympathovagal balance derived from HRV, indicating sympathetic predominance [9]. Also, leptin has shown to be positively related to SNS activity [10], and therefore is another potential candidate to affect SNS activity during a hypercaloric diet. Another hormone that might be involved in overfeeding-induced changes in ANS is the stomach derived ghrelin as intravenous ghrelin infusion reduces SNS activity in lean humans [11]. However, studies on the effects on ghrelin during hypercaloric diets in lean subjects are not available. Finally, it might be the nutrients themselves that cause alterations in ANS activity because a single meal causes an increase in SNS activity [4-6]. One study showed an increase in SNS activity measured with HRV after a high-carbohydrate meal but not after a high-fat meal indicating a role for specific macronutrients [12].

Besides the relationship between feeding and its hormonal responses and ANS activity, food intake is also associated with alterations in the brain. One of the brain regions related to food intake is the striatal dopamine system, which is involved in rewarding aspects of eating [13]. In humans striatal dopamine D₁/D₃ receptor binding decreases after feeding, indicating endogenous dopamine release [14]. An increase in striatal dopaminergic signalling is related to the rewarding properties of food as well as to the motivation to obtain food [15]. Interestingly, previous studies indicate a role for dopamine in the control of ANS activity. In animals, stimulating the ventral tegmental area (VTA), a brain area where dopamine is produced, results in increased blood pressure and deple-
tion of brain dopamine reduces hypertension in rats (16), indicating a relationship between the brain dopamine system and the SNS. More recently, Yeh et al showed that striatal dopamine D2/3 receptor binding negatively correlates with heart rate in healthy humans (17). Although it has repeatedly been shown that obese subjects have lower striatal D2/3-receptor binding availability (18-20), the short-term effects of overeating on the striatal dopamine system are not known.

We studied the effect of a short term hypercaloric diet on both the ANS and the striatal dopaminergic system by measuring HRV and striatal dopamine transporter (DAT) binding using SPECT in lean men. DAT regulates available synaptic dopamine for dopamine signaling (21). In addition we measured insulin, leptin and ghrelin to correlate the expected changes in these hormones to the changes in ANS.

**MATERIALS AND METHODS**

The study subjects described in this study participated in a hypercaloric diet intervention trial. The effects of the hypercaloric diets on dienecephalic serotonin transporter availability and on intrathoracic triglycerides and glucose metabolism are recently published (22,23).

**PARTICIPANTS**

We included 36 healthy male Caucasian subjects [age 22 (19-27) years, BMI 22.5 (19.5-24.5) kg/m²] in the main study. Inclusion criteria were good physical health, no family history of type 2 diabetes mellitus (T2DM) and a normal glucose tolerance test (24). Exclusion criteria were use of any medication, substance abuse (nicotine or drugs, alcohol > 2 units/day), history of eating or psychiatric disorders, exercise > 3 hours/week, and an unhealthy diet. To assess whether the diet was balanced, we evaluated dietary intake according to the Dutch guidelines for a healthy diet macronutrient composition (25). Body weight had to be stable in the six months prior to inclusion, thereby excluding a hypocaloric or hypercaloric state when entering the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Medical Ethics Committee of the AMC Amsterdam. Written informed consent was obtained from all study participants before start of study participation.

**STUDY DESIGN**

After inclusion, subjects were randomised into 1 of the 4 hypercaloric diet groups (N=31) or a control group (N=5). Subjects in the 4 hypercaloric diet groups consumed 40% extra calories on top of their normal ad libitum diet for six consecutive weeks. During the diet period, subjects visited the research unit weekly for diet monitoring and for measurement of body weight and resting energy expenditure (REE). Subjects daily reported their ad libitum intake online. When ad libitum caloric intake was lower than caloric need (1.4 x REE), subjects were instructed to increase their ad libitum intake. The excess calories of the 4 hypercaloric diets were provided either as high-fat-high-sugar liquids (Nutridrink Compact; Nutricia Advanced Medical Nutrition, Zoetermeer, the Netherlands) or as high-sugar liquids (commercially available sugar sweetened beverages) to be consumed either with or in between the meals (22,23).

**HEART RATE VARIABILITY**

In a subset of 32 participants (N=5 controls, N=27 on the hypercaloric diet) heart rate and blood pressure were continuously recorded for 30 minutes using a Nexfin system with a pressure cuff on the middle finger (BMeye, Amsterdam, The Netherlands). Measurements were performed after an overnight fast and after at least 30 minutes of acclimatization in the supine position after entering the hospital. Blood pressure and heart rate were continuously recorded for 1.5 minutes in the supine position. Subjects were then asked to stand up to stimulate the SNS and blood pressure and heart rate were continuously recorded for another 1.5 minutes in the upright position. Two subjects on the hypercaloric diet had a vasovagal collapse after standing up and their data had to be excluded from the analyses. Power spectral analysis was performed with an in-house developed MATLAB based software as described previously (26). In short, a stable part of at least 300 data points of the heart rate recording that did not contain spikes or other confounders was selected for both the supine and the upright position. These selected data were Fourier transformed by a MATLAB Digital Fourier Transform, which does not require zero padding to the nearest power of 2. From the spectrum two signals were derived: a low frequency (LF) signal ranging from 0.04 to 0.15 Hz and a high frequency (HF) signal ranging from 0.15 to 0.40 Hz. The area under the curve of the LF and the HF signals was calculated. Variability in the LF power mainly reflects sympathetic activity and variability in the HF power reflects parasympathetic activity (27). The LF/HF ratio [LF signal/HF signal] was calculated representing the sympathovagal balance.

**IMAGING OF STRIATAL DOPAMINE TRANSPORTER (DAT) BINDING**

On a different study day, after an overnight fast, participants underwent single photon emission computed tomography (SPECT) imaging with the radioligand [123I]FP-CIT (DaTSCAN) for the visualization of striatal DAT. A total dose of 115 MBq (range: 110-120 MBq; specific activity > 750 MBq/nmol; radiochemical purity > 98%, produced according to GMP criteria at GE Healthcare, Eindhoven, The Netherlands) was given intravenously. Participants were scanned 3 hours after injection of the tracer, a time point at which the specific striatal DAT binding is stable (28). Each participant was pre-treated with potassium iodide to block thyroid uptake of free radioactive iodide. SPECT imaging was performed using a 12-detector, single slice brain-dedicated scanner (Neurofocus, 810, Strichman Medical Equipment, Cleveland, OH, USA) using an acquisition protocol as described previously with slight modifications (interslice distance: 5 mm; acquisition time: 210 sec/slice) (29). Due to technical problems with the SPECT scanner, scans of 6 subjects on the hypercaloric diet were unavailable and therefore analyses were performed on data of 25 subjects.
on the hypercaloric diet and 5 control subjects. All scans were reconstructed in 3D mode and corrected for attenuation. For quantification, a region-of-interest (ROI) analysis to determine specific binding activity in the striatum was performed by a well-trained researcher as described previously [28]. Briefly, the 4 consecutive slices with the highest binding were selected to assess binding to DAT. Activity in the cerebellum (3 consecutive slices) was assumed to represent non-displaceable activity (nonspecific binding and free radioactivity). The outcome measure was specific-to-nonspecific binding ratio (SNS-BR), calculated as [ROI-binding minus cerebellar-binding / cerebellar-binding].

LABORATORY ANALYSES

On the same day HRV was measured, we collected blood samples for laboratory analyses in a fasting condition (overnight fast). Plasma glucose concentrations were measured with the glucose oxidase method using a Biosen C Line plus glucose analyzer (EKF Diagnostics, Barleben/Magedeburg, Germany). Insulin was determined on an IMMULITE 2000 system (Siemens Healthcare Diagnostics B.V., Breda, The Netherlands). Insulin was determined with a chemiluminescent immunometric assay (intra-assay variation 3-10%, total-assay variation 4%, detection limit 15 pM). Leptin was determined with a human leptin radioimmunoassay kit (Millipore) (intra-assay pairs test was performed. Data on striatal DAT availability were normally distributed and analyzed using a Wilcoxon matched pairs test. HRV data before and after the diet were not normally distributed, therefore a Wilcoxon matched pairs test was performed. For the current manuscript, we performed an additional power analysis using a Wilcoxon test with a 0.05 two-sided significance level. Hence, we can only analyze HRV in the pooled hypercaloric diet groups and not in the subgroups. The control group was included to exclude a study effect on our primary outcome measures [22,23] but is not analyzed with regard to the secondary outcome measures as presented here.

STATISTICS

HRV data before and after the diet were not normally distributed, therefore a Wilcoxon matched pairs test was performed. Data on striatal DAT availability were normally distributed and analyzed using a paired student T-test. LF and LF/HF were not normally distributed, therefore we used Pearson’s correlation test. A priori sample size calculation was performed for the primary outcome measures of the study, namely diencephalic serotonin transporter binding and insulin sensitivity. The sample size calculations resulted in a number of 8 subjects per hypercaloric diet group and was previously published [22,23]. For the current manuscript, we performed an additional power analysis based on a study that describes LF/HF ratio in lean women before and after a carbohydrate-rich meal [112]. A sample size of 16 is required for 80% power to detect a probability of 0.794 that an observation at baseline is significantly different from an observation after the dietary manipulation using a Wilcoxon test with a 0.05 two-sided significance level. Hence, we can only analyze HRV in the pooled hypercaloric diet groups and not in the subgroups. The control group was included to exclude a study effect on our primary outcome measures [22,23] but is not analyzed with regard to the secondary outcome measures as presented here.

RESULTS

The baseline characteristics of the 31 subjects that followed one of the four hypercaloric diets are presented in table 1.

| Table 1. Characteristics, anthropometric and laboratory data. |
|-----------------|-----------------|-----------------|
|                | Before diet     | After diet      | p     |
| N               | 31              | 31              |       |
| Age (y)         | 22.0 ± 2.4      | 22.3 ± 1.3      | 0.66  |
| BMI (kg/m²)     | 23.3 ± 1.4      | 23.3 ± 1.4      | 0.84  |
| Body weight (kg)| 79.5 ± 2.75     | 79.0 ± 2.75     | 0.38  |
| Caloric intake (kCal/day)| 2922 ± 4318 | 3706 ± 3811 | 0.01  |
| Glucose (mmol/L)| 4.77 ± 0.22     | 4.76 ± 0.27     | 0.89  |
| Insulin (pmol/L)| 470 ± 199       | 525 ± 160       | 0.04  |
| Leptin          | 2.91 ± 1.36     | 4.12 ± 2.20     | 0.04  |
| Ghrelin         | 784 ± 179       | 725 ± 310       | 0.1   |

Data presented as mean and SD. BMI = body mass index. * Cerebral cortex during the diet average of the 6 weeks. Blood samples collected in fasted condition. ** p < 0.01; *** p < 0.001; # p = 0.10; ns = not significant

HRV RATIO, BLOOD PRESSURE AND HEART RATE VARIABILITY

Systolic blood pressure and heart rate, as well as HF power and LF/HF ratio, were unaltered after a six-week hypercaloric diet in the supine or the upright position. LF power in the supine position significantly increased (table 2).

The expected increase in LF power after standing up at baseline was attenuated after the hypercaloric diet. The difference in LF power (upright-supine) was significantly lower at the end of the diet compared to baseline [0.66 [-10.74-5.37] vs 0.07 [-13.01-3.85] Hz/ms; p=0.02). HF power decreased and LF/HF ratio increased from the supine to the upright position with no differences in response after the hypercaloric diet (table 2).

STRIATAL DAT BINDING AFTER THE SIXWEEK HYPERCALORIC DIETS

Striatal DAT binding tended to decrease after the hypercaloric diet (p=0.08, figure 1A).

| Table 2. Blood pressure and heart rate measures before- and after a hypercaloric diet |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Before diet     | After diet      | p       | Before diet     | After diet      | p       |
| Systolic Blood Pressure (mmHg) | 118.4 [109.1-130.4] | 118.6 [109.8-141.9] | 0.52  | 118.6 [109.8-141.9] | 124.6 [112.8-141.9] | 0.52  |
| Heart rate (beats/min)            | 54.5 [62.0-68.7]  | 59.3 [64.3-76.3]  | 0.52  | 59.3 [64.3-76.3]  | 71.9 [68.8-99.1]  | 0.71  |
| LF/HF Ratio                       | 0.00 [0.09-1.29]  | 0.74 [0.00-2.28]  | 0.95  | 0.74 [0.00-2.28]  | 4.28 [0.10-9.02]  | 0.18  |
| LF variance (Hz/ms)               | 1.92 [3.33-8.78]  | 1.93 [3.18-10.75] | 0.96  | 1.93 [3.18-10.75] | 1.97 [2.66-8.80]  | 0.18  |
| HF variance (Hz/ms)               | 1.93 [2.93-7.29]  | 1.89 [2.93-7.29]  | 0.96  | 1.89 [2.93-7.29]  | 1.89 [2.93-7.29]  | 0.96  |

Data expressed as median and range. LF: low frequency (sympathetic activity); HF: high frequency (parasympathetic activity); LF/HF ratio: sympathovagal balance
BIOCHEMICAL DATA AFTER THE SIX WEEK HYPERCALORIC DIET.

Leptin increased significantly while ghrelin and glucose concentrations remained stable and insulin tended to increase \((p=0.08)\) after the hypercaloric diet period.

CHANGE IN LF POWER NEGATIVELY CORRELATES WITH CHANGE IN STRIATAL DAT AVAILABILITY

Data for both HRV and striatal DAT binding were available in 22 subjects. At baseline and after the diet, no correlations were found between striatal DAT availability and supine LF power \((p=0.40 \text{ and } p=0.23 \text{ resp.})\), supine HF power \((p=0.78 \text{ and } p=0.88 \text{ resp.})\) or supine LF/HF ratio \((p=0.11 \text{ and } p=0.22 \text{ resp.})\). At baseline, plasma leptin correlated with LF power only \((p=0.046, r=0.40, F=4.46)\) while insulin \((p=0.89)\), ghrelin \((p=0.30)\) and body weight \((p=0.99)\) did not correlate with either LF power, HF power and LF/HF ratio. After the diet no associations were present between any of the metabolic parameters and ANS. Furthermore, a significant negative correlation was found between change in supine LF power and the change in striatal DAT binding \((p=0.015, r=-0.51, F=7.03)\), figure 1B) but not between changes in body weight \((p=0.62)\) or changes in leptin \((0.95)\).

DISCUSSION

We here describe the effect of 6-week 40% hypercaloric diets on heart rate variability as a measure for ANS activity in lean men. LF power, representing sympathetic activity, increased in the supine position and the response of LF power to a sympathetic stimulation, i.e. standing up, was blunted. Sympathetic activity is the main determinant of LF power, parasympathetic activity also contributes. In our study subjects, parasympathetic-determined supine HF power did not change which suggests that an increase in sympathetic activity rather than a decrease in parasympathetic activity causes the increase in LF power. In spite of the increase in LF power, we did not find a difference in LF/HF ratio. This might have several reasons: the increase in LF power might be too small to result in significant changes in the LF/HF ratio or, although it was not significant, the small increase in HF power might blunt the effect.

Our findings are in line with previous studies showing that overfeeding increases basal SNS activity, measured using MSNA, in rodents (1-3) and in humans (30). However, these studies could not distinguish between the direct effect of food intake on ANS activity or other factors related to weight gain such as an increase in leptin. In our subjects changes in body weight or leptin were not related to the differences in ANS activity although baseline leptin concentrations correlated with LF power. Since this association was no longer present after the hypercaloric diet, leptin might only have a regulatory role in ANS in eucaloric conditions while during forced hypercaloric conditions, either leptin resistance occurs or other regulatory factors become more prominent. Insulin has frequently been pointed out as a possible candidate to alter ANS activity in obesity (e.g. [9,31]). Insulin levels in our subjects were measured in the fasting state only and did not correlate with measures of ANS, but this does not exclude a role for postprandial insulin in the observed changes in LF power in our subjects. An increase in sympathetic activity might be a compensatory mechanism to expend the excess calories through increasing energy expenditure. In obese humans, weight loss is predicted by sympathetic activity (32) and sympathetic activity has been related to energy expenditure in lean Caucasian men (33). Resting energy expenditure was unaltered in the current study (data not shown) (23) but this does not exclude subtle changes which might have beneficial effects in the longer term. Moreover, we used indirect calorimetry while doubly labelled water might be a more appropriate technique to measure total longer-term energy expenditure. Taken together our data show that consumption of calories in excess of caloric need results in increased sympathetic activity not related to weight gain or hormonal factors in lean men. Therefore increased sympathetic activity could be part of the early adaptive response to caloric overload. Whether increased sympathetic activity leads to reduced food intake or increased energy expenditure was not assessed in the current study.

In addition to changes in sympathetic activity, we found a trend towards a decrease in striatal DAT binding after the hypercaloric diets. The dopaminergic signalling system consists of DAT and dopamine receptors and the complex regulation of DAT in humans is only partly understood. The presynaptically localized DAT exerts dynamic spatial and temporal control of extracellular dopamine concentrations via re-uptake of released dopamine from the synaptic cleft (34). The short term hypercaloric diet in our subjects might have resulted in increased dopamine release with sub-
sequest lower DAT since substrates for DAT, including increased levels of endogenous dopamine, may induce down-regulation of DAT (34). Alternatively, Romamoorthy et al. showed that activation of dopamine D2 receptors play a role in the expression of DAT and activation of dopamine receptors by endogenous dopamine may induce reduced striatal DAT expression.

Interestingly, the increase in LF power after the hypercaloric diet was associated with the trend towards the decrease in striatal DAT binding. A connection between striatal dopamine and blood pressure, a cardiovascular outcome regulated by the ANS, has previously been shown in rodents (16). Our data therefore suggest that the increased sympathetic tone after the hypercaloric diet might in part be explained by the changes in striatal DAT and thus increased extracellular dopamine. This requires further detailed studies.

The current study has a number of limitations. First, heart rate variability is a derivative of sympathetic activity. This requires further detailed studies.

CONCLUSION

A hypercaloric diet increases resting sympathetic activity and reduces responsiveness to a sympathetic stimulation in lean men, a combination that has previously been linked to obesity. Changes in LF power were not related to changes in plasma concentrations of insulin, leptin or ghrelin, suggesting a possible direct effect of food or food intake on SNS activity. In addition, the increase in resting sympathetic activity was negatively correlated with alterations in striatal DAT binding indicating a link between changes in striatal dopaminergic signalling and sympathetic activity.

REFERENCES

<table>
<thead>
<tr>
<th>Reference</th>
<th>Title</th>
</tr>
</thead>
</table>
Summary and General discussion
SUMMARY

The main objective of this thesis was to study cerebral serotonin transporters (SERT) in the diencephalon and striatal dopamine transporters (DAT) in humans in different metabolic conditions (i.e. lean, obese and insulin resistant state) in relation to feeding behavior. In addition, we aimed to investigate the early consequences of overeating on diencephalic SERT and striatal DAT, on a number of metabolic parameters including fat accumulation and insulin sensitivity, and on the autonomic nervous system in lean humans.

Previous studies assessed the optimal time point to assess striatal DAT binding using 123I-FP-CIT in humans. However, the optimal time point to assess extrastriatal SERT binding with this imaging tool had not been determined yet. We therefore first determined the optimal time point for imaging extrastriatal SERT binding using 123I-FP-CIT in the human brain in vivo. From two hours after bolus injection, the specific to non-specific 123I-FP-CIT binding in the SERT-rich midbrain and diencephalon was stable, and it was concluded that the optimal time frame for assessing 123I-FP-CIT binding to extrastriatal SERT was 2 hr post-injection (chapter 2). Next, using immunocytochemistry on postmortem human hypothalamic tissue, we confirmed that SERT protein was abundantly present in the human hypothalamus (chapter 3). Moreover, SERT protein was most markedly expressed in the infundibular nucleus (IFN) of the hypothalamus and overweight/obese subjects showed lower IFN SERT protein expression than lean subjects. We next studied SERT in lean and obese women in vivo and found no difference between lean and obese subjects. However, when comparing SERT between insulin resistant obese and insulin sensitive obese women, the former showed lower SERT (chapter 4), suggesting that SERT in obese women is related to glucose metabolism rather than body weight per se. In contrast, striatal DAT binding did not differ between lean and obese women, nor between insulin resistant obese and insulin sensitive obese women (chapter 4).

In lean humans, we showed that diencephalic SERT and striatal DAT are related to food motivation (chapter 5) since visual attention bias for food, which is a measure of food motivated behavior in humans, inversely correlated with both striatal DAT and diencephalic SERT binding. Moreover, visual attention bias positively correlated with actual food intake, indicating that food motivated behavior predicts food intake or vice versa. We next aimed to study how hypercaloric feeding affects diencephalic SERT and striatal DAT and whether increasing meal size versus meal frequency may have differential effects. We showed that a high fat high sugar (HFHS), but not a high sugar (HS) only, snacking diet, reduces diencephalic SERT binding within 6 weeks without significantly affecting striatal DAT. Moreover, this effect was not observed in the subjects consuming the same macronutrients during their 3 major meals, i.e., by increasing meal size (chapter 6). This indicates that besides calories and dietary content, eating pattern in itself is able to modulate SERT in the human brain. Whether this is a direct effect or indirect effect through diet-induced changes in glucose metabolism is at present unknown. Surprisingly, hepatic insulin sensitivity tended to decrease after the hypercaloric HFHS snacking diet only, further showing that eating pattern, per se in combination with macronutrient content in a hypercaloric setting differentially affects metabolism and likely independently contributes to insulin resistance next to the effects on the serotonergic pathways (chapter 7). Lower diencephalic SERT induced by a HFHS snacking diet might promote on-going caloric intake through modulation of extracellular serotonin and since our current obesogenic environment is characterized by snacking behavior, our findings might explain part of overeating in obesity. Striatal DAT binding on the other hand tended to be affected by hypercaloric feeding in general, without a differential effect of eating pattern or macronutrient composition of the diet (chapter 8) pointing to a more general relationship with excess calories or body weight gain. We also observed that sympathetic nervous system (SNS) activity was increased by the hypercaloric diet and this was related to the change in striatal DAT binding, suggesting a link between SNS activity and the striatal dopamine system in response to overfeeding/weight gain (chapter 8).

Finally, we describe in chapter 7 that hypercaloric snacking, irrespective of dietary macronutrient composition, resulted in increased liver- and abdominal fat whereas iso-calorically increasing meal size did not. Since BMI increased to a similar extent in all groups, this shows an independent effect of meal pattern on the accumulation of liver and abdominal fat. Further setting the stage for the complexity of the underlying mechanisms of the metabolic complications of weight gain/obesity.

In summary, we here show that SERT and DAT in the human brain are involved in food motivated behavior and that SERT is reduced in the hypothalamus of overweight/obese subjects and particularly in obese women with insulin resistance. Moreover we provide evidence that consuming HFHS snacks in excess of caloric need reduces SERT in the diencephalon and reduces hepatic insulin sensitivity in addition to the negative effects of snacking on liver and abdominal fat accumulation. Increasing meal size only did not show these effects pointing to an independent effect of hypercaloric snacking behavior on peripheral glucose metabolism and on brain circuitries involved in feeding behavior and central control of glucose metabolism. Further studies are needed to elucidate the underlying mechanisms explaining these associations in humans and to disentangle the relationship between glucose metabolism and brain serotonergic pathways. This might lead to novel treatment modalities and dietary advice with the aim to combat obesity and insulin resistance.

GENERAL DISCUSSION

Our brain orchestrates energy metabolism by responding to signals from peripheral organs including nutrients, peptides and afferent autonomic nerve signals. Within the hypothalamus this information on energy status is integrated with signals from higher brain centers involved in reward processes resulting in neuroendocrine responses and behavioral adaptations to regulate energy intake and expenditure [for reviews (1,2)]. Within this network, serotonin and dopamine are important neurotransmitters and, as can be appreciated from this thesis, their regulatory role extends beyond their long established effects on feeding behavior [3-9]. We demonstrate associations between serotonin and dopamine transporter binding, motivation to eat, obesity and insulin resistance. Furthermore, diencephalic serotonin transporter binding and hepatic insulin sensitivity both can be modulated in the short term by intake of specific macronutrients when consumed in between meals and in excess to caloric need, suggesting mutual interactions between diet composition, diet timing pattern, glucose metabolism and central serotonin signaling.
In general, genetically reduced SERT expression is associated with obesity and type 2 diabetes mellitus [T2DM] [10,11] and SERT knockout mice are (abdominally) obese and insulin resistant [12,13]. Studying the serotonergic system in humans is challenging because the brain is not readily accessible for in vivo studies. So far it was known that serotonin and its metabolites are lower in cerebrospinal fluid (CSF) of obese compared to lean women [14] and SERT binding in subcortical regions is negatively correlated with BMI [15], though a recent study showed a positive correlation between (hypothalamic) SERT binding and BMI [16]. However, these studies could not determine a specific brain region responsible for the relationship with BMI/obesity. In this thesis, we show in chapter 3 that SERT protein is highly expressed in the hypothalamic infundibular nucleus (IFN), and expression is lower in overweight/obese subjects compared to lean subjects while we show in chapter 6 that a hypercaloric HFHS snacking diet reduces SERT binding by 30% in the diencephalon of lean healthy men. This effect was independent of body weight gain and thus suggests a direct effect of nutrients and eating pattern on the serotonergic system in the brain, and this may in turn facilitate further excessive caloric consumption. Since in rodents neurochemical depletion of hypothalamic serotonin leads to hyperphagia and obesity [5-7] and tryptophan (and thus serotonin) depletion lowers SERT binding [17], these findings indicate that overconsumption of fat and sugar snacks in-between meals reduces extracellular serotonin, which may predispose to obesity because of serotonin’s inhibiting effects on food intake. In line, in humans serotonin is lower in CSF in the obese state [14]. Interestingly in chapter 5, we show that lower diencephalic SERT binding is associated with higher visual attention bias for food, and in turn, higher visual attention bias for food correlates positively with actual food intake, again suggesting that a reduction in SERT binding predisposes to weight gain through its effects on food intake. Finally, the recently approved serotonin receptor 2c agonist lorcaserin has shown a weight reducing effect through lowering of food intake without affecting energy expenditure [18]. Thus the serotonergic system in the human brain is involved in food intake and body weight regulation and is affected by nutrients and eating pattern.

The brain serotonin system might also affect glucose metabolism. Indeed, diencephalic SERT binding was reduced in obese individuals with insulin resistance [IR] compared to equally obese individuals without IR [chapter 4] while there were no differences in SERT binding between the lean and obese group, contradicting our IFN SERT data in post mortem hypothalamus as well as previous findings by others [15]. This indicates that the relation between diencephalic SERT/serotonin and obesity is less straightforward than suggested and might be influenced by glucose metabolism as well. This is in line with rodent studies showing alterations in glucose metabolism in the absence of obesity in a SERT knockout model [12] and in mice lacking the serotonin 2C receptor specifically in POMC neurons [19]. The spatial resolution of the current imaging techniques does not allow for further characterization of different hypothalamic nuclei and, although serotonin within (the POMC neurons of) the ARC is heavily involved in regulation of food intake and glucose metabolism [19], other nuclei might be responsible for the association we describe in chapter 4. One candidate is the suprachiasmatic nucleus (SCN), as SCN-lesioned rats have higher plasma insulin levels compared to sham operated rats [20] and glucose intolerant hamsters have markedly reduced SCN serotonin metabolite levels compared to glucose tolerant hamsters [21]. Moreover, SERT protein is present in the human hypothalamic SCN [chapter 3], but whether this expression relates to glucose metabolism is unknown. Moreover, additional cell populations in the hypothal-
D2/3 receptors are reduced in obese humans (37;40-42) and rats (43;44), and DAT expression (49;50), supporting that lifestyle factors besides genetics play a role in reduced dopamine via increased DAT function (61;62) and insulin receptors are present on the hypothalamus regulates glucose metabolism but whether this is also true for humans is unknown. On the other hand, circulating factors associated with the insulin resistant obese state might affect serotonergic pathways and we show that diet composition and eating pattern both affect serotonin transporters in the diencephalon, including the hypothalamus and thalamus, in non-obese individuals. This indicates that dietary habits are independent modulators of serotonergic pathways in the human brain. In view of these considerations, serotonin signaling might be a promising target to treat both obesity and insulin resistance/diabetes in humans. This could be achieved by either pharmacologically targeting serotonin transporters or receptors or by designing hypocaloric diets aimed at increasing serotonin transporters.

The striatal dopamine system is involved in signaling the rewarding properties of food. Extracellular dopamine (DA) is regulated by presynaptic dopamine transporters (DAT) that facilitate reuptake of dopamine from the synaptic cleft. Inhibiting or genetically knocking down DAT results in enhanced DA release in the striatum is related to experienced pleasure after consuming a meal (34) and to ratings of hunger and desire for food in humans (35). In line, as described in chapter 5, lower striatal DAT binding, which could reflect higher extracellular dopamine (36), is associated with a higher visual attention bias for food and in turn, visual attention bias for food is associated with actual food intake, thus providing further evidence for a role of dopamine in food motivated behavior in lean healthy humans. Obesity has been related to lower responsiveness of the reward circuit as result of a reduced dopaminergic tone (referred to as the reward deficit theory) (37), supported by the findings that a) when food is consumed or consumption is imagined, obese humans show less striatal activation compared to lean individuals (38;39), b) dopamine release to an amphetamine challenge is diminished in obese humans (40), c) striatal dopamine D2/3 receptors are reduced in obese humans (37;40;42) and rats (43;44), and d) DAT expression (42) has been shown to be reduced in obesity-prone rats. The data on striatal DAT binding in humans is less convincing as only one out of 3 studies reported an association between striatal DAT binding and BMI (44-46). In line we could not demonstrate a difference in striatal DAT binding between lean and obese individuals (chapter 4). It is not clear whether the reduced dopaminergic tone is a cause or a consequence of obesity. Genetic studies show that adults with a polymorphism in the dopamine D2 receptor gene, associated with compromised striatal dopamine signaling, are at higher risk of becoming obese (47) suggesting that genetically low dopaminergic tone might lead to obesity. In contrast, children with this polymorphism show enhanced striatal responsiveness to consumption of palatable food (48), suggesting that the assumed reward deficiency is acquired later in life. Rodent studies show that high fat diets reduce striatal dopamine D2/3 receptor binding (49;50), supporting that lifestyle factors besides genetics play a role in reducing striatal dopaminergic tone. These D2/3 receptor changes might subsequently influence feeding behavior because it was shown that lentivirus-mediated knockdown of striatal D2 receptors rapidly accelerated the development of addiction-like reward deficits and the onset of compulsive-like behavior in rats (51). In chapter 8, we show that hypercaloric diets tend to reduce striatal DAT in healthy humans while we found a clear effect on diencephalic SERT in those individuals that consumed hypercaloric high fat high sugar snacks in between meals, suggesting that in the short term striatal DAT is less affected than SERT by caloric overconsumption or specific macronutrients and eating pattern. Whether the hypercaloric diet intervention affected D2/3R binding availability is unknown because we were not able to image DAT and D2/3R simultaneously. In addition, short term clinically significant weight loss through bariatric surgery did not restore lower striatal D2/3 receptor availability in obese humans (52) pointing to a less straightforward relationship between the striatal dopamine system and body weight. Reduced dopaminergic tone is probably a consequence of chronic caloric overconsumption and/or obesity itself rather than a cause of obesity, although genetic factors might contribute. The exact underlying molecular mechanisms and series of events taking place in the striatal dopaminergic system upon weight gain remains to be elucidated. In consequence, the changes that occur in striatal dopaminergic signaling might differ during the course of the weight gain with an initial rise in extracellular dopamine and subsequent decrease, since a positive correlation between BMI and dopamine release was described in lean and overweight/mildly obese subjects (53) while dopamine release is reduced in obese compared to lean subjects (40). Longitudinal studies are needed to explore this further in humans. In addition, it should be studied in rodents how lower DAT and lower D2/3R affect dopamine signaling. This will potentially enable the development of therapeutic strategies aimed to modulate dopaminergic pathways in the setting of obesity (prevention).

During weight gain and in overt obesity, the activity of the sympathetic nervous system (SNS) is altered. Excess body fat is associated with increased SNS activity measures in humans (54). In addition, striatal dopamine D2/3 receptors correlate positively with sympathetic activity in healthy volunteers (55). In chapter 8 we show enhanced sympathetic activity in lean males after a 6 weeks hypercaloric diet. Interestingly, the increase in sympathetic activity after the hypercaloric diet was associated with a trend towards a decrease in striatal DAT binding. A connection between striatal dopamine and blood pressure, a cardiovascular outcome regulated by the autonomic nervous system (ANS), has previously been shown in rodents (56). Therefore, our data might point to a diet-induced increase in extracellular dopamine, resulting in increased sympathetic activity. This remains speculative and more detailed studies aimed at unravelling the relation between striatal dopamine and cardiovascular outcomes are needed.

Finally, striatal dopamine has been implicated in the regulation of glucose metabolism (57). Modulation of central dopamine by D2 receptor agonists has an insulin sensitizing effect on glucose metabolism in hamsters (58). Reports on the relation between striatal D2/3R availability and measures of insulin sensitivity in obese women are somewhat contradictory (52;59) but treatment of diabetic patients with dopamine agonists showed improved glucose tolerance (60). Reciprocal-ly, insulin also affects dopamine signaling by acutely enhancing the clearance of synaptically released dopamine via increased DAT function (61;62) and insulin receptors are present on dopaminergic neurons in the VTA (63). Moreover, Zucker fatty rats that are hyperinsulinemic also show increased DAT mRNA expression (64) and dopamine antagonists are associated with insu-
lin resistance independently of obesity (65). It was therefore unexpected that striatal DAT binding did not correlate with insulin sensitivity and that there were no differences between insulin resistant obese and insulin sensitive obese individuals (chapter 4). Based on the work presented in this thesis, the focus of future studies on the role of the dopamine system in peripheral glucose metabolism should be on extrastriatal dopaminergic brain areas or on striatal dopamine receptors or dopamine release instead of on DAT. Specific dopamine receptor agonists might improve insulin resistance besides lowering food intake, and this might lead to novel medical treatment strategies for T2DM.

In conclusion, as for the serotonergic system, dopaminergic pathways are involved in food intake and glucose metabolism. Many questions regarding underlying molecular mechanisms, pathways and causes explaining the changes observed in obesity and during caloric overconsumption remain to be answered before new safe treatment modalities can be developed. Our data and available other data point towards the brain as a potential new therapeutic target in the battle against obesity.

CRITICAL NOTES AND FUTURE STUDIES

The conclusions drawn from the measurements of diencephalic SERT and striatal DAT binding need some critical appraisal. First, when visualizing SERT and DAT binding with \( ^{11} \text{C} \)-FP-CIT, we measure binding potential of the tracer to the transporter. Binding potential is related to the density of available transporters when a secular equilibrium is reached (66), but we cannot differentiate if a change in binding potential is due to a change in density of available SERT or DAT or due to a change in the affinity (K_C) of the radiotracer for the transporter. In addition, after bolus injection of \( ^{11} \text{C} \)-FP-CIT a transient equilibrium is reached 2 h later (chapter 2) which might lead to an overestimation of SERT and DAT densities (66,67). Second, \( ^{11} \text{C} \)-FP-CIT is a non-selective ligand which gives access besides lowering food intake, and this might lead to novel medical treatment strategies for T2DM.

The conclusions drawn from the measurements of diencephalic SERT and striatal DAT binding need some critical appraisal. First, when visualizing SERT and DAT binding with \( ^{11} \text{C} \)-FP-CIT, we measure binding potential of the tracer to the transporter. Binding potential is related to the density of available transporters when a secular equilibrium is reached (66), but we cannot differentiate if a change in binding potential is due to a change in density of available SERT or DAT or due to a change in the affinity (K_C) of the radiotracer for the transporter. In addition, after bolus injection of \( ^{11} \text{C} \)-FP-CIT a transient equilibrium is reached 2 h later (chapter 2) which might lead to an overestimation of SERT and DAT densities (66,67). Second, \( ^{11} \text{C} \)-FP-CIT is a non-selective ligand which gives the opportunity to measure diencephalic SERT and striatal DAT simultaneously, but might also lead to an over- or underestimation of the binding potential to SERT or DAT. Moreover, it does not allow us to measure striatal SERT or diencephalic DAT. Therefore, our studies should be repeated using selective ligands to confirm our findings and to investigate SERT and DAT binding in other brain regions. Third, measurement of SERT and DAT binding with SPECT is static, whereas the regulation of SERT and DAT in response to stimuli is spatial and dynamic (68,69). Therefore, dynamic measurement of SERT and DAT in response to, for instance, food or food cues in vivo would be of great interest. Unfortunately, the neuroimaging techniques for that are not yet available. Fourth, there might be a gender effect as has been demonstrated for the relationship between central SERT expression and body weight: female SERT knockout rats become abdominally obese but the males do not [13], and in humans obese females have higher thalamus/hypothalamus SERT binding than their lean monozygotic co-twins, with no difference for male twin pairs [70]. In lean subjects gender differences in SERT binding have been described [71], but not consistently [72,73], and whether the relationship between insulin sensitivity and diencephalic SERT is affected by gender is unknown. Similarly for striatal DATs, an effect of gender in humans has been demonstrated with females having higher striatal DAT binding (as measured with \( ^{11} \text{C} \)-FP-CIT SPECT) than males [74,75] but this is not a consistent finding [44]. Nevertheless, our studies were performed in either males or females only and extrapolation to the other gender should be done with caution. The relationships between SERT and extracellular serotonin, and between DAT and extracellular dopamine are not clear which limits the explanation of our findings. Unfortunately at present, local serotonin and dopamine concentrations in the brain cannot be measured in vivo in humans and therefore, it is necessary to repeat the hypercaloric diet experiments in rodents to further explore underlying mechanisms. This would also allow for determining in which specific nuclei within the diencephalon SERT (and DAT) is changed upon a hypercaloric diet intervention. In humans, using positron emission tomography (PET) as a neuroimaging technique would allow to visualize smaller brain areas than when using SPECT. Still, it is not sensitive enough to visualize distinct hypothalamic nuclei. Furthermore, studies in rodents are needed to elucidate which pathways are involved in the consequent finding of lower striatal D2/3R in obese humans. In addition, longitudinal studies in humans and rodents are needed to follow the sequence of events occurring during the course of weight gain. Finally, studying insulin resistant versus insulin sensitive obese humans and rodents might reveal which brain circuits protect from metabolic deterioration.

CLINICAL IMPLICATIONS

The relationship between diencephalic SERT and insulin resistance suggests that the brain serotonergic system is a potential future pharmacological target in the treatment of T2DM. Indeed, it has been shown that pharmacologically increasing extracellular serotonin concentrations by short-term SSRI treatment or by agonizing the serotonin 2C receptor is beneficial for glycemic control [76,77]. However, as mentioned before, there are differences in outcome between short-term and long-term pharmacological targeting of the serotonin system and more insight in these differences is necessary before any conclusions on therapeutic implications can be drawn. Moreover, thorough research of possible side effects is necessary as fenfluramine and fenfluramine-like derivatives, drugs that induce a massive release of serotonin and were previously on the market for the treatment of obesity, have been withdrawn due to serious cardiovascular side effects [78].

In addition, dopamine agonists targeted at the striatal D2 and D3 receptors or modulating striatal DAT might be beneficial in reducing food intake and ameliorating insulin resistance in the obese state.

Obviously, weight loss is a major goal in the treatment of insulin resistance and obesity. Dieters often advice obese individuals, that it is best to eat frequent smaller meals to reduce the feelings of hunger that occur in a hypocaloric state. Indeed, when this advice is strictly followed, eating more frequent smaller meals results in more weight loss compared to eating fewer larger meals [79] although this has recently been challenged in patients with T2DM [80]. However, when consuming food in between the main meals the extra calories from the snack are rarely compensated for in the next meal [81], which suggests that it may not be free of risk to advice obese humans who clearly have problems regulating their caloric intake, to consume meals more frequently. In fact, we showed that eating pattern and macronutrient composition are an intertwined independent factor of increasing liver and abdominal fat and reducing diencephalic SERT in a hypercaloric setting. Overall this suggests that reintroducing cycles of hunger and satiety (instead of exposing our body to ingested nutrients more frequently during the day) might be beneficial in restoring diencephalic SERT and reducing abdominal and liver fat. However this has to be studied and verified in future studies.
REFERENCES


Summary & General discussion

February 3;357(9253):354-7.


No association between striatal dopamine transporter binding and body mass index in a multicenter European study in healthy volunteers. Neuroimage 2013 January;1;64:61-7.


**NEDERLANDSE SAMENVATTING**

De studies in dit proefschrift beschrijven de serotonine transporters (SERT) in het diencephalon (thalamus en hypothalamus) en de dopamine transporters (DAT) in het striatum in verschillende metabole condities in slanke en obese mensen alsook hun relatie met eetgedrag. Om de rol van deze transporters in het ontstaan van obesitas en stoornissen in de suikerstofwisseling te bestuderen hebben we SERT en DAT gevisualiseerd voor en na een hoogcalorisch dieet in slanke mensen.

**SAMENVATTING**

Eerdere studies toonden aan dat het optimale tijdsinterval om DAT beschikbaarheid in het striatum te meten met $^{123}$I-FP-CIT SPECT drie uur na toediening van de tracer is. $^{123}$I-FP-CIT SPECT is een nucleaire technologie met een hoge tracer die aan SERT en DAT bindt. Het tijdsinterval waarop SERT beschikbaarheid in het diencephalon met deze techniek het best gemeten kan worden met deze techniek was echter nog onbekend en werd in de eerste fase van ons onderzoek bepaald. Vanaf twee uur na toediening van een bolus injectie $^{123}$I-FP-CIT was de specifieke tot niet-specifieke bindingsratio in de SERT-rijke mid-doven hersenen stabiel, en daarom werd gecombineerd met het optimale tijdsinterval om binding van $^{123}$I-FP-CIT aan SERT buiten het striatum te meten een optimale tijdsinterval voor de binding van $^{123}$I-FP-CIT aan SERT buiten het striatum te meten twee uur na toediening was (hoofdstuk 2). Vervolgens hebben we door middel van immunocytochemie in de post-mortem humane hypothalamus bevestigd dat SERT eiwit uitgebreid aanwezig was (hoofdstuk 3). SERT eiwit was het meest prominent aanwezig in de nucleus fusiformis en mensen met overgewicht/obesitas hadden significant minder SERT eiwit in hun IFN dan slanke mensen. Vanaf twee uur na toediening van een bolus injectie $^{123}$I-FP-CIT was de specifieke tot niet-specifieke bindingsratio in de SERT-rijke mid-doven hersenen stabiel, en daarom werd gecombineerd met het optimale tijdsinterval om binding van $^{123}$I-FP-CIT aan SERT buiten het striatum te meten een optimale tijdsinterval voor de binding van $^{123}$I-FP-CIT aan SERT buiten het striatum te meten twee uur na toediening was (hoofdstuk 2). Vervolgens hebben we door middel van immunocytochemie in de post-mortem humane hypothalamus bevestigd dat SERT eiwit uitgebreid aanwezig was (hoofdstuk 3). SERT eiwit was het meest prominent aanwezig in de nucleus fusiformis en mensen met overgewicht/obesitas hadden significant minder SERT eiwit in hun IFN dan slanke mensen. Vervolgens onderzochten we SERT binding in vivo (met SPECT) in het diencephalon van slanke en obese mensen. Daarbij vonden we geen verschil tussen slanke en obese proefpersonen. We vonden echter wel dat obese proefpersonen met insulineresistentie significant lagere SERT beschikbaarheid in hun diencephalon hadden dan obese proefpersonen zonder insulineresistentie (hoofdstuk 4). Dit zou kunnen betekenen dat SERT in obese mensen gerelateerd is aan glucosemetaboïsme en niet per se aan lichaamsgewicht. In tegenstelling tot SERT vonden we dezelfde DAT beschikbaarheid in het striatum bij obes en slanke proefpersonen en bij obese insuline resistentie en obese insuline gevoelige proefpersonen (hoofdstuk 4). Verder toonden we aan dat in slanke mensen SERT beschikbaarheid in het diencephalon en DAT beschikbaarheid in het striatum gerelateerd is aan motivatie om te eten. Dit bleek uit het feit dat de visuele aandachtsbias voor voedsel, een maat voor de motivatie voor voedsel, negatief correleerde met zowel SERT beschikbaarheid in het diencephalon, als DAT beschikbaarheid in het striatum (hoofdstuk 5). Bovendien correleerde de visuele aandachtsbias voor voedsel positief met voedselintake, wat er op wijst dat motivatie om te eten de voedselintake voorspelt, of andersom. Het volgende doel was om te bevestigen dat het effect is van hypercalorisch eten op SERT binding in het diencephalon en op DAT binding in het striatum, en of er verschillende effecten zijn van maaltijdgrootte en maaltijdfrequentie. We toonden aan dat een snack dieet ($^{123}$I-FP-CIT SPECT) in het diencephalon van slanke en obese mensen alsook hun relatie met eetgedrag. Om de rol van deze transporters in het ontstaan van obesitas en stoornissen in de suikerstofwisseling te bestuderen hebben we SERT en DAT gevisualiseerd voor en na een hoogcalorisch dieet in slanke mensen.

Tenslotte beschrijven we in hoofdstuk 7 dat hypercalorisch eten een direct effect is of een indirect effect via dieet geïnduceerde veranderingen in het glucose metaboïsm. Verrassend genoeg zagen we een trend tot verlaging van de hepatische insulinegevoeligheid alleen na het hypercalorische HFHS snack dieet (hoofdstuk 7), wat blijkt dat in een hypercalorische setting het eetpatroon in combinatie met de macronutriënt samenstelling van het dieet effect hebben op glucose metabolisme naast de effecten op het serotonin systeem in de hersenen. De lagere SERT beschikbaarheid in het diencephalon die veroorzaakt wordt door een HFHS snack dieet zou voortdurende calorische intake kunnen bevorderen via veranderingen in de concentratie van extracellular serotonine. Aangezien onze huidige obesogene omgeving wordt gekarakteriseerd door de aanwezigheid van veel snacks zouden onze bevindingen een deel van de verklaring kunnen zijn voor het chronisch overeten leidend tot obesitas.

Naast de effecten op SERT, resulterde een 6-weeks hypercalorisch dieet in een trend tot verlaging van DAT beschikbaarheid in het striatum, echter zonder een afzonderlijk effect van eetpatroon of specifieke macronutriënten (hoofdstuk 8). Wat wijst op een meer algemene relatie met overtollige calorieën en gewichtstoename. Verder zagen we dat de activiteit van het sympathisch zenuwstelsel verhoogd was na een hypercalorisch dieet en dat dit in verhouding stond tot de verlaging in DAT beschikbaarheid in het striatum, wat suggereert dat er een verband is tussen de activiteit van het sympathisch zenuwstelsel en het striatal dopamine systeem in reactie op overeten en/of aankomen (hoofdstuk 8).

Samenvattend is uit het onderzoek gebleken dat SERT en DAT in de hersenen van de mens gerelateerd zijn aan de motivatie voor voedsel en dat SERT verminderd is in de hypothalamus van mensen met een BMI > 25 kg/m², met name in obese mensen met insulineresistentie. Bovendien vermindert het eten van extra HFHS snacks SERT in het diencephalon en de hepatische insulinegevoeligheid. Het verhogen van de maaltijdgrootte liep geen van deze effecten zien, wat erop maaltijdfrequentie met veel vet en suiker (high fat high sugar; HFHS) de SERT beschikbaarheid in het diencephalon binnen 6 weken verlaagt maar een dieet met alleen veel suiker (high sugar; HS) niet. Het vergrepen van de maaltijdgrootte met dezelfde macronutriënten en dezelfde hoeveelheid calorieën had geen effect op SERT beschikbaarheid in het diencephalon (hoofdstuk 6). Dit wijst er op dat naast calorieën en de samenstelling van het dieet, het eetpatroon zelf een rol speelt bij de verandering in SERT beschikbaarheid in het diencephalon. Het is op dit moment onbekend of dit een direct effect is of een indirect effect via dieet geïnduceerde veranderingen in het glucose metaboïsm. Verrassend genoeg zagen we een trend tot verlaging van de hepatische insulinegevoeligheid alleen na het hypercalorische HFHS snack dieet (hoofdstuk 7), wat blijkt dat in een hypercalorische setting het eetpatroon in combinatie met de macronutriënt samenstelling van het dieet effect hebben op glucose metabolisme naast de effecten op het serotonin systeem in de hersenen. De lagere SERT beschikbaarheid in het diencephalon die veroorzaakt wordt door een HFHS snack dieet zou voortdurende calorische intake kunnen bevorderen via veranderingen in de concentratie van extracellular serotonine. Aangezien onze huidige obesogene omgeving wordt gekarakteriseerd door de aanwezigheid van veel snacks zouden onze bevindingen een deel van de verklaring kunnen zijn voor het chronisch overeten leidend tot obesitas.

Maaltijdgrootte en maaltijdfrequentie (met voedselintake) voor voedsel, een maat voor de motivatie voor voedsel, negatief correleerde met zowel SERT beschikbaarheid in het diencephalon, als DAT beschikbaarheid in het striatum (hoofdstuk 5). Bovendien correleerde de visuele aandachtsbias voor voedsel positief met voedselintake, wat er op wijst dat motivatie om te eten de voedselintake voorspelt, of andersom. Het volgende doel was om te bevestigen dat het effect is van hypercalorisch eten op SERT binding in het diencephalon en op DAT binding in het striatum, en of er verschillende effecten zijn van maaltijdgrootte en maaltijdfrequentie. We toonden aan dat een snack dieet ($^{123}$I-FP-CIT SPECT) in het diencephalon van slanke en obese mensen alsook hun relatie met eetgedrag. Om de rol van deze transporters in het ontstaan van obesitas en stoornissen in de suikerstofwisseling te bestuderen hebben we SERT en DAT gevisualiseerd voor en na een hoogcalorisch dieet in slanke mensen.

Maaltijdgrootte en maaltijdfrequentie (met voedselintake) voor voedsel, een maat voor de motivatie voor voedsel, negatief correleerde met zowel SERT beschikbaarheid in het diencephalon, als DAT beschikbaarheid in het striatum (hoofdstuk 5). Bovendien correleerde de visuele aandachtsbias voor voedsel positief met voedselintake, wat er op wijst dat motivatie om te eten de voedselintake voorspelt, of andersom. Het volgende doel was om te bevestigen dat het effect is van hypercalorisch eten op SERT binding in het diencephalon en op DAT binding in het striatum, en of er verschillende effecten zijn van maaltijdgrootte en maaltijdfrequentie. We toonden aan dat een snack dieet ($^{123}$I-FP-CIT SPECT) in het diencephalon van slanke en obese mensen alsook hun relatie met eetgedrag. Om de rol van deze transporters in het ontstaan van obesitas en stoornissen in de suikerstofwisseling te bestuderen hebben we SERT en DAT gevisualiseerd voor en na een hoogcalorisch dieet in slanke mensen.
wijst dat hypercalorisch snacken een onafhankelijk effect heeft op perifere glucose metabolisme en op de hersengebieden die betrokken zijn bij regulatie van voedingsgedrag en regulatie van glucose metabolisme. Verdere studies zijn nodig om te ontrafelen welke mechanismen ten grondslag liggen aan deze associaties in mensen en om het onderscheid te kunnen maken tussen de relatie met glucose metabolisme en het serotonerge systeem in de hersenen. Dit zou op den duur kunnen leiden tot nieuwe behandelingen en dieetadviezen, met als uiteindelijk doel obesitas en insulineresistentie te bestrijden.
Chapter 10

Author Affiliations

Dr. Ir. Mariëtte T. Ackermans
Department of Clinical Chemistry, laboratory of Endocrinology, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Anneke Alkemade
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Cognitive Science Center Amsterdam, University of Amsterdam, Amsterdam, The Netherlands.

Dr. Peter H. Bisschop
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Prof. Dr. Jan Booij
Department of Nuclear Medicine, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Anke J. Borgers
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Matthan W.A. Coan
Department of Radiology, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Danne C.E. Elbers
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Prof. Dr. Eric A. Elbers
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Susanne E. La Fleur
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Prof. Dr. John M. Karemaker
Department of Physiology, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Karin E. Koopman
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Ir. Aart J. Nederveen
Department of Radiology, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Anouk Pels
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Dr. Anne Roels
Department of Clinical Psychological Science, Maastricht University, Maastricht, The Netherlands.

Dr. Mireille J. Serlie
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

Prof. Dr. Dick F. Swaab
Department of Neuropsychiatric Disorders, Netherlands Institute for Neuroscience, An Institute of the Royal Netherlands Academy of Arts and Sciences, Amsterdam, The Netherlands.

Drs. Ruud J. Versteeg
Department of Endocrinology & Metabolism, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.
DANKWOORD

Eindelijk is het dan nu tijd om datgene te schrijven waar ik al vanaf het begin naar uiltjek het dankwoord. Waarschijnlijk het meest gelezen, voor velen zelfs het enig gelezen, stuk van een proefschrift. Uiteindelijk zijn het de mensen die een proefschrift en een promotietraject kleur geven, en er is veel te zeggen tegen de vele mensen die mogelijk hebben gemaakt dat mijn proefschrift in de huidige vorm heeft kunnen ontstaan.

Als eerste richt ik graag het woord tot alle proefpersonen die hebben deelgenomen aan mijn onderzoeken, met name de slanke jonge mannen die ik heb overvloed. Zonder jullie was het tot stand komen van dit proefschrift niet mogelijk geweest. De hypercalorische studie was echt pittig, zowel voor jullie om te volgen als voor mij om uit te voeren, maar dankzij jullie enthousiasme en belangstelling waren de vele metingen en bezoekenjes altijd een plezier en ik kijk mede dankzij jullie terug op een leuke periode.

De volgende personen die ik graag wil bedanken zijn mijn beide co-promotoren, Mireille en Suzanne. Jullie zeggen altijd dat ik jullie eerste gemeenschappelijke project was en dus jullie proefkonijn. In de context van een promotietraject waarbij ik diverse “proefkonijnen” aan allerhande metingen onderwerp is, dat eigenlijk best ironisch. Hoewel de weg af en toe best hobbelig was en jullie onderling moesten zoeken naar het beste samenwerkingsformat, ben ik heel tevreden met de begeleiding. Jullie zijn een ijzersterk team dat het nog ver kan schoppen, ik ben benieuwd wie en jullie onderling moesten zoeken naar het beste samenwerkingsformat, ben ik heel tevreden met de overige leden van mijn promotiecommissie, Prof. dr. R.A.H. Adan, Prof. dr. A. Kalsbeek, Prof. dr. W. van den Brink, Prof. dr. A.T.M. Jansen, Prof. dr. H. Pijl en Dr. M. Nieuwdorp, dank ik voor het kritisch beoordelen van mijn manuscript, ik ben erg blij dat u in mijn promotiecommissie zitting wilt nemen en kijk er naar uit over het proefschrift van gedachten te wisselen.

Een belangrijke factor in het overleven van je promotie zijn je collega’s en dat geldt zeker voor mijn collega’s op de afdeling Endocrinologie en Metabolisme van het AMC. We begonnen met een klein groepje op F5, volgens mij was ik destijds de 8e onderzoeker, en inmiddels is de groep minstens verdubbeld. Mede dankzij jullie ging ik elke dag weer met plezier naar mijn werk. We konden enorm met elkaar lachen en ouwehoeren over niets, maar ook scherpe discussies voeren over werk en onze projecten. Niet zelden kwam het voor dat iemand even moest afzlaan of dat er een proef moest worden overgenomen, wat altijd mogelijk was. Allereerst richt ik graag het woord tot mijn beide paranimfen. Barbara, wij gaan inmiddels al weg terug en ik koester nog steeds alle moments in de stad en bij de lelijke oranje keet om onze onderzoeken en het leven te relativeren. Uiteraard gaat dat beter met bier! Weet je nog hoe we er achter kwamen dat we eigenlijk best veel gemeen hadden, tijdens de barrel bij de Blauwe Engel bij het Endo-uitje? Jij kent als geen ander alle hoogtepunten en dieptepunten van mijn promotietraject en andersom. Hopelijk duurt het niet meer lang voordat we het in omgekeerde setting kunnen overdoen :-). AnNEGreet, mede door onze gezamenlijke zwangerschap en bevalling zijn wij in korte tijd erg naar elkaar toegegroei. Tijdens onze afspraken heeft er niet zozeer bier maar des te meer Nutrilon gevloed, maar de gezelligheid was er niet minder om! Vorig jaar was voor ons allebei een lastig jaar en ik kon altijd bij je terecht om te klagen over de tegenslagen die ik op mijn pad tegenkwam. Gelukkig is het voor ons allebei helemaal goed gekomen. En uiteraard was dit proefschrift niet geworden wat het nu is zonder jouw enorme kennis en kunde omtrent statistiek :-) (ps: ik heb ook geen syntax). Bedankt Dirk Jan, ik moet nog steeds af en toe stiekem griffelen over de sexy secretaresse op de NVDO. Eveline en Bouwien: het was me een genoegen samen zwanger te zijn en daar heel dik ook wel over te kunnen kletsen! Ruth, jij valt eigenlijk in twee dankwoordcategorieën aangezien je als student tijdens mijn zwangerschap een deel van de data van de BIO studie hebt verzameld, waarvoor veel dank. Inmiddels ben je vooral een collega en ik ben benieuwd wat er uit je projecten zal komen! Mijn overige roomies Sam, Marieke, Eelke, Pin, Murat, Laura en Linda: bedankt voor alle koffies, trouwerijen, goede gesprekken, bierjes op congressen, en het overnemen van proeven zodat ik ook soms met vakantie kon. Kasper, Martine, Charlotte, Yvonne, Arvid, Nicolette, Myrte, Hamid, Anke en Saskia: bedankt voor alle leuke moments! Birgit en Martine, de afdelingsmoeders van iedere AIO, bedankt voor de ondersteuning bij mijn projecten.

Daarnaast heb ik ook het genoegen gehad te werken met twee geweldige promotoren, Prof. dr. E. Fliers en Prof. dr. J. Boon. Beste Eric en Jan, jullie expertise was onmisbaar en jullie nimmer aflatende belangstelling heeft mij veel goed gedaan. Jullie waren te allen tijde beschikbaar voor een luisterend oor, raad en daad en dit heeft mij geholpen vooruit te komen en goed te plannen. De overige leden van mijn promotiecommissie, Prof. dr. R.A.H. Adan, Prof. dr. A. Kalsbeek, Prof. dr. W. van den Brink, Prof. dr. A.T.M. Jansen, Prof. dr. H. Pijl en Dr. M. Nieuwdorp, dank ik voor het kritisch beoordelen van mijn manuscript, ik ben erg blij dat u in mijn promotiecommissie zitting wilt nemen en kijk er naar uit over het proefschrift van gedachten te wisselen.

Een belangrijke factor in het overleven van je promotie zijn je collega’s en dat geldt zeker voor mijn collega’s op de afdeling Endocrinologie en Metabolisme van het AMC. We begonnen met een klein groepje op F5, volgens mij was ik destijds de 8e onderzoeker, en inmiddels is de groep minstens verdubbeld. Mede dankzij jullie ging ik elke dag weer met plezier naar mijn werk. We konden enorm met elkaar lachen en ouwehoeren over niets, maar ook scherpe discussies voeren over werk en onze projecten. Niet zelden kwam het voor dat iemand even moest afzlaan of dat er een proef moest worden overgenomen, wat altijd mogelijk was. Allereerst richt ik graag het woord tot mijn beide paranimfen. Barbara, wij gaan inmiddels al weg terug en ik koester nog steeds alle moments in de stad en bij de lelijke oranje keet om onze onderzoeken en het leven te relativeren. Uiteraard gaat dat beter met bier! Weet je nog hoe we er achter kwamen dat we eigenlijk best veel gemeen hadden, tijdens de barrel bij de Blauwe Engel bij het Endo-uitje? Jij kent als geen ander alle hoogtepunten en dieptepunten van mijn promotietraject en andersom. Hopelijk duurt het niet meer lang voordat we het in omgekeerde setting kunnen overdoen :-). AnNEGreet, mede door onze gezamenlijke zwangerschap en bevalling zijn wij in korte tijd erg naar elkaar toegegroei. Tijdens onze afspraken heeft er niet zozeer bier maar des te meer Nutrilon gevloed, maar de gezelligheid was er niet minder om! Vorig jaar was voor ons allebei een lastig jaar en ik kon altijd bij je terecht om te klagen over de tegenslagen die ik op mijn pad tegenkwam. Gelukkig is het voor ons allebei helemaal goed gekomen. En uiteraard was dit proefschrift niet geworden wat het nu is zonder jouw enorme kennis en kunde omtrent statistiek :-) (ps: ik heb ook geen syntax). Bedankt Dirk Jan, ik moet nog steeds af en toe stiekem griffelen over de sexy secretaresse op de NVDO. Eveline en Bouwien: het was me een genoegen samen zwanger te zijn en daar heel dik ook wel over te kunnen kletsen! Ruth, jij valt eigenlijk in twee dankwoordcategorieën aangezien je als student tijdens mijn zwangerschap een deel van de data van de BIO studie hebt verzameld, waarvoor veel dank. Inmiddels ben je vooral een collega en ik ben benieuwd wat er uit je projecten zal komen! Mijn overige roomies Sam, Marieke, Eelke, Pin, Murat, Laura en Linda: bedankt voor alle koffies, trouwerijen, goede gesprekken, bierjes op congressen, en het overnemen van proeven zodat ik ook soms met vakantie kon. Kasper, Martine, Charlotte, Yvonne, Arvid, Nicolette, Myrte, Hamid, Anke en Saskia: bedankt voor alle leuke moments! Birgit en Martine, de afdelingsmoeders van iedere AIO, bedankt voor de ondersteuning bij mijn projecten.
Daarnaast veel dank aan de collega’s op F2, die misschien iets verder weg zaten maar ik door het ‘la Fleur groepje’ (mooi blauw is niet lelijk), de AIO lunch en diverse congressen toch goed heb leren kennen. Charlene, Jose, Merel, Leslie, Joëlle, Rianne, Evita, Myrtille, Hannah en Emmely: bedankt voor alle drankjes, etentjes, goede gesprekken, slechte grappen en nimmer allatende belangstelling.

Dan zijn er nog diverse andere mensen in het AMC met wie ik samen heb gewerkt bij het uitvoeren van mijn proeven. Mariette Ackermans, dank voor alle isotopen bepalingen, de ultrasensitieve insulinekit en vele andere labtechnische dingen. Anneke Alkmade, met jou heb ik voor het eerst in een lab met een pipet gewerkt, nog dank voor de uitleg van capillaire werking. Het was leuk om met je samen te werken en te publiceren ook al hou je niet van dokters ;). John Karemaker, dank voor de leuke samenwerking bij de analyse van de Nexfin data en je feedback bij het schrijven van het stuk. Op de afdeling nucleaire geneeskunde dank ik Ehsan en Astrid voor het inwerken op de SME en Matthijs voor de medewerking bij de altijd ingewikkelde planning voor research op de SME. Op de afdeling radiologie dank ik Aart Nederveen en Matthew Caan voor de leuke samenwerking en Sandra en Raschel voor het inwerken en de ondersteuning bij de 3T. Tenslotte dank ik Elsmanrieke van de Giessen voor het aanleveren van en de uitleg over de computerstoeken en de vragenlijsten en Anne Roefs van de afdeling klinische psychologie van de Universiteit van Maastricht voor de samenwerking bij het schrijven van het artikel hierover.

Door de jaren heen heb ik diverse studenten begeleid bij hun bachelor of masterstage en ieder een heeft op zijn of haar manier bijgedragen aan het verzamelen of uitwerken van data voor dit proefschrift. Nouras, Danne, Ranzma, Jet, Danielle, Anouk en Jesper: bedankt voor jullie inzet, belangstelling en gezelligheid!

Een groot woord van dank gaat ook uit naar mijn ‘nieuwe’ collega’s in het Medisch Centrum Alkmaar: jullie hebben veel geklaag aan moeten horen over het laatste staartje van mijn promotietraject, wat niet makkelijk was, maar hopelijk heb ik ook kunnen overbrengen dat onderzoek doen en promoveren erg leuk is! Ik was in ieder geval erg blij met jullie belangstelling en support en ik heb me in Alkmaar vanaf het eerste moment welkom gevoeld. Het is een genoegen met iedereen samen te werken en ik heb enorm veel van jullie geleerd (en leer nog steeds iedere dag). Door dit proefschrift hoop ik doctor te worden maar door jullie ben ik een dokter geworden! Frank Stam, ik wil je erg bedanken voor de extra tijd die je me gegeven hebt om mijn proefschrift af te maken, want ik weet niet of ik het anders had gered. Veel dank!

Ewout en Jeroen van drukkerij Terts, heel erg bedankt voor de soepete en gezellige samenwerking bij het drukken van mijn proefschrift en uiteraard het geweldige resultaat. We weten jullie weer te vinden bij het volgende major life event!

Zonder een geweldig netwerk van familie en vrienden was het afronden van dit proefschrift in combinatie met een full-time baan in de kliniek en een opgroeien kind nooit gelukt. Vooral mijn ouders en schoonfamilie hebben hierin een belangrijke rol gespeeld. Lieve papa, mama, Jo, Peet, Sanne en Arjen: ik ben jullie enorm dankbaar voor jullie steun en goede zorgen voor Mads in de laatste fase van mijn promotie. Papa en mama, ik ben trots op jullie en al jullie goede eigen schappen die aan mij zijn doorgegeven, waaronder doorzettingsvermogen, eigenwijsheid, relatiesvermogen en een nuchter boerenverstand. Al deze eigenschappen waren belangrijke ingrediënten bij het doorlopen van mijn promotietraject. Jullie onvoorwaardelijke leden en steun, ook al is het soms ver van jullie bed wat ik doe, is voor mij onmisbaar in het leven. Ook mijn broers, Wout en Lars, bedankt voor jullie belangstelling. Een wijs en grijs man zei ooit tegen me: gaat er enkel wierder iemand in de familie zijn studie echt afmaken? Frank, ik ben blij dat ik op deze manier iets aan de familie heb kunnen toevoegen en gelukkig weet ik dat er meer dingen zijn waar ik om word gewaardeerd, maar daar heb ik het met Sas onder het genot van een wijn tje nog wel eens over.

Ook een belangrijk woord van dank aan mijn ‘nieuwe’ vriendinnen, al was het alleen al omdat ze me nog steeds aardig lijken te vinden na een jaar van extreme verwaarlozing. Lynne, wij kennen elkaar al zo lang dat we snel van elkaar weten wat er door de andere heen gaat. Dat we allebei promoveren en moeder zijn van een aantal fantastische kinderen heeft dit alleen maar versterkt. Ik ben blij dat we nog steeds zulke goede vriendinnen zijn en zo veel kunnen delen! Anne, Margot, Merle, Marj hinge, Tessa, Eva, Connel, Janine en Noor: bedankt voor het al en toe aanzorgen van mijn geklaag en jullie steun en vriendschap voor het leven. Het is nu klaar en ik heb eindelijk weer tijd om lekker te eten, drankjes te doen en dansjes te maken (in binnen- of buitenland). Laten we dat snel doen!

Tenslotte richt ik het woord tot de twee belangrijkste personen in mijn leven. Mijn allergrootste vriend en liefde, mijn steun en toeverlaat: Roeland, zonder jou had ik het niet gered. Niet alleen heb je praktisch geholpen door het contact met de drukker op je te nemen en je te bemoeien met de lay-out maar ook heb je, vooral in het laatste stuk van mijn promotie toen ik ook al in Alkmaar werkte, de organisatie van ons huishouden overgenomen. Het was niet altijd makkelijk maar je was er altijd voor me, voor better and for worse, en ik weet zekerder dan ooit dat wij samen oud gaan worden. Zoals ze zeggen: je weet pas dat het goed zit met je relatie als je samen een promotie hebt overleefd!

Mads, mijn zonnetje, jij bent belangrijker dan ik je ooit zal kunnen zeggen. Jij bent geboren tijdens het tot stand komen van dit proefschrift en hoewel mijn proefschrift mooi en goed is, ben jij het mooiste en beste wat ik de afgelopen jaren heb voortgebracht. Ik hou van jullie!
PhD period: August 2010 - November 2014
Name PhD student: Karin Eva Maria Koopman
Name PhD supervisors: Prof. dr. E. Fliers & Prof. dr. J. Bors
Name PhD co-supervisors: Dr. S.E. la Fleur & Dr. M.J.M. Selle

PhD training

General courses
- Basic course Law and Organization for clinical researchers (BROK)
- Research methods
- Scientific writing and presentation in English

Specific courses
- Research Protection SB
- Practical biostatistics
- Critical Reading
- Educational Skills Training

Seminars, workshops and master classes
- Weekly department seminars Endocrinology & Metabolism
- Weekly department seminars Clinical Diabetology
- Master Classes (2012/2013)

Natural sciences
- Serotonin Transporter Availability within the Diencephalon of Healthy Lean Male Subjects does not correlate with insulin sensitivity. NASO Annual Meeting
- A Hypercaloric High-Fat-High-Sugar diet decreases hypothalamic Serotonin Transporters, Dutch Neuroscience Meeting (formerly known as ENP meeting).
- Effects of different hypercaloric diets on liver fat, abdominal fat and insulin sensitivity in lean humans. ADDM/NVDO Annual Meeting
- The effect of a hypercaloric diet on Dopamine- and Serotonin Transporters. Annual Meeting Dutch Endocrine Society (NVE)
- A Hypercaloric Snacking Diet Increases Liver Fat in Lean Men within 6 Weeks, NASO Annual Meeting
- The effects of nutrients and BMI on the brain dopamine and serotonin system. Dutch Neuroscience Meeting (formerly known as ENP meeting), invited speaker.
- Brain serotonin and dopamine transporter binding correlate with visual attention for food in healthy, lean men. Dutch Neuroscience Meeting (formerly known as ENP meeting), invited speaker.

International Presentations
- Serotonin Transporter Availability within the Diencephalon of Healthy Lean Male Subjects does not correlate with insulin sensitivity, 72° ADA Scientific Sessions.
- Serotonin Transporters in the Brain: the effect of hypercaloric diets and relationship with insulin sensitivity. 72° ADA Scientific Sessions.
- Nutrition, Metabolism and the Brain (NMB) Meeting
- The effect of hypercaloric diets on Serotonin Transporters and Dopamine Transporters in the in Human brain, 73° ADA Scientific Sessions.
- The effect of hypercaloric diets on different diet composition and pattern on insulin sensitivity, 73° ADA Scientific Sessions.
- Hypercaloric Snacking Increases Liver Fat in Lean Men within 6 Weeks, 73° ADA Scientific Sessions.
- The effects of a hypercaloric snack on liver fat and insulin sensitivity in lean men, 49° EASD Annual Meeting.
- Hypercaloric High-Fat-High-Sugar Snacking decreases Serotonin Transporters in the hypothalamic region, but not striatal Dopamine Transporters, in lean Men, 49° EASD Annual Meeting.
- Striatal dopamine transporter binding correlates with body composition, energy expenditure, and visual attention bias for food cues, 74° ADA Scientific Sessions.
- Serotonin Transporter binding in the diencephalon is associated with insulin sensitivity in obese women, 74° ADA Scientific Sessions.

Publications


Appendix

OVER DE AUTEUR