

Central Integration of Dietary Protein Signalling

Jessica Schwarz

▶ **To cite this version:**

Jessica Schwarz. Central Integration of Dietary Protein Signalling. Food and Nutrition. AgroParisTech, 2010. English. NNT : 2010AGPT0003 . pastel-00769585

HAL Id: pastel-00769585
https://pastel.hal.science/pastel-00769585

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THESIS

submitted to obtain the degree of

Doctor of Philosophy

at

L'Institut des Sciences et Industries du Vivant et de l'Environnement (AgroParisTech)

Speciality: Nutrition Science

Presented and defended in public
by

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on 7th January 2010

CENTRAL INTEGRATION OF DIETARY PROTEIN SIGNALLING

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Acknowledgements

At the end of this period, I would like to thank all the people who supported and encouraged me during the past three years of my PhD:

- Professor Daniel Tomé for having given me the great opportunity to conduct my research in his lab and for offering me the occasion to continue working together.

-The members of the jury, Prof. Mihai Covasa and Prof. Thomas Lutz for accepting being rapporteur as well as Patricia Parnet for being examiner of my thesis.

- My supervisors Gilles Fromentin and Nicolas Darcel for their support, guidance and patience in working with a German who does not always function the French way.

- My collaborators at the NOPA in Jouy en Josas, Phillippe Andrey, Jasmine Burguet, Yves Maurin and Olivier Rampin for their copious practical help and numerous scientific discussions to develop my work.

- Professor Jimmy Bell for accepting to supervise my 6-month secondment in his lab and all members of the Metabolic and Molecular Imaging Group at Imperial College London, in particular Jelena and Nad.

- The NuSISCO team, Hannelore Daniel, Alice François, Gary Frost, Luc Ozanne, Harry Peters, Manuela Rist as well as my co-PhDs Boris, Camilla, Charles, Christina, Laure, Magda, Nadine and Véro, for our interesting discussions and support of each other during the last three years.

- All colleagues from the PNCA, especially Sylvette Gougis and my students Anne-Ruth, Justine and Florence for their help as well as my office mates Anne-So, Fanny, Géraldine, Magda, Nattie, Senghua and Wahiba for their endless moral support and good mood.

- Last but not least a warm thank you to my family and friends for their encouragement and support. I especially want to thank the extended ‘coloc’ Antoine, Béné, Ben, Clem, Gim, Julie, Martin, Nico, Patou and Renaud for welcoming me in Paris, for their great company and all the fun I had outside work.

Abstract

Protein is accepted as the nutrient with the most satiating effect. During digestion it acts at different levels. In the stomach it delays gastric emptying and maintains the effect of gastric distension on satiety. In the gut amino acids (AA) and peptides are detected and this leads to an activation of the vagus nerve and the release of gut hormones. The vagus nerve then transmits the signals to the dorsal vagal complex (DVC), responsible for meal termination. Circulating gut peptides and absorbed AA are also detected in the hypothalamus, which is the main structure controlling energy balance.

The exact mechanisms by which dietary protein influences hunger and satiety signalling are not yet fully understood. To gain insight, in this thesis we carried out 3 projects. Firstly, concerning transmission of protein signals by the vagus nerve, our study showed that vagal capsaicin sensitive fibres are not necessary for high protein (HP) diet induced hypophagia, and thus that compensatory mechanisms probably exist which enable adaptation. Secondly we compared the activity of DVC regions in response to a protein or carbohydrate load. Using 3D reconstruction neurones were found in distinct though partially overlapping positions in the NTS. This suggests that short-term protein signalling is specifically transmitted to the DVC. In the third project, we discovered that mice fed a HP diet for 6 weeks had altered body composition but not food intake or body weight. These results suggest an effect of protein on energy metabolism independent from the satiating effect. Further studies may help to understand the role of protein in meal size control, energy balance and body weight regulation.

Résumé

Les protéines sont considérées comme le macronutriment avec le pouvoir de satiété le plus fort. Pendant la digestion les protéines agissent à différents niveaux: dans l'estomac, elles retardent la vidange gastrique et prolongent donc la distension gastrique agissant ainsi sur le rassasiement; dans l'intestin des acides aminés (AA) et les peptides sont détectés et entraînent l'activation du nerf vague et la libération de médiateurs gastro-intestinaux. Le nerf vague transmet ces signaux vers le complexe vagal dorsal (DVC), responsable du contrôle de l'ingestion. Les peptides gastro-intestinaux ainsi que les AA absorbés sont également détectés au niveau l'hypothalamus, en charge de la régulation de l'équilibre énergétique.

Les mécanismes exacts par lesquels les protéines alimentaires influencent la faim et la satiété ne sont pas encore compris. Pour mieux comprendre ces phénomènes, nous avons réalisé 3 études qui constituent le présent travail de doctorat. Tout d'abord, concernant la transmission des informations relatives à la présence de protéines via le nerf vague, une première étude a montré que les afférences vagales de la zone hépato-portale ne sont pas nécessaires pour la dépression de la prise alimentaire induite par un régime HP. Deuxièmement, nous avons comparé les motifs d'activité neuronale dans le DVC en réponse à une charge protéique ou glucidique. L'utilisation de techniques de modélisation et de reconstruction 3D a permis de montrer que les motifs d'activité neuronale par la présence de protéines et de glucides sont spatialement distinctes. Dans une troisième étude nous avons testé l'effet des protéines sur le métabolisme énergétique et sur la réponse à une stimulation inhibitrice de la faim, ce dernier paramètre ne semble pas être significativement modulé par une augmentation de l'apport en protéine de la ration chez la souris.

Keywords

Dietary protein, food intake control, satiety, satiation, nutrient sensing, vagus nerve, nucleus tractus solitarius, area postrema, hypothalamus, gut peptides, three-dimensional neuronal activity mapping, magnetic resonance imaging

Mots clés

Protéines alimentaires, régulation de la prise alimentaire, satiété, rassasiement, détection des nutriments, nerf vague, noyau du faisceau solitaire, area postrema, hypothalamus, peptides gastro-intestinaux, cartographie d'activation neuronales tridimensionnelles, imagerie par résonance magnétique

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Abbreviations

3V	3rd ventricle
5-HT	5-hydroxytryptamine / serotonin
AA	Amino acids
ACTH	Adrenocorticotropic hormone
AgRP	Agouti-related protein
α-MSH	α-melanocyte-stimulating hormone
AMPK	AMP-activated protein kinase
AP	Area postrema
ARC	Arcuate nucleus
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CART	Cocaine- and amphetamine-regulated transcript
CC	Central channel
CCK	Cholecystokinin
CCK1-R	CCK1 receptor
CGRP	Calcitonin gene-gene related peptide
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
DMX	Dorsal motor of vagus
DVC	Dorsal vagal complex
FOV	Field of view
fsems	Fast spin-echo multislice sequence
FT	Fourier transformation
GI tract	Gastrointestinal tract
GLP-1	Glucagon-like peptide 1

GLUT	Glutamate transporter
GPCR	G protein-coupled receptor
GRPP	Glicentin-related pancreatic peptide
HP	High protein
IHCL	Intrahepatocellular lipid
IMCL	Intramyocellular lipid
LHA	Lateral hypothalamic area
ME	Median eminence
MEMRI	Manganese enhanced magnetic resonance imaging
mGluR	Metabotrophic glutamate receptor
MPGF	Major proglucagon fragment
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
mTOR	Mammalian target of rapamycin
NDS	Normal donkey serum
NP	Normal protein
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
NuSISCO	Nutrient sensing in satiety control and obesity
OXM	Oxyntomodulin
PBS	Phosphate-buffered saline
PEPT1	Peptide transporter 1
PFA	Perifornical area
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
ppm	Parts per million

press	Point resolved spectroscopy sequence
PVN	Paraventricular nucleus
PYY	Peptide tyrosin-tyrosin / peptide YY
RF	Radiofrequency
ROI	Region of interest
SEM	Standard error of mean
sems	Spin-echo multislice sequence
SGLT1	Sodium-glucose co-transporter 1
SI	Signal intensity
SON	Supraoptic nucleus
spuls	Single-pulse sequence
SW	Spectral width
TE	Echo time
TR	Repetition time
VMH	Ventromedial hypothalamus
VR1	Vanilloid receptor subtype 1
WHO	World Health Organisation

Publications and communications

Publications:

Schwarz, J., Burguet, J., Rampin, O., Fromentin, G., Andrey, P., Tomé, D., Maurin, Y., Darcel, N. *Three-dimensional Macronutrient-associated Fos Expression Patterns in the Mouse Brainstem.* PLoS One 2010 Feb 1; 5(2): e8974.

Tomé D., **Schwarz, J.**, Darcel N., Fromentin, G. *Protein, amino acids, vagus nerve signaling and the brain.* American Journal of Clinical Nutrition 2009 Sep; 90(3): 838S-843S. Epub 2009 Jul 29.

Foltz M., Ansems, P., **Schwarz, J.**, Tasker, M., Lourbakos A., Gerhardt C. *Protein Hydrolysates Induce CCK Release from Enteroendocrine Cells and Act as Partial Agonists of the CCK1 Receptor.* Journal of Agriculture and Food Chemistry 2008 Feb 13; 56(3): 837-843. Epub 2008 Jan 23.

Scientific communications:

Schwarz, J., Darcel, N., Rampin, O., Andrey, P., Burguet, J., Fromentin, G., Tomé, D, Maurin, Y. *Nutrient-dependent neuronal activation patterns in the nucleus of the solitary tract: a study using 3D modelling and neuronal density maps.* Poster communication at the International Congress of Nutrition (ICN) October 2009, Bangkok, Thailand (Abstract published in Annals of Nutrition and Metabolism 2009, 55, Suppl. 1).

Schwarz, J., Darcel, N., Rampin, O., Andrey, P., Burguet, J., Fromentin, G., Tomé, D, Maurin, Y. *Nutrient-dependent neuronal activation patterns in the nucleus of the solitary tract: a study using 3D modelling and neuronal density maps.* Poster presentation for scientist of Unilever R&D September 2009, Vlaardingen, The Netherlands.

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Schwarz, J., Darcel, N., L'Heureux-Bouron, D., Gougis, S., Rampin, O., Tomé, D., Fromentin, G. *Hepatic portal vein deafferentation has no effect on the satiating effect of a HP diet.* Oral communication at the Annual meeting of the Society for the Studies of Ingestive Behaviour (SSIB) July 2008 in Paris, France (Abstract published in Appetite 2008; 51 (2): 398).

Schwarz, J., Darcel, N., Rampin, O., Andrey, P., Burguet, J., Fromentin, G., Maurin, Y., Tomé, D. *Activation maps in the nucleus of the solitary tract (NTS) in response to internal stimuli.* Poster communication at the Annual meeting of the Society for the Studies of Ingestive Behaviour (SSIB) July 2008 in Paris, France (Abstract published in Appetite 2008; 51 (2): 398).

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Schwarz, J., Darcel, N., Rampin, O., Burguet, J., Andrey, P., Tomé, D., Maurin, Y., Fromentin, G. *Central cartography of macronutrients' internal sensing.* Poster presentation for scientist of Unilever R&D September 2007, Vlaardingen, The Netherlands.

General Introduction: Obesity, a worldwide epidemic

This work is funded by the European Community's Sixth Framework Programme (FP6) under the contract NuSISCO ('Nutrient Sensing in Satiety Control and Obesity').

The World Health Organisation (WHO) estimates that over a billion adults worldwide are currently overweight (WHO 2007). In Europe 30-80 % of the population are considered as overweight and up to one third as obese (WHO 2007). In addition to social and professional stigmatisation (Puhl and Brownell 2001), obesity is causally associated with cardiovascular disease, type 2 diabetes mellitus, obstructive sleep apnoea, hypertension, stroke as well as some forms of cancer (Kopelman 2000). In Europe as well as in the rest of the world including nowadays even developing countries, the prevalence of obesity as a major cause of premature death is accelerating dramatically (Ofei 2005). Shortened life expectancy compared to normal weight people can reach from 2-5 years in overweight subjects to 5-20 years in severe obese persons (Fontaine et al. 2003). Obesity is also an increasing economic problem as obesity related diseases cause 2-8% of the total health costs per year for the national health care systems (WHO 2007).

There are two most popular causes for developing obesity, genetic susceptibility and behavioural changes. From the genetic point of view, during evolution it has always been an advantage to be able to develop fat stores in order to survive during nutrient shortage. Nowadays, since food shortage is no longer an issue, exactly this former advantage causes problems as the modern, energy-dense diet leads to an increased energy intake while physical activity, and therefore energy expenditure, is decreasing. In order to keep body weight stable, energy intake must match energy expenditure. The state of positive energy balance caused by overeating and missing compensating energy expenditure is well accepted as one of the main reasons for this epidemic. However, public health initiatives promoting a healthy diet and increased energy expenditure by exercise did not have the desired effect on the population and could not stop the increasing weight.

Wide ranges of medical and behavioural interventions as anti-obesity treatment have already been tried in obese patients, however only a few were successful. Pharmacological compounds often had to be withdrawn due to severe undesired side effects (Farrigan and

Pang 2002). At present bariatric surgery is the most successful treatment in strongly obese persons, but also here adverse effects do not make it tolerable for wide-range use (Sjostrom et al. 2004). It is therefore still a huge challenge for the scientific community to search for more effective and better tolerable treatment targets against obesity.

It is commonly accepted that energy homeostasis is under control of a complex signalling system. Many different parameters, involving adiposity signals, whose secretion is proportional to body fat, and satiety signals generated in the gastrointestinal (GI) tract during meals as well as neurotransmitters signalling are processed in a limited number of feeding centres within the central nervous system (CNS) (Woods et al. 2004).

However, there is still a big lack of knowledge in the exact understanding of feeding behaviour and appetite control. The NuSISCO programme covers the whole area of satiety, food intake control and obesity from the molecular and cellular level to human intervention trials. This thesis focuses particularly on understanding the mechanisms of protein-induced satiety signalling to the brain. Therefore it was investigated if sensory vagal afferents in the area of the hepatic portal vein are necessary for protein induced satiety. Food intake and body weight gain of rats were compared that did or did not undergo chemical deafferentation. Moreover, the macronutrient specificity of post-ingestive satiety signalling in the dorsal-vagal complex of mice was studied by comparing density maps of neuronal activity in response to a either protein or carbohydrate load. At last the effect of a high protein diet on the body weight and body composition of mice was studied as well as if the diet can affect the action of the anorexigenic gut peptide oxyntomodulin in hypothalamic areas involved in food intake regulation.

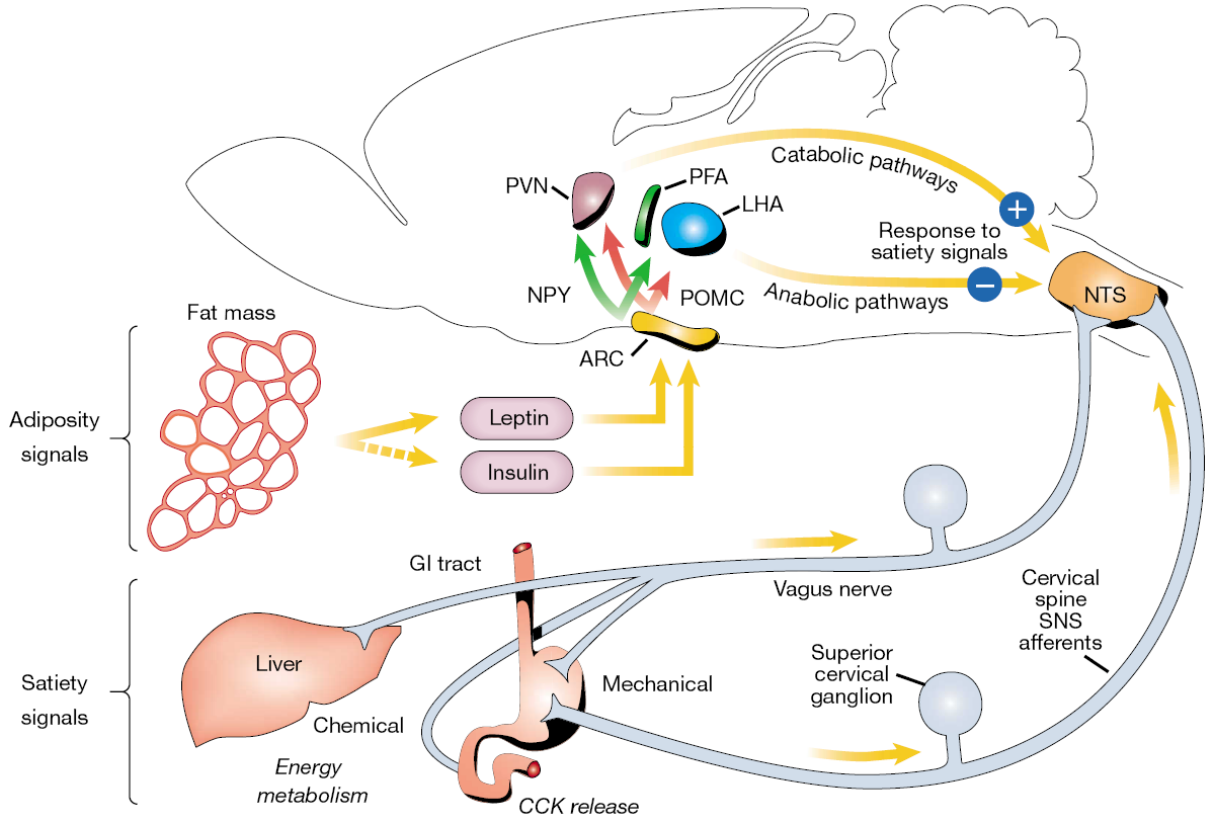


Figure 1.1 Neuroanatomical model for pathways involved in peripheral and central satiety signalling.

Long-term adiposity signals (leptin, insulin) interact with hypothalamic structures responsible for energy balance where they stimulate catabolic and inhibit anabolic pathways. Short-term chemical and mechanical satiety signals from the gastrointestinal (GI) tract and liver are transmitted to the NTS, the satiety centre located in the caudal brainstem. There signals are integrated with descending hypothalamic input which leads to meal termination. ARC, arcuate nucleus; CCK, cholecystokinin; LHA, lateral hypothalamic area; NPY, neuropeptide Y; NTS, nucleus tractus solitarius; PFA, perifornical area; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus (Schwartz et al. 2000).

1 Scientific background

1.1 Appetite, satiety and the control of food intake

Appetite is the internal driving force for search, choice and ingestion of food in order to maintain energy homeostasis and body weight and therefore responsible for meal initiation (de Graaf et al. 2004). The opposite of appetite is satiation, which is the major determinant of meal size. The meal size is defined by ingestion rate and meal termination. 'Satiation' is therefore understood as the physiological process that leads to meal termination (Schwartz et al. 2000). In contrast 'satiety' defines the period after a meal before the onset of hunger. Here the word 'satiety' will be used for expressing the absence of hunger in general. Even food intake varies widely from day to day, regarded at long-term a fine regulation can be observed.

Hunger and satiety signals from the periphery responsible to maintain energy balance and regulate feeding behaviour can be categorised in pre- and post-ingestive signals and among the latter in pre- and post-absorptive signals. Pre-absorptive signals are derived in the intestine at the moment nutrients enter the GI tract. Post-absorptive signals occur after nutrients crossed the gut wall and enter the circulation. Adiposity signals are also part of the peripheral signals, so do hormones such as insulin and leptin reflect the levels of energy stores and are regulating body weight and ingestive behaviour. On the other hand, satiety as well as meal termination occurs partly due to gastrointestinal satiety signals, like mechanical as well as chemical signals from metabolites and gut hormones (Figure 1.1) (Schwartz et al. 2000).

All these signals are subsequently integrated in the CNS where structures in both the hypothalamus and brainstem are important for the regulation of energy homeostasis as well as the onset of appetite and satiety (Figure 1.1). The negative feedback control of meal size transmitted by gastrointestinal signals takes place in the dorsal vagal complex (DVC) in the brainstem, while hypothalamic nuclei rather process adiposity signals responsible for energy stores (Schwartz et al. 2000). Both centres involved in appetite and food intake regulation are reciprocally connected in order to control energy balance.

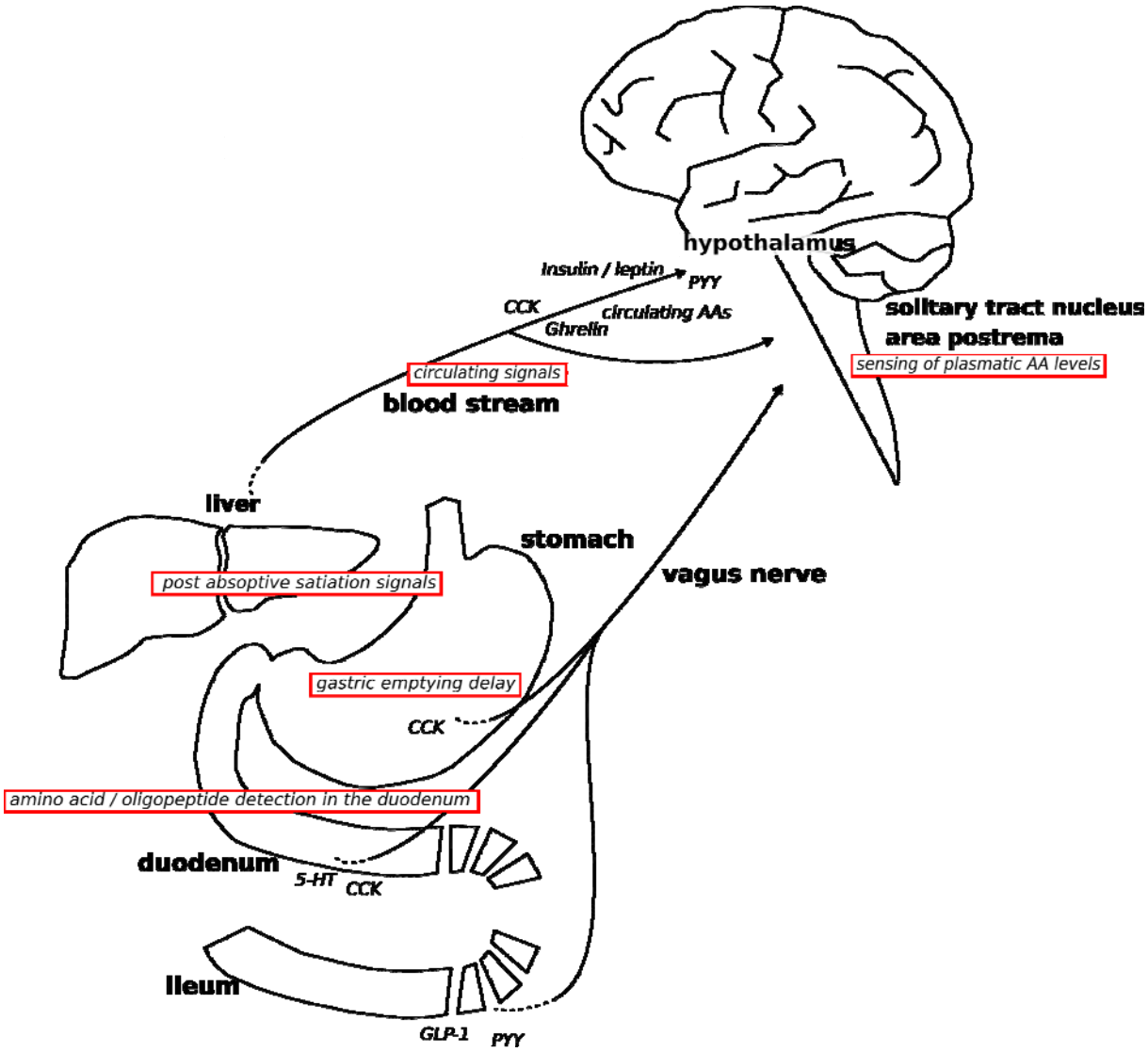


Figure 1.2 Mechanisms responsible for the protein-induced suppression of food intake.

Dietary protein is detected in the gastrointestinal tract. Signals are generated in the stomach, duodenum, and ileum via the release of gut hormones that activate peripheral nerves (particularly the vagus nerve). These gut hormones are also carried by the bloodstream and act directly on brain centres such as the area postrema and hypothalamus to suppress food intake. Post absorptive anorexigenic signals are also generated by the liver. PYY, peptide YY; CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; 5-HT, 5-hydroxytryptamine; AA, amino acid
(Adapted from Tomé et al. 2009).

1.2 Peripheral signals involved in protein induced satiety

Within the three macronutrients our daily food consists of, protein has been shown to have the most satiating effect. The effects are based on different levels from which satiety signals are derived.

Pre-absorptive signals, originated in the intestine after nutrient ingestion are integrated in the brain's satiety centres after being transmitted via the vagus nerve. Those gut derived signals are for instance gastric distension, but are also occurring due to intestinal detection of nutrients such as amino acids, peptides, fat and carbohydrates as well as gut peptides like cholecystokinin (CCK) or glucagon-like peptide 1 (GLP-1) released from enteroendocrine cells in response to those macronutrients (Figure 1.2).

Post-absorptive signals, arising from the nutrients having passed the intestinal barrier and entering circulation are transported to the brain via the blood stream. An elevated plasma amino acid concentration is able to reduce food intake, amongst others due to amino acid sensing in specific areas of the brain (Mellinkoff et al. 1956; Harper et al. 1970). Likewise the increasing circulating blood glucose detected in critical brain regions induces satiety. In case of a high protein and low carbohydrate diet for instance gluconeogenesis occurs, in order to maintain blood glucose levels (Jungas et al. 1992). Also thermogenesis is suggested to be responsible for protein induced satiety (Westerterp-Plantenga et al. 2004).

1.2.1 Sensory information from lingual detection

Orosensory information provides animals with valuable information about the nature and quality of food (Nelson et al. 2002). Mammals can recognize and respond to a wide variety of chemical products, including sugars, salts, acids and a broad range of toxic substances.

According to Zhao et al., several amino acids taste sweet or delicious (umami) to humans, and are attractive to rodents and other animals (Zhao et al. 2003). Umami is one of the basic taste sensations, induced solely and synergistically by free glutamate and 5-mononucleotides. To sense umami, several receptors such as taste receptors (T1R1/T1R3

heterodimer) and metabotrophic glutamate receptors (mGluR1 and mGluR4) can be found in the taste buds on the tongue.

Nelson et al identified and characterized another mammalian amino acid taste receptor, T1R1+3, which combines the T1R1 and T1R3 G-protein coupled receptors (GPCR) to function as a broadly tuned L-amino-acid sensor; this responds to most of the 20 standard amino acids (Nelson et al. 2002).

Gustatory detection via taste receptor cells on the tongue and palate is one of the key processes in protein and amino acid signalling to the brain centres that control ingestion and hunger.

1.2.2 Pre-absorptive signals generated in the gastrointestinal tract

Pre-absorptive satiety signals are generated during ingestion of nutrients at their appearance in the gastrointestinal (GI) tract but prior to absorption. They might play an indirect role influencing in which way nutrients are metabolised later on, as different metabolic pathways might be used when duration of absorption is increased or shortened.

1.2.2.1 The stomach is sensitive to gastric distension

Gastric distension occurs when the nutrients reach the stomach and is therefore one of the first pre-absorptive satiety signals (Figure 1.1). Mechanoreceptors in the stomach sense this distension and are transmitting the volumetric signal to the satiety centres (Mathis et al. 1998). Proteins acting as mechanoreceptors are intraganglionic laminar endings, which innervate the myenteric ganglia and are distributed throughout the stomach and duodenal tract, as well as intramuscular arrays which are limited to the stomach and adjacent sphincters (Fox et al. 2000; Fox et al. 2001). Nutrients sensing in the stomach was suggested to be macronutrient-independent and therefore only volumetric (Phillips and Powley 1996; Mathis et al. 1998).

The longer the food bolus stays in the stomach, the longer mechanoreceptors are activated and satiety signals transmitted. The timing of gastric emptying is therefore a second important factor which influences satiety. Some dietary protein can delay gastric emptying as it coagulates at the acidic gastric pH (Hall et al. 2003). Gastric emptying can also

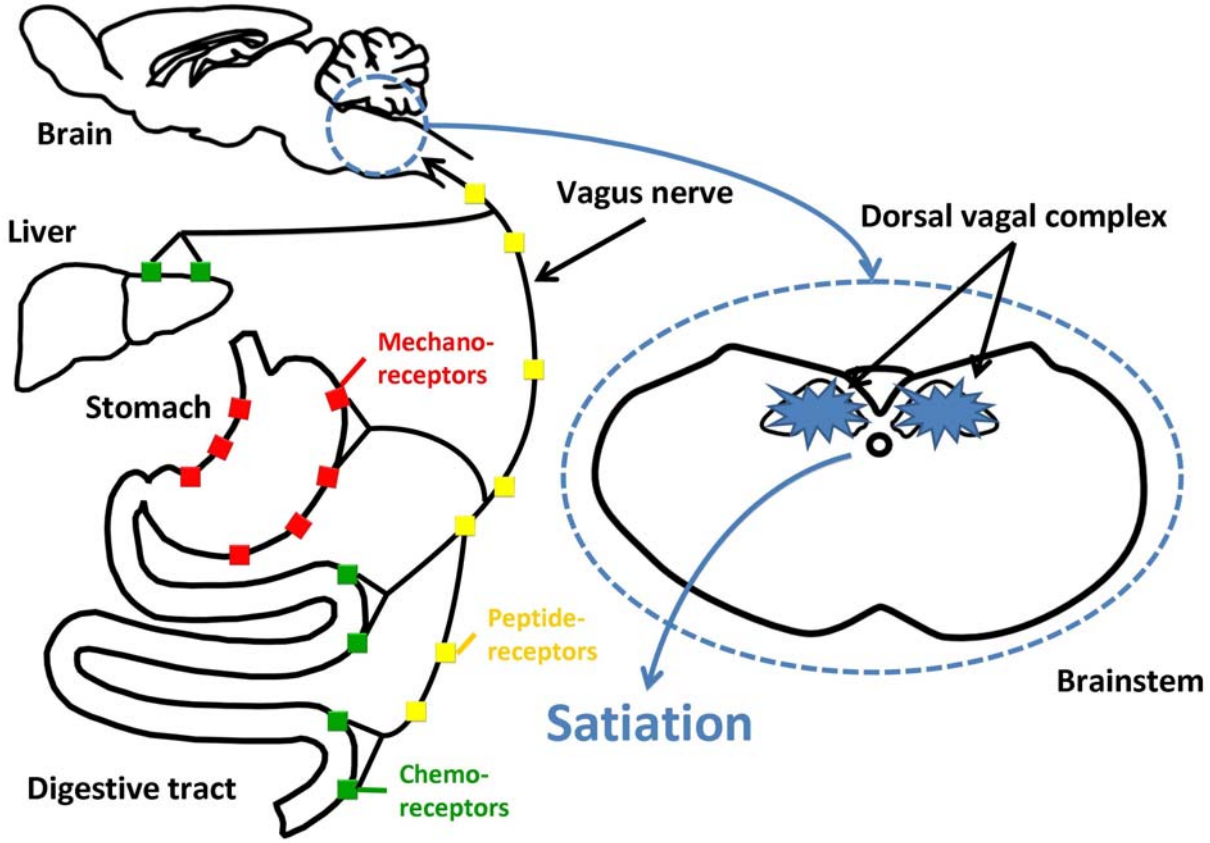


Figure 1.3 The vagus nerve connects the brain and the gastrointestinal tract (GIT).

The vagus nerve innervates the GIT and the liver expressing chemo- and mechanoreceptors on its sensory endings. Additionally receptors for various gut peptides released during ingestion can be found all along the vagal afferents. Generated signals are then transmitted to the dorsal vagal complex in the caudal brainstem where they are integrated and lead to satiation sensation (Parts of figure kindly provided by Nicolas Darcel).

be slowed down in response to the satiety hormone CCK, released from enteroendocrine cells in the duodenum after stimulation by peptides and amino acids (Zhao et al. 1997). Both these examples lead to a longer maintenance of the nutrients in the stomach and hence a maintenance of gastric volume. However, there are a number of other factors influencing gastric emptying, such as energy level of the meal and the type and amount of carbohydrates and fat or dietary fibre (Stubbs 1999).

1.2.2.2 The vagus nerve builds the gut-brain axis

The vagus nerve innervates multiple target organs such as stomach, duodenum and liver and builds therefore one major neuro-anatomic link between the GI tract and the brain for transmitting nutrient induced signals (Figure 1.3) (Fox et al. 2000). Each branch of the vagus nerve consists of an ascending sensitive afferent nerve strand from the GI tract to the brain and a motor efferent nerve strand descending from the brain to the GI tract. The cell bodies of afferent fibres are gathered in the left and the right nodose ganglion, from where in turn axons arise and terminate in second order neurones in the DVC located in the brainstem (Norgren and Smith 1988). In its role as signal transmitter between visceral organs and the brain, the vagus nerve helps additionally regulating various physiological functions such as heart rate, gastrointestinal peristaltic, sweating and muscle movement in the mouth (Berthoud 2004a).

Within this thesis, we are interested in the contribution of vagal afferents to the central feeling of satiation and satiety within the control of food intake. Prior to transmitting their satiety signals to the DVC, sensory terminal ends of vagal afferents become stimulated all along the GI tract. This meal related vagal activation is not only caused by mechanical stimulation such as gastric distension, but also from chemical sensing of metabolites of the ingested meal by chemosensory enterocytes. In addition, gut peptides, which have receptors expressed all along the vagal afferents, are released when macronutrients appear in the intestine (Berthoud 2004a).

1.2.2.3 Chemoreceptors sense nutrients in the intestine and activate the vagus nerve

Chemosensitivity of vagal afferents to luminal nutrients occurs due to the presence of chemoreceptors located on enterocytes (Mei 1978). In response to nutrients appearance in the intestinal lumen endocrine and enterochromaffin cells activate nearby afferent terminals, for instance by releasing gut peptides or other signalling substances (Raybould et al. 2006).

In the case of dietary protein, the intestinal peptide transporter 1 (PEPT1), located on the apical membrane of intestinal epithelial cells has been shown to be involved in the sensory transduction process of duodenal vagal afferents (Darcel et al. 2005b). It has been demonstrated that this activation is mediated by the release of CCK in response to protein hydrolysates and the binding to the CCK1 receptor on vagal afferents (Eastwood et al. 1998; Daniel 2004). As PEPT1 can only process short chain oligopeptides, larger polypeptides have first to be broken down enzymatically. It was likewise suggested that also other GPCRs on enteroendocrine cells might be responsible for the detection and signal transmission of protein hydrolysates (Choi et al. 2007a; Choi et al. 2007b).

As for carbohydrates, different GPCRs are involved in the sensing and signal transmission. Sweet taste receptors of the T1R family, usually present on the tongue, were recently identified on enterocytes (Dyer et al. 2005; Bezencon et al. 2007). After enzymatic digestion of complex carbohydrates, monosaccharides such as glucose and galactose are sensed and transported from the lumen into the cell with the means of the sodium-glucose co-transporter 1 (SGLT1) located on apical surface of enterocytes. The glutamate transporter GLUT-2 is responsible for the exocytosis of these molecules and was demonstrated to be regulated by T1R (Mace et al. 2007). Fructose in contrast crosses the membrane by facilitated diffusion of the GLUT-5 (Mueckler 1994). After uptake of those monosaccharides, enterochromaffin cells release GLP-1 (Shima et al. 1990; Ritzel et al. 1997) and serotonin (5-HT) (Racke and Schworer 1991) which in turn bind to their equivalent receptors on the vagal nerve endings (Raybould et al. 2003).

Dietary fat does not necessarily need a specific uptake mediator as monoglycerides and free fatty acids can freely diffuse into enterocytes from which they transfer into the lymph as chylomicrones. Similar to proteins and carbohydrates, luminal free fatty acids are

sensed by GPCRs, for short chain fatty acids these receptors were shown to be members of the GPR40 family (Covington et al. 2006). Moreover, triglycerides and long chain fatty acids, but not short chain fatty acids, were demonstrated to stimulate the release of CCK and 5-HT (Raybould 1999). Dietary fat has likewise been shown to stimulate GLP-1 secretion which is regulated by binding of free fatty acids to GPR120 (Hirasawa et al. 2005).

1.2.2.4 Peptide hormones act as humoral peripheral signals

Up to now a wide range of peptide hormones have been identified which are released in response to nutrients appearing in the gut and subsequent act either indirectly on vagal afferences or directly on central structures such as the dorsal vagal complex and the hypothalamic area.

Table 1.1 Summary of **the release and action of peptide hormones**

Peptide	**Nutrient stimulating release**	**Release from**	**Action site**
5-HT	Carbohydrate	Enterochromaffin cells in small intestine	Vagus nerve, brain
CCK	Protein and fat	I-cells in small intestine	Vagus nerve, brain, stomach, gall bladder
Ghrelin	Absence of nutrients, calorie restriction	P/D1 cells in gastric mucosa, pancreas	Vagus nerve, brain
GLP-1	Macronutrients	L-cells in small intestine, α-cells of pancreas, brain	Vagus nerve, pancreas, brain
OXM	Macronutrients	L-cells in small intestine, brain	Vagus nerve, brain

Cholecystokinin

Cholecystokinin (CCK) was the first peptide hormone that was shown to alter appetite and therefore being involved in the regulation of meal termination (Gibbs et al. 1973). The main stimulation for the release of CCK from endocrine I-cells of the small intestine is the presence of dietary fat and protein in the intestine in response to apolipoprotein A-IV and

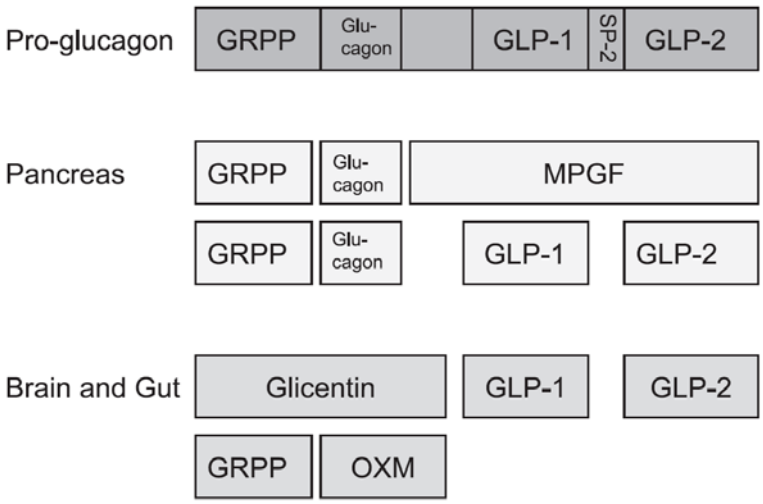


Figure 1.4 The processing of proglucagon in different tissues of the body.

GRPP, glicentin-related pancreatic peptide; GLP, glucagon-like peptide; MPGF, major proglucagon fragment; OXM, oxyntomodulin (Stanley et al. 2004).

PEPT1 activation (Liddle et al. 1985; Darcel et al. 2005; Foltz et al. 2008). Even it is predominantly released in the duodenum and jejunum, CCK is widely distributed in the GI tract (Larsson and Rehfeld 1978). CCK is acting via binding to the G-protein coupled receptors, CCK-1 and CCK-2. The CCK-1 receptor was found on afferent fibres of vagus nerve, in brainstem as well as in the dorsomedial nucleus of the hypothalamus (Moran 2000; Moran and Kinzig 2004). CCK-sensitive vagal afferents activate POMC neurones in the nucleus tractus solitarius (NTS) and were shown to stimulate c-Fos expression dose-dependently (Zittel et al. 1999; Fan et al. 2004; Appleyard et al. 2005). Moreover CCK is involved in several other regulatory processes in the periphery such as gall bladder contraction, enzyme release from the pancreas, stimulation of intestinal peristaltic and inhibition of gastric motility and emptying (Raybould 1991; Schwartz et al. 1991; Schwartz et al. 1993; Schwartz et al. 1997). The effect of CCK had been shown to be potentiated by leptin (Liddle et al. 1985; Raybould 1991).

Oxyntomodulin and glucagon-like peptide as products of the proglucagon gene

Both oxyntomodulin (OXM) and glucagon-like peptide (GLP) are cleavage products of the expression of proglucagon that takes place in the L-cells in the distal part of the small intestine, the α-cells of the pancreas and the brain (Stanley et al. 2004). Depending on the tissue the gene is expressed, the two enzymes prohormone convertase 1 and 2 cleave the 160-amino acid peptide into different products (Figure 1.4). OXM has only been found in the brain and the intestine while GLP-1 and GLP-2 are present in all organs where the gene is expressed (Holst 1999). The peptides are subsequently post-translationally modified in order to gain their biological activity (Mojsov et al. 1986). Both peptides are released 5-30 minutes postprandial in proportion to the caloric intake.

GLP-1 has been demonstrated to act both on the brainstem and the vagus nerve, but also on several hypothalamic regions and on ß-cells in the pancreas where it stimulates postprandial insulin release and inhibits glucagon in order to lower blood glucose (Orskov et al. 1996). GLP-1 receptors have been found to be present on neurones in the NTS and the AP, where GLP-1 also stimulates the expression of c-Fos (Wei and Mojsov 1995; Van Dijk et al. 1996), but also in several nuclei of the hypothalamus including the ARC, PVN and supraoptic nucleus (SON).

OXM consists of the amino acid sequence of pancreatic glucagon, but has an eight amino acid long C-terminal extension. Up to now, no specific OXM receptor has been cloned; however OXM seems to bind, whether with a two-fold lower affinity, to the GLP-1 receptor (Baggio et al. 2004). Yet, as both OXM and GLP-1 appear to exert a comparable effect on the reduction of food intake it is thus likely another OXM specific receptor exists (Stanley et al. 2004). The hypothalamus has been proposed as the principle action site of OXM in the CNS (Wei and Mojsov 1995; Stanley et al. 2005).

In fasted state plasma OXM levels are low; however, an increase in plasma levels inhibits gastric acid secretion and motility and leads to postprandial satiety (Bataille et al. 1982; Stanley et al. 2004). Studies have shown the involvement of OXM in long-term body weight regulation but it also acts as short-term satiety signal. Administered centrally or peripherally OXM leads to decreased weight gain in mice and rats due to its inhibiting effect on energy intake (Baggio et al. 2004; Dakin et al. 2004; Parkinson et al. 2009). Similarly, OXM reduces appetite and caloric intake in lean and obese humans and promotes significant weight loss (Cohen et al. 2003; Wynne et al. 2005). Part of its effect is suggested to be the depression of plasma ghrelin levels which was demonstrated in both rodents and humans (Cohen et al. 2003; Dakin et al. 2004) and an increase in activity and so energy expenditure (Wren and Bloom 2007). Better understanding the mechanisms of action of OXM in the CNS in the control of food intake may therefore help to study its potential role as anti-obesity treatment.

Serotonin

Serotonin (5-HT) is released from enterochromaffin cells, located in the epithelium of the small intestine, in the presence of carbohydrates in the GI tract and the activation of SGLT1 (Raybould et al. 2003; Freeman et al. 2006). Injection of 5-HT in turn reduces glucose intake (Hayes and Covasa 2005). After stimuli such as gastric distension, pressure sensors are activated and were demonstrated to be involved in the 5-HT dependent c-Fos expression in the NTS and the AP (Mazda et al. 2004). There are many different 5-HT receptors known. The 5-HT3 receptor was found on vagal and spinal afferent neurones (Raybould et al. 2003; Tome 2007), but 5-HT was also shown to act directly on the AP. A 5-HT receptor agonist activates catecholaminergic neurones in NTS at level of AP (Lam et al. 2009). 5-HT is also

suggested to mediate intestinal CCK and GLP-1 satiety signals (Hayes et al. 2006; Asarian 2009).

Ghrelin

The only peripheral orexigenic peptide known yet that increases appetite when administered peripherally and centrally is Ghrelin, which is synthesised in stomach (Date et al. 2005; Valassi et al. 2008). Ghrelin receptors can be found all along the vagus nerve as well as in the DVC (Guan et al. 1997). Ghrelin levels decrease after ingestion of a meal and thereafter increase in correlation with the amount of calories ingested (Weigle et al. 2003). Additional Ghrelin levels increase according to an endogenous rhythm with a maximum concentration at 01:00 am (Cummings et al. 2001). It is suggested that the satiating effect of protein also occurs due to a longer maintenance of postprandial low plasma ghrelin levels (Blom et al. 2006; Bowen et al. 2006b).

1.2.3 Pre-absorptive signals specifically induced by protein ingestion

Ingestion of a high protein diet leads to an increase in gastric volume and a delay in gastric emptying (Bowen et al. 2006b; Faipoux et al. 2006). Gastric distension was demonstrated to participate in meal termination by activating vagal afferent fibres (Schwartz et al. 1991; Phillips and Powley 1996, 1998). Rats with a pyloric cuff terminate their food intake as soon as they reach a certain volume (10-12 ml), independent from the sort of nutrient. In rats that have no pyloric cuff, meal termination occurs after ingestion of an even smaller volume as nutrients can leak into the intestine and stimulate the release of CCK. CCK in turn slows down gastric emptying and maintains gastric distension for longer what leads to meal termination after a smaller meal size (Raybould 1991; Schwartz et al. 1991; Schwartz et al. 1993; Schwartz et al. 1997). However, some studies suggest that the caloric value of the ingested meal is the determining factor for gastric emptying rather than the nature of the nutrients (McHugh and Moran 1979; Maerz et al. 1994).

Electrophysiological studies demonstrated that protein is able to generate pre-absorptive signals in vagal afferents (L'Heureux-Bouron et al. 2004). Also gastric or intestinal distension was shown to stimulate certain afferent fibres. However, vagal response to duodenal infusion with amino acids or protein was delayed by 3 minutes, suggesting that

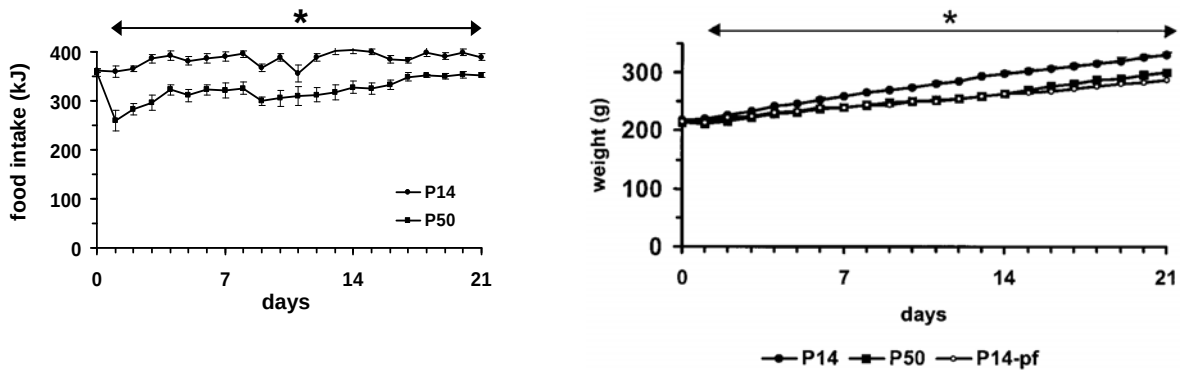


Figure 1.5 Daily energy intake and body weight of rats.

Daily energy intakes (left panel) and body weight (right panel) of rats fed either a 14 g/100 g protein diet (P14), a 50 g/100 g protein diet (P50) or a 14 g/100 g protein diet pair-fed to the P50 group in energy (P14-pf) for 21 days. Values are means ± SEM, n=24. *P50 value is different from P14, $P < 0.05$. From d 1 to 21, the P14 rats consumed more energy than the P50 and the P14-pf rats. Additionally, during this period, the P50 rats consumed more protein energy than the rats from the other groups. There was no difference between the body weights of the P14-pf rats and P50 rats (Jean et al. 2001).

these nutrients do not directly stimulate the intestinal sensory endings of vagus nerve. They are rather activated indirectly, for instance by the release of CCK. Moreover, parts of the hydrolysates might have been absorbed and lead to stimulation of hepatic afferent fibres.

Depression of food intake in response to a high protein diet can hence be explained by a larger gastric volume due to higher water intake as well as delay of gastric emptying and an amplification of vagal signalling in response to an increased release of CCK.

1.2.4 Post-absorptive peripheral signals

Additional to the pre-absorptive signals emerging during the passage of nutrients in the intestinal lumen, post-absorptive signals occur when nutrients or their metabolites enter the blood stream. Numerous metabolic events have also been hypothesised as signals in protein-induced satiety, including an increase in plasma amino-acid level, energy expenditure, thermogenesis and the production of glucose through gluconeogenesis.

1.2.4.1 Direct sensing of dietary protein

After the nutrient breakdown and absorption, the increase in circulating amino acids levels is detected by the brain and influence in this way satiety (Mellinkoff et al. 1956). Studies in rats have shown that replacing a normal protein diet with a high protein diet, animals immediately decrease their food intake (Figure 1.5). This depression in food intake has been demonstrated not to be due to a taste aversion to the protein diet, but indeed due to the protein content (Bensaid et al. 2002; Bensaid et al. 2003). Additionally a massive increase in the amino acid concentration could be detected in the first hours of a high protein diet which went back to baseline after adaptation to the new diet (Peters and Harper 1985, 1987). Together with the decrease in blood amino acid concentration, the protein induced satiety signalling becomes weaker (Long et al. 2000).

There is also some evidence that circulating leucine levels may influence food intake. An increase in dietary leucine (Ropelle et al. 2008) or the intra-cerebroventricular administration of either amino acids or only leucine reduced food intake and body weight (Cota et al. 2006; Morrison et al. 2007). These findings seem to be leucine-specific, as leucine alone exerts the same effect on food intake as a mixture of amino acids (Ropelle et al. 2008). Indeed, leucine is associated with mechanisms involving AMP-activated protein kinase

(AMPK) and the mammalian target of rapamycin (mTOR), both of which are energy sensors active in the regulation of energy intake, at least in the arcuate nucleus (ARC) but probably also in other brain areas such as the paraventricular nucleus (PVN).

It was suggested that not only the amino acids themselves act on brain satiety centres, but they stimulate the synthesis of the neurotransmitter 5-HT which is derived from the amino acid tryptophan (Latham and Blundell 1979). Nowadays this hypothesis has been abandoned (Harper and Peters 1989; Stubbs 1999). Still, other amino acids such as tyrosine and histidine are precursors of the neurotransmitters noradrenalin and histamine, respectively which can then indirectly influence hunger and satiety (Mercer et al. 1990). However, Bassil failed to demonstrate any effect of a diet supplemented with 5 % of either histidine or tyrosine on the levels of food intake by Sprague-Dawley rats (Bassil et al. 2007).

1.2.4.2 Glucose-mediated sensing of dietary protein

Blood glucose is not only detected by glucosensitive cells in the hypothalamus (Fioramonti et al. 2007) and the NTS (Ritter et al. 2000), but also at the level of the liver (Russek 1971). The longer a high blood glucose level can be maintained, the longer the sensation of satiety occurs (Holt et al. 1996).

As glucose is an important substrate for many body functions, especially performance of the brain, in a hypoglycaemic state the metabolism is forced to synthesise de novo glucose in order to maintain glucose homeostasis. This metabolic pathway is called gluconeogenesis and uses gluconeogenic amino acids such as alanine, glutamine, serine or glycine as precursor for glucose synthesis. During the postprandial state after ingestion of a high protein diet, due to the gluconeogenesis from dietary protein, the decrease in blood glucose concentration is more efficiently delayed (Blouet et al. 2006).

1.2.4.3 Protein-induced thermogenesis can influence satiety

Increased energy expenditure was observed after a high protein load or meal and was proposed as another mechanism of protein satiety (Porrini et al. 1997; Westerterp-Plantenga et al. 1999). There was also a correlation noticed between increased energy expenditure and elevated satiety after a high protein meal (Crovetti et al. 1998; Westerterp-

Plantenga et al. 1999; Lejeune et al. 2006). The satiating effect could be explained by the feeling of oxygen deprivation caused by elevated body temperature and greater use of oxygen in protein metabolism, used for absorption, storage and oxidation (Tappy 1996; Westerterp 2006). In contrast to lipids and carbohydrates, which can be stocked easily, proteins have to be metabolised prior to storage. Gluconeogenesis in the liver and building of muscle mass expend energy and in this way produce heat (Johnston et al. 2002; Westerterp-Plantenga et al. 2004). However, there is no consensus on this theory, as several studies failed to show a relation between the intake of a high protein diet, elevated body temperature or energy expenditure and satiety (Luscombe et al. 2003; Raben et al. 2003).

1.2.4.4 The role of hormones as peripheral adiposity signals

Other important factors among peripheral signals influencing satiety are hormones released from adipose tissue and the pancreas which are responsible for the control of energy homeostasis (Stanley et al. 2005). Most of them have an action site on both the level of the GI tract by activation of the vagus nerve and central on the hypothalamus and the dorsal vagal complex via the blood stream. Postprandial hormone profiles have been studied intensively in order to investigate in which way protein influences satiety (Al Awar et al. 2005; Bowen et al. 2006a; Bowen et al. 2006b).

Leptin

Leptin is the product of the *ob* gene and that is mainly produced and secreted from adipocytes but also in lower levels from gastric epithelium and placenta. Amongst others leptin influences energy homeostasis as well as neuroendocrine and immune functions. The plasma levels of leptin are direct proportional to adiposity and total fat mass (Considine et al. 1996); it likewise enters the brain in proportion to those plasma levels (Valassi et al. 2008). Leptin expresses direct action on neurones of the nucleus of the solitary tract and therefore short-term energy intake. Neurophysiologic studies revealed that central administration of leptin significantly increased NTS responses to gastric loads in unconscious rats (Schwartz and Moran 2002). Moreover, using current-clamp records on hindbrain sections, superfusion with leptin was demonstrated to induce a dose-dependant hyperpolarisation of the resting membrane potential in neurons in the nucleus of the solitary tract (Williams and Smith 2006). Together with insulin, leptin is also regulating the activity of

first order neurones in the ARC of the hypothalamus. There it stimulates the expression of the pro-opiomelanocortin (POMC) gene and the release of the anorexigenic α-melanocyte-stimulating hormone (α-MSH) while at the same time it inhibits neurones which are usually expressing neuropeptide Y (NPY) and the agouti-related protein (AgRP) (Schwartz et al. 2000). Up to now no study could show a correlation of a high protein load on plasma leptin levels compared to a high fat or high carbohydrate charge (Raben et al. 2003).

The importance of leptin in the regulation of body weight can be seen in the absence of either the hormone itself or the leptin receptor, leading to hyperphagia and severe obesity in both animals and humans (Licinio et al. 2004). In humans, however, leptin or leptin receptor deficiency is extremely rare and in most obese subjects a contrary problem occurs as elevated leptin levels sooner or later result in leptin resistance (Considine et al. 1996). Leptin is therefore an anorexigenic hormone, peripherally administered it is very powerful in decreasing both spontaneous and long-term food intake resulting in loss of fat mass and body weight (Halaas et al. 1995). However, due to the increased leptin resistance in overweight subjects, anti-obesity treatment with this hormone is often not successful (Considine et al. 1996).

Insulin

Insulin, secreted from pancreatic β-cells, was the first hormonal signal that was shown to be involved in the central control of food intake and body weight (Wynne et al. 2005). Proportional to the body fat content, plasma levels of this hormone are rapidly raised in response to blood glucose. It reaches the brain through receptor-mediated transport via the blood-brain barrier (BBB) as for leptin in proportion to its circulating levels (Baura et al. 1993). Insulin achieves the reduction in food intake by acting on neurones in the ARC which express the insulin receptor (Cone et al. 2001; Benoit et al. 2002). A clear relation between a high protein meal and plasma insulin concentration was not shown yet (Veldhorst et al. 2009a, 2009b).

In overweight subjects, insulin resistance is a common phenomenon as both in basal state and in response to a meal. Insulin plasma concentration has to increase in order to maintain stable glucose levels. As a result of long-term overproduction of insulin, pancreatic

β-cells can not raise insulin concentrations high enough anymore to decrease elevated plasma glucose levels. Subsequently hyperglycaemia occurs leading to type 2 diabetes.

Adiponectin

As for leptin, adiponectin is released from adipose tissue, but in a 1000 fold higher concentration than leptin or insulin whereby its plasma concentration is negatively correlated with adiposity (Stanley et al. 2005). In peripheral tissue such as muscle and liver, adiponectin was shown to be involved in the regulation of energy homeostasis by modulating glucose and fatty acid metabolism (Berg et al. 2002). In contrast, the action of adiponectin in the brain is still controversially discussed. On the one hand, two receptors were identified which are located not only in peripheral tissue but also in regions of the hypothalamus and the brainstem, including the area postrema (AP), ARC and the PVN (Dridi and Taouis 2009). On the other hand, only low concentrations of adiponectin can be detected in the cerebrospinal fluid. Adiponectin is suggested to be transported from the blood by receptor-mediated transcytosis or via circumventricular organs such as the median eminence (ME) or the AP. A diet high in protein was shown to result in decreased plasma adiponectin levels (Stroubini et al. 2009).

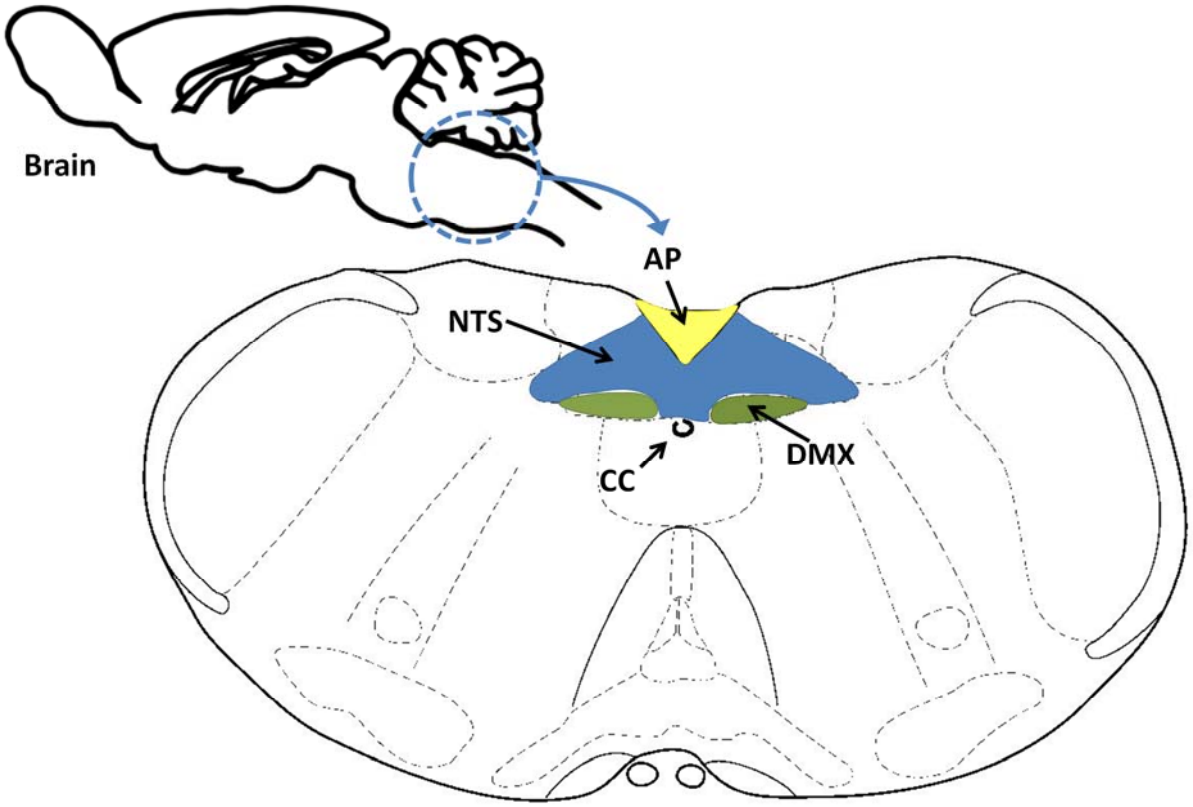


Figure 1.6 Coronal section of the brainstem showing the dorsal vagal complex (DVC).

Structures building the DVC are: AP, area postrema; CC, central channel; DMX, dorsal motor of vagus; NTS: nucleus tractus solitarius (Adapted from Paxinos and Franklin 2001, Figure 96, Bregma -7.76).

1.3 Central signalling of dietary protein

Even there is no doubt that the CNS plays an important role integrating information which is related to the amount of ingested protein, many studies using electrolytic lesions carried out in the past years failed to identify a brain region which is especially responsible for the control of protein intake (Fromentin et al. 2000). It is commonly accepted that hunger and satiety are correlated with the activation of different brain regions (Berthoud 2004b). The main centres for the control of food intake are on the one hand the dorsal vagal complex responsible for the regulation of meal size and termination and on the other hand the hypothalamus which is responsible for the indirect, long-term regulation of energy homeostasis. In this context 'direct control' stands for the afferent pathways activated in response to the food stimuli contacting pre-absorptive receptors along the surface of the gut from the tip of the tongue to the end of the small intestine. Indirect controls are all those that are not directly affected by food stimuli acting at sensory receptors along the mucosal surface of the gut during a meal (Smith 1996).

1.3.1 Short-term regulation of food intake: the dorsal vagal complex controls meal size

The dorsal vagal complex lies within the caudal brainstem, which builds the lower part of the brain and is the connection with and prolongation of the spinal cord (Figure 1.6). The DVC is a neuronal network that consists of three major structures which integrate inhibitory and excitatory peripheral signals and are strongly suggested to be involved in the direct regulation of food intake and meal size. The NTS processes visceral sensation and taste from afferent sensory fibres of the trigeminal (V), facial (VII), glossopharyngeal (IX) and vagus (X) cranial nerve. Moreover it also processes primary afferent signals from a variety of visceral regions and organs via the The AP having a deprived BBB mainly receives blood borne signals. The third structure within the DVC, the dorsal motor of vagus (DMX), in turn is the primary location of motor neurones. From here vagal efferents arise which then respond to processed signals by causing gastric and intestinal peristaltic (Norgren and Smith 1988).

The essential role of the DVC in the regulation of meal size was demonstrated in the way that meal termination induced by short-term satiety signals occurred, even when all

neuronal connections between forebrain and brainstem were severed in rats (Grill and Smith 1988).

1.3.1.1 The blood-brain barrier

The blood-brain barrier prevents the entrance of potentially unwanted circulating factors into the brain. Cells building the BBB form specialised capillaries, blood vessels consisting of a single layer of endothelial cells whose one side is facing the blood and the other side is facing the cerebrospinal fluid surrounding the brain (Hawkins et al. 2006). While small lipophilic molecules can pass the BBB by diffusion, hydrophilic substances can only enter central structures by active transporters, channels or at areas, where the BBB is deprived such as the AP and the ME (Maolood and Meister 2009).

1.3.1.2 The area postrema mainly responding to humoral signals

The area postrema as part of the DVC lies in the brainstem, directly above the NTS (Figure 1.6) and is involved in the short-term regulation of food intake and individual meal size. It is a circumventricular organ built of fenestrated blood vessels and it is known for its incomplete BBB, what gives it the possibility to control the entrance of blood-borne substances to the neurones of DVC (Maolood and Meister 2009). Therefore, the NTS being places next to this organ with deficient BBB can also respond to peripheral circulating signals (Sawchenko 1983). The AP expresses a large number of receptors for gut peptides being involved in food intake control, including CCK1-R, GLP-1R, NPY (Wei and Mojsov 1995; Moran 2000; Stanley et al. 2005). In addition to its tasks in food intake regulation, the AP is also involved in the control of cardiovascular functions (Saito et al. 2003).

1.3.1.3 The NTS receives signals from vagal afferents and the blood stream

The NTS, the second important component of the DVC involved in the short-term regulation of food intake, consists of a dense vascular network and is located in the caudal brainstem (Norgren and Smith 1988) (Figure 1.6). Initiations of satiety signals reaching the NTS via vagal afferents during food ingestion are derived from mechanical or chemical stimulation of the GI tract and abdominal viscera as well as taste information from the oral cavity and from neural input related to energy metabolism in the liver (Travers et al. 1987; Emond et al. 2001; Powley and Phillips 2004). Due to its location in direct neighbourhood of

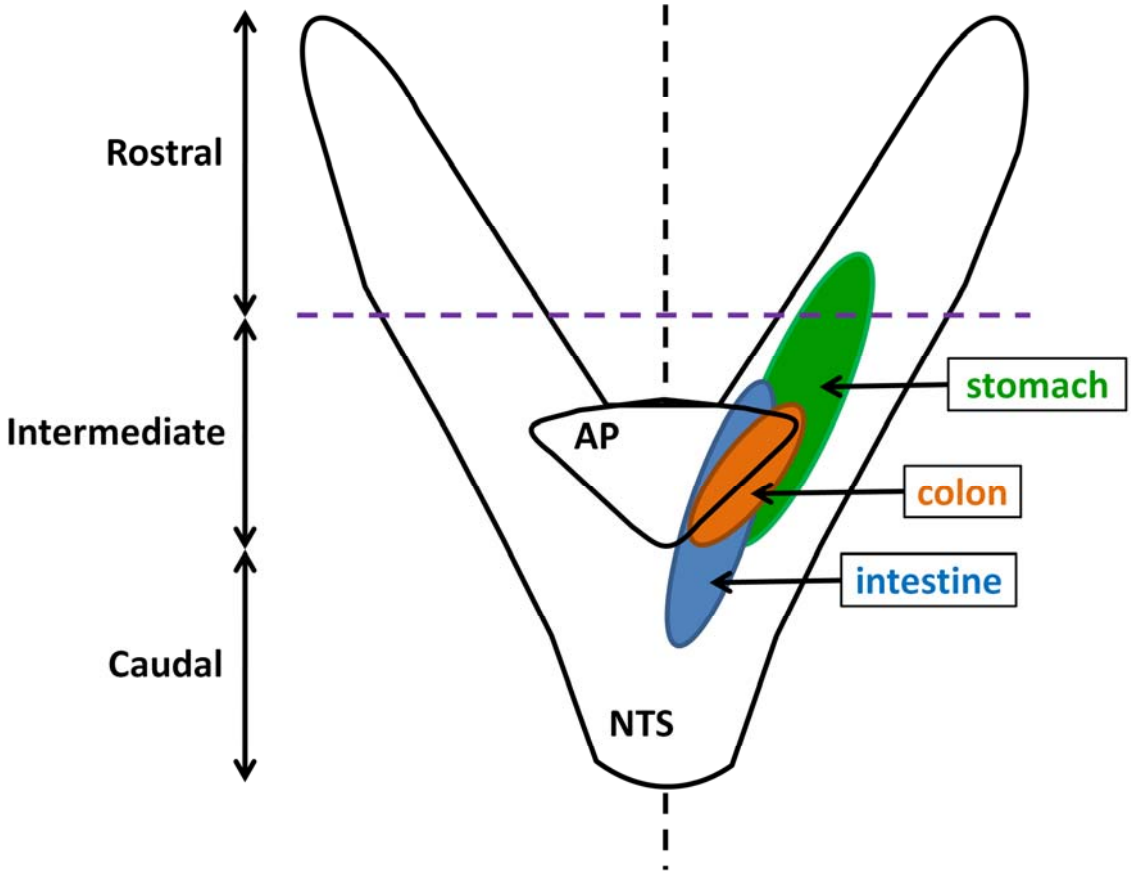


Figure 1.7 Projection sites of the different vagal branches in the nucleus tractus solitarius (NTS).

Vagal afferents ascending from different sites within the gastrointestinal tract projecting in the NTS. AP, area postrema (Adapted from Méi 1998, page 115).

the AP, which is missing BBB, also the entrance of humoral signals in the NTS is possible, specifically peptides and substrates which in turn can bind to specific receptors and activate NTS neurones (Maolood and Meister 2009). The GLUT-1 transporter system for instance, which is sensitive to glucose, was found in the NTS (Maolood and Meister 2009), but also receptors for gut peptides which are released upon nutrient stimulation from neuroendocrine cells in the intestinal lumen during ingestion, such as CCK1-R, GLP-1R, NPY (Wei and Mojsov 1995; Moran 2000; Stanley et al. 2005). NMDA and AMPA glutamatergic receptors are as well expressed in NTS neurones (Berthoud et al. 2001). All this peripheral sensory information is subsequently integrated in second order neurones.

The neuroanatomic distribution of the NTS had been suggested after retrograde labelling studies (Figure 1.7). The most rostral part of the NTS receives vagal afferents from the oral cavity, while the most caudal part receives those from the hepatic branches and coeliac. The intermediate part of the NTS, which lies next to the AP, mainly receives projections from the stomach and intestine (Norgren and Smith 1988).

As part of the entire network involved in appetite regulation, afferent fibres of the NTS project, amongst others, to the ARC and PVN within the hypothalamus and in turn receive information from this second important satiety centre what makes it possible to integrate satiety signals with adiposity signals like insulin and leptin (Schwartz et al. 2000; Berthoud et al. 2006; Valassi et al. 2008).

Even there is still a big lack of knowledge in understanding the exact mechanisms of how the DVC controls meal size, it is hypothesised that neurones in the NTS can be activated macronutrient specific (Rinaman et al. 1998; Yamamoto and Sawa 2000; Berthoud et al. 2001; Emond et al. 2001).

1.3.2 Long-term regulation of energy balance: the hypothalamic area is responsible for maintaining body weight through meal initiation

While the DVC is known to control short-term regulation of food intake, integration of peripheral long-term hunger and satiety signals as well as body weight control mainly takes place in the hypothalamic area. Being the negative feed-back regulator for energy metabolism, the hypothalamus integrates humoral adiposity signals so as leptin and insulin

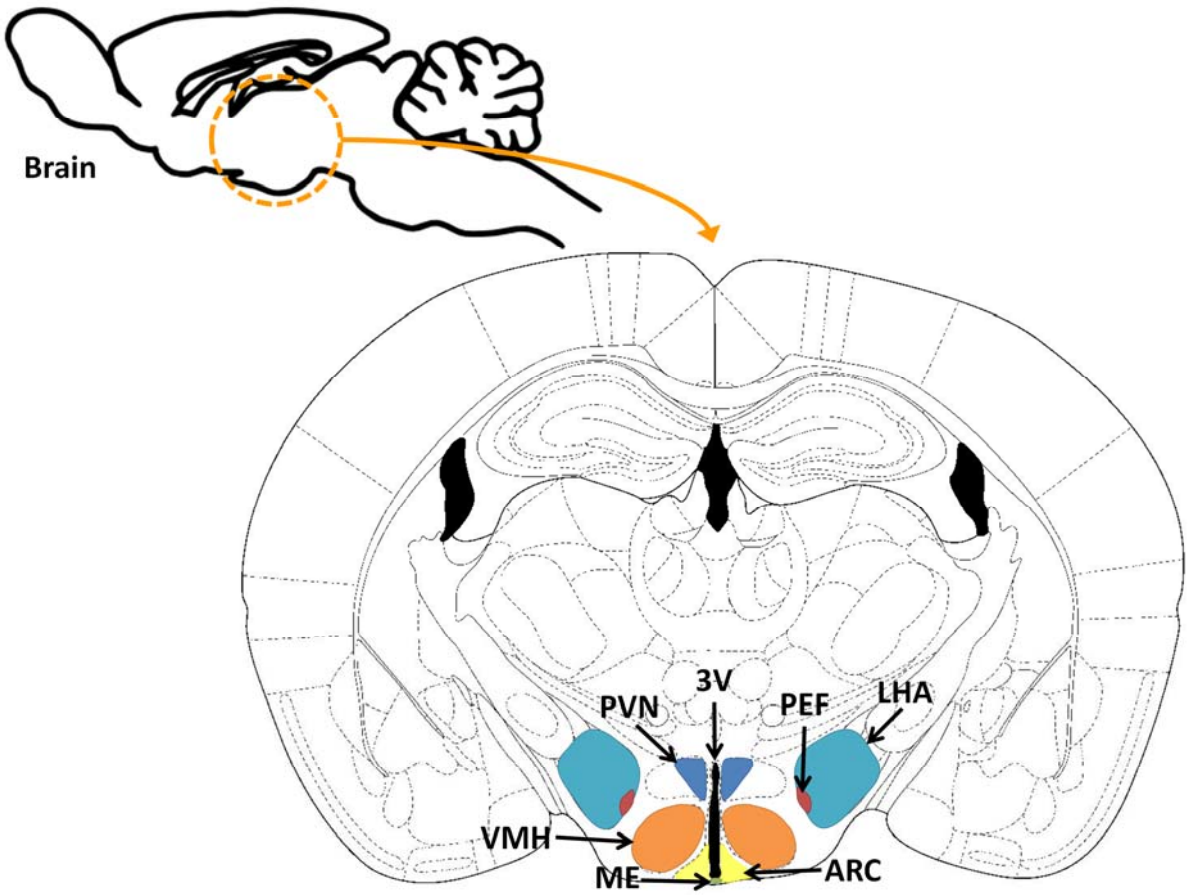


Figure 1.8 Coronal section of the hypothalamus summarising hypothalamic nuclei involved in long-term food intake regulation.

3V, 3rd ventricle; ARC, arcuate nucleus; LHA, lateral hypothalamic area; ME, median eminence; PFA, perifornical area; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus (Adapted from Paxinos and Franklin 2001, Figure 44, Bregma -1.58).

which are released in direct proportion to the level of energy stores, specifically to the amount of energy stored as fat (Schwartz et al. 2000). There are a number of hypothalamic areas involved in the integration and transmission of signals, in particular the arcuate and paraventricular nucleus as well as the perifornical area and lateral hypothalamus (Figure 1.8). It was suggested that a high protein diet can effect hypothalamic gene expression (Kinzig et al. 2007; Ropelle et al. 2008) and neuronal activity (Simkova et al. 1979; Darcel et al. 2005a).

1.3.2.1 The arcuate nucleus

The arcuate nucleus (ARC) is situated at the basis of the third ventricle and is extending over around a half of the length of the hypothalamus (Figure 1.8). Due to its neighbourhood to ME, which lacks BBB, the ARC is able to receive signals by circulating hormones (Peruzzo et al. 2000; Hawkins et al. 2006). The ARC consists of two types of first order neurones indicating the highly specialised roles of the here present brain circuits (Bagnol et al. 1999). As described above, NPY and AgRP are co-localised in one subset of neurones, while POMC and CART (cocaine- and amphetamine-regulated transcript) are co-expressed in a second subset (Figure 1.9). When rodents are fed a high protein diet, catabolic pathways are supported as NPY is down regulated while POMC is up regulated in both the fed and fasted state (Kinzig et al. 2007; Ropelle et al. 2008). Both sets of neurones were shown to express the leptin receptor (Elmquist et al. 1998); however leptin does have an opposite effect on them. While NPY/AgRP neurones are inhibited by leptin, POMC/CART neurones are activated. The catabolic pathway is activated and anorexigenic peptides are released. In contrast low levels of circulating leptin result in activation of the anabolic pathway, including inhibition of POMC/CART neurones and activation of NPY/AgRP leading to NPY and AgRP gene expression, release of orexigenic peptides and setting-on of food intake (Schwartz et al. 2000). A high concentration of insulin receptors was also found in the ARC and it is suggested that low insulin levels also activate NPY/AgRP neurones and inhibit POMC/CART neurones. Not only humoral signals are reaching the hypothalamus via the ARC, but also afferent fibres of the NTS project to the ARC (Valassi et al. 2008). The ARC is therefore suggested to be responsible for transducing afferent input from circulating leptin and insulin into a neuronal response which is subsequently projected to second order neurones in other

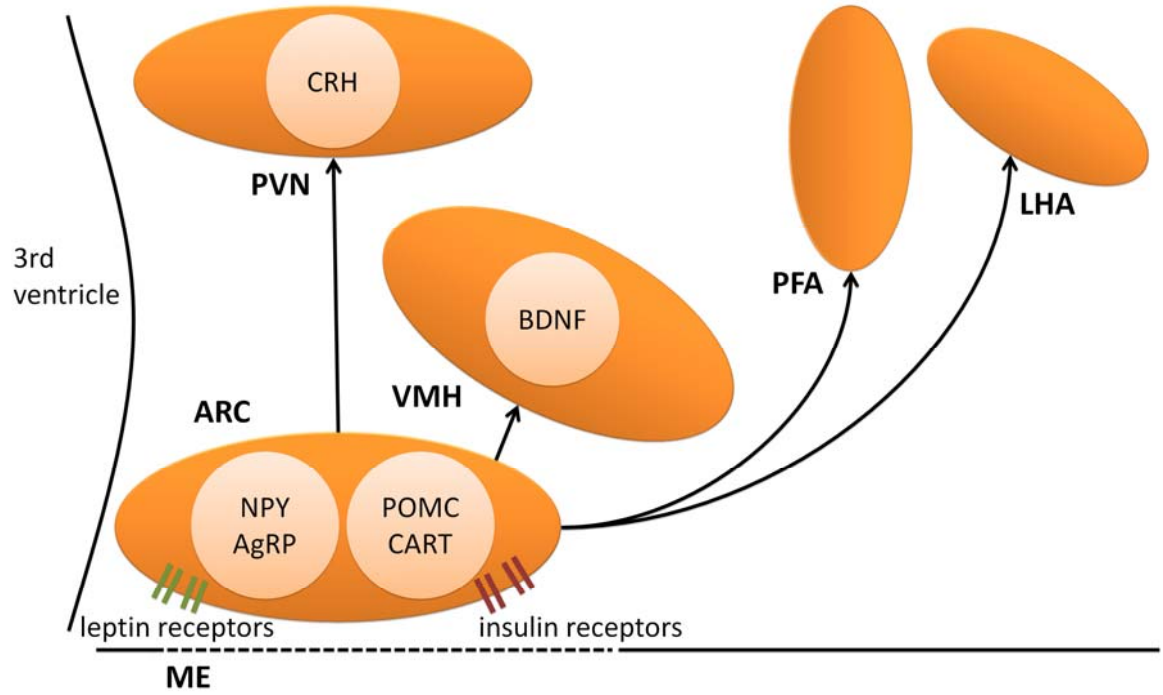


Figure 1.9 Hypothalamic projections.

Projections from NPY/ AgRP and POMC/ CART neurones of the ARC to other hypothalamic areas. ARC, arcuate hypothalamic nucleus; BDNF, brain-derived neurotrophic factor; CRH, corticotrophin-releasing hormone; LHA, lateral hypothalamus; NPY/ AgRP, neurones co-expressing neuropeptide Y and agouti-related protein; PFA, perifornical area; POMC/ CART, neurones co-expressing pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus (Adapted from Wynne et al. 2005).

hypothalamic nuclei such as the PVN, the perifornical area (PFA) and the lateral hypothalamic area (LHA).

1.3.2.2 The paraventricular nucleus

The paraventricular nucleus (PVN) is located next to the superior part of the third ventricle (Figure 1.8). Its second order neurones integrate signals from various regions of the CNS, for instance from the ARC and the brainstem and express the central neuromediator corticotropin-releasing hormone (CRH) also involved in food intake control and energy expenditure (Figure 1.9). The PVN in turn is projecting to sites such as the ME and the pituitary gland. In the ME neurosecretory nerve terminals originating in the PVN release peptides into the blood vessels of the hypothalamo-pituitary portal system where they regulate hormone secretion into the systemic circulation. Being additionally richly supplied with neuronal projections from the ARC, microinjection of nearly all known orexigenic signals like NPY and ghrelin into the PVN stimulates feeding, whilst microinjection of anorexigenic compounds such as CCK, GLP-1 and leptin inhibits feeding (Stanley et al. 2005).

1.3.2.3 The ventromedial hypothalamus

The ventromedial hypothalamus (VMH) can be divided into a superior and an anterior region and is located above the ARC (Figure 1.8). The superior part of the VMH is suggested to be the “satiety centre” within the hypothalamus in contrast to the “feeding centre” located in the lateral hypothalamus. The VMH receives projections in form of NPY, AgRP and α-MSH from ARC neurones and is in turn sending signals to other hypothalamic nuclei and the brainstem (Figure 1.9). Brain-derived neurotrophic factor (BDNF) was demonstrated to be present in high concentrations in the VMH which might built another downstream pathway for modulation energy homeostasis over the nutritional status (Stanley et al. 2005). A modulation of the neuronal activity in the VMH was demonstrated by feeding animals a high protein diet (Simkova et al. 1979; Darcel et al. 2005a).

1.3.2.4 Central neurotransmitters involved in hypothalamic projections

Neuropeptide Y

Neuropeptide Y (NPY) is an anabolic signalling molecule, belonging to the pancreatic polypeptide family, the expression and secretion of which is increased during depletion of body fat stores as well as reduced insulin and leptin signalling to the brain (Schwartz et al. 2000). Other members of this family are pancreatic polypeptide (PP) (Whitcomb et al. 1990) and peptide tyrosin-tyrosin (PYY). The main sites of expression are specific NPY neurones in the ARC of the hypothalamus which subsequently project to second order neurones in other hypothalamic areas and brain regions starting the anabolic pathway. Hypoglycaemia, low insulin and leptin levels and negative energy balance activate these neurones of which 90% co-express the AgRP (Schwartz et al. 2000).

Administered centrally, the NPY inhibits thermogenesis, enhances food intake and promotes adipogenesis (Williams et al. 2004). There have been several GPCRs identified which can bind NPY; they are mainly expressed in the brain whereby some subtypes found in the hypothalamus are suggested to be responsible for the orexigenic effect on food intake and increase in energy expenditure (Stanley et al. 2005).

The melanocortin system

Melanocortins such as α-MSH, β-endorphin or adrenocorticotropic hormone (ACTH) are bioactive peptides cleaved tissue-specifically from a common precursor molecule, pro-opiomelanocortin. POMC is primarily expressed in the brain, precisely in another subset of ARC neurones than NPY and AgRP in co-localisation with CART. Other sites of expression are the anterior and intermediate pituitary, the skin and the immune system (Smith and Blalock 1981; Bertagna 1994; Castro and Morrison 1997).

Melanocortins are the anorexigenic counter players of NPY and AgRP; they are secreted in response to positive energy balance, elevated fat cell mass and high circulating leptin and insulin levels and signal the inhibition of food intake. Up to now five G protein-coupled melanocortin receptors have been identified. Two of them are suggested to play a role in energy homeostasis as they are expressed in several areas of the hypothalamus such as the ARC, PVN and VMH. The agouti protein as well as the AgRP was demonstrated to act

as antagonists to the melanocortin receptors (Fong et al. 1997; Tota et al. 1999; Yang et al. 1999).

1.3.3 Integration of short-term satiation signals and long-term hunger signals in the DVC and the hypothalamus

In order to ensure a fine regulation of meal initiation and termination, interaction of forebrain and brainstem areas is necessary. There are extensive reciprocal connections between the DVC and the hypothalamic area, particularly between the NTS on the one hand and the PVN and LHA on the other hand (Ter Horst et al. 1989). The ARC receives and responds to projections from the NTS via afferent fibres (Valassi et al. 2008). At the same time NTS neurones integrate afferent information related to satiety with descending information from forebrain neurones, located for example in the VMH, which are involved in energy homeostasis (Schwartz et al. 2000). Additionally receptors for satiety signals such as leptin as well as peptide hormones, e.g. GLP-1 and CCK, are found in both the DVC and in hypothalamic areas. Furthermore, synergistic mechanisms were shown to take place, for instance the adiposity signals leptin and insulin were demonstrated to enhance the satiating effect of CCK (Figlewicz et al. 1986; Emond et al. 1999; Matson and Ritter 1999).

1.4 Effect of a diet high in protein on body composition

A diet high in protein does not only have a beneficial effect on the depression of food intake, but also leads to changes in energy metabolism which are independent from the satiating effect. These changes can be observed in the form of differences in body composition of animals fed a high protein diet. For instance rats fed a high protein diet for a period of two to four weeks had a lower of body weight gain and food intake compared to animals fed the control diet (Jean et al. 2001; Pichon et al. 2006; Kinzig et al. 2007). Moreover, rats fed a high protein diet were shown to have less adipose tissue compared to animals receiving less protein (Jean et al. 2001; Lacroix et al. 2004). Another study has shown that also adipocyte size was smaller (Pichon et al. 2006). A diet high in protein is recently also proposed to prevent liver steatosis. When feeding rats either a high protein diet or a normal protein control diet for 6 months, only the control animals developed liver steatosis while no hints for that were found in the high protein fed animals (Lacroix et al. 2004).

Mechanistic explanations for these observations might be increased postprandial energy expenditure due to a high cost of protein metabolism (e.g. ureogenesis, protein synthesis, gluconeogenesis) and thus lower energy efficiency of HP diet. Moreover, there is indication that under the condition of a high protein diet glycolysis and lipogenesis are decreased (Pichon et al. 2006; Stepien 2010).

1.5 Summary and objective of this thesis

Within the context of avoiding and treating obesity, the concept of a high protein diet to decrease weight gain and adiposity could be a solution. Ingestion of a high protein meal is commonly accepted to decrease food intake at the following meal in both rodents and humans which appears possibly due to satiety mechanisms. It is also agreed that high protein diets lead to a depression in food intake over a longer period of time. However, the exact mechanisms of the effects of protein and amino acids on food intake regulation at the level of the periphery as well as in central structures are still not well known.

In this thesis we wanted to investigate those brain areas and peripheral systems which are involved in direct as well as indirect food intake depression, caused by transmission and integration of protein intake signalling.

- First we investigated if sensory vagal afferents located within the hepato-portal vein area are involved in protein induced food intake depression. Therefore deafferentation using capsaicin was carried out and food intake was measured.

- Second, in order to examine if post-ingestive satiety signalling in the dorsal-vagal complex is macronutrient specific, we were comparing statistical activity maps created in response to either protein or carbohydrates or a volume control in mice.

- At last, concerning the indirect control of energy homeostasis, we were then interested in the effect of a high protein diet on body weight and body composition. Moreover, we investigated if the effect of the anorexigenic gut peptide oxyntomodulin on hypothalamic hunger and satiety centres can be modulated by a high protein diet.

2 Hepatic portal vein deafferentation has no effect on the satiating effect of a high protein diet in rats

2.1 Introduction

2.1.1 High protein diet altering food intake

High protein (HP) diets are known to reduce body weight and improve energy balance (Tome 2004; Kinzig et al. 2007) by inducing both short-term decrease of food intake (Poppitt et al. 1998; Williams et al. 2006) and long-term maintenance of body weight. The effect of protein was shown in rodents (Bensaid et al. 2003) as well as in humans (Porrini et al. 1995; Long et al. 2000). Today, this strategy is widely used in humans in terms of weight management (Raben 2002; Kushner and Doerfler 2008). The beneficial effect, although poorly understood yet, is likely to partially result from the ability of dietary proteins to affect the central structures orchestrating the control of ingestion (Phifer and Berthoud 1998; Darcel et al. 2005a). During protein digestion protein-derived free amino acids and peptides are released in the intestinal lumen and are believed to generate pre- and post-absorptive signals that contribute to the control of gastric kinetics, pancreatic secretion and food intake (Harper and Peters 1989; Jean et al. 2001; L'Heureux-Bouron et al. 2004). The process is known to be the consequence of the central signal integration of macronutrient detection, including dietary protein (Appleyard et al. 2005).

The detection of dietary protein occurs potentially in two internal sites: pre-absorptive specific macronutrient detection in the upper intestine (Jeanningros 1982; Mei 1985; Raybould et al. 2006) and post-absorptive detection of amino acid and glucose in the hepatic portal vein area (Tanaka et al. 1990; Saitou et al. 1993; Niijima and Meguid 1995; Berthoud and Neuhuber 2000). Both these detections processes have previously been reported to activate vagal afferents located either in the close vicinity of enteroendocrine cells of the intestine or within hepatic portal zone (Broberger 2005).

The vagus nerve, which connects peripheral organs such as the GI tract to the brain (Berthoud et al. 2006), has been extensively shown to be one of the major vectors conveying information relative to peripheral macronutrient detection towards central structures

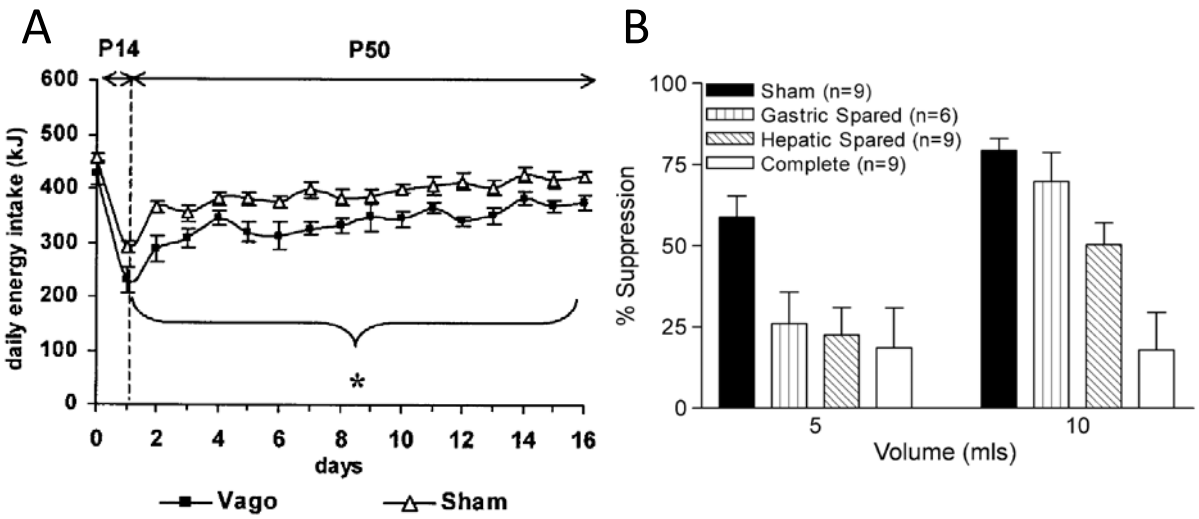


Figure 2.1 Energy intake of vagotomised and sham-operated rats.

A) Rats consumed a normal protein diet (P14) and thereafter a high protein diet (P50) for 16 days. Values are means ± SEM, n = 8–9. *Groups differed, $P < 0.005$ (L'Heureux-Bouron et al. 2003). B) Mean (± SEM) percent suppression of 30 min consumption, following distension of the stomach with the pyloric cuff closed, in rats with selective nerve cuts. Four groups were studied: rats with their vagal nerves intact (shams), rats with their gastric branches intact (gastric spared), rats with their hepatic branch intact (hepatic spared), and rats with all of their vagal nerves transacted (complete). The ability to detect gastric distension after 5-ml infusions of saline was blunted in completely vagotomised rats and in selectively vagotomised animals regardless of the branch(s) spared. A differential response to gastric distension with 10 ml of saline was found, and was correlated with the amount of residual nervous innervation provided to the stomach by vagal nerves (Powley and Phillips 2004).

controlling food intake (Yox and Ritter 1988; Yox et al. 1991). However, its implications in protein induced hypophagia has not been entirely clarified yet. Recent studies from both our group and other laboratories have shown that the vagus nerve is not indispensable for long-term protein induced food intake depression. So does total subdiaphragmatic vagotomy not suppress high protein diet induced food depression in rats (Figure 2.1) (L'Heureux-Bouron et al. 2003) nor does selective gastric branch vagotomy eliminate gastric satiation (Powley and Phillips 2004).

Nevertheless, several pieces of evidence are in favour of vagus nerve implication in protein induced reduction in short-term food intake, since protein, amino acids and peptides activate vagal endings (Tanaka et al. 1990; Niijima and Meguid 1995; Darcel et al. 2005b) and trigger activity in the NTS (Phifer and Berthoud 1998), the primary central projection of the vagal afferents. The NTS is generally been considered as being responsible for the control of ingestion. However, luminal protein-derived peptides do not directly act on vagal afferents but induce the release of gut peptides by mucosal enteroendocrine cells. For instance CCK modulates exocrine pancreatic secretion, gastric kinetics and activates the vagus nerve (Green et al. 1986; Moran et al. 1993; Kimura et al. 1997).

It has recently been suggested that hepato-portal deafferentation is sufficient to abolish the anorexigenic effect of proteins (Mithieux et al. 2005). Since protein digestion leads to an enhancement of vagal electrophysiological activity (L'Heureux-Bouron 2004) and since this activity is associated with short-term modulation of eating behaviour, capsaicin-sensitive vagal afferents located within hepato-portal vein area are potential key elements in protein induced short-term food intake control.

2.1.2 Capsaicin for selective deafferentation

Capsaicin, 8-methyl-N-vanillyl-6-nonenamide, is the active compound in chilli peppers and binds to the vanilloid receptor subtype 1 (VR1) (Caterina et al. 1997). Binding of capsaicin to the receptor leads to an influx of sodium and calcium into the neurone, which in turn, exposed at a high dose, leads to the death of the neurone (Raybould 1991; Schwartz 2000).

Vagal afferent neurones are subdivided into two categories, believed to be functionally distinct myelinated a-fibres and unmyelinated c-fibres. It has been well documented that c-fibres building the spinal afferents at the level of the portal vein are capsaicin sensitive primary sensory neurones expressing VR1 (Patterson et al. 2003; Fujita et al. 2007). Topical application of capsaicin directly on the nerve was shown to permanently degenerate the function of these capsaicin-sensitive neurones (Blackshaw et al. 2000).

The VR1 was shown to co-localise with sensory neurones expressing calcitonin gene-related peptide (CGRP), a 37-amino acid peptide which is derived from the calcitonin gene. CGRP is widely distributed in neural tissue of the brain, gut, perivascular nerves, and other tissue. CGRP expressing fibres have been shown to strongly innervate the portal vein (Barja and Mathison 1984). Portal vein immunoreactivity for CGRP is substantially reduced by systemic capsaicin pre-treatment in neonatal rats (Goehler and Sternini 1996; Fujita et al. 2007). For these reasons, CGRP is commonly accepted as a reliable marker for dorsal root sensory neurones and can be used to detect their distribution in various tissues (Berthoud 2004a) as well as to verify the success of deafferentation by capsaicin.

In contrast to surgical vagotomy it is therefore possible to specifically disrupt the transmission of ascending signals to the brain by capsaicin-sensitive vagal afferents without affecting the descending efferent branch of the vagus nerve. In this way undesired side effects being related to vagal efferents, such as destruction of gastrointestinal peristaltic or regulation of energy homeostasis, are limited.

2.1.3 Aims of the study

In this study we specifically investigated the implications of vagal afferents located within hepato-portal vein area on daily food intake of rats fed a high protein diet. Glucose and amino acids levels were demonstrated to be detected in this area. Moreover vagal electrophysiological activity is associated with short-term modulation of eating behaviour and can be enhanced during protein digestion. We therefore hypothesised that the vagal afferents located within hepato-portal vein area are key elements in protein induced changes in food intake control. In order to test this hypothesis we performed hepato-portal vein deafferentation using localised capsaicin contact (van de Wall et al. 2005a; Fujita et al. 2007) and measured food intake in free behaving animals with free access to food.

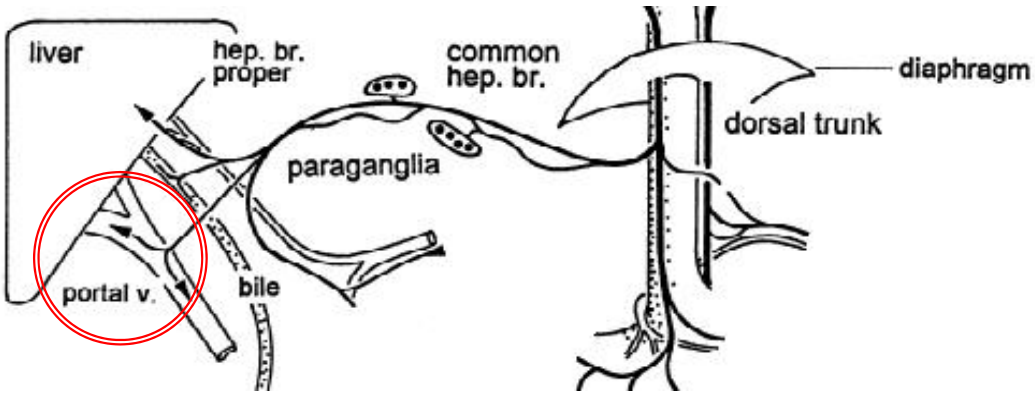


Figure 2.2 Location of capsaicin solution application on the isolated hepatic portal vein.

(Adapted from Berthoud and Neuhuber 2000)

2.2 Materials and Methods

2.2.1 Materials

All chemicals were purchased from Sigma-Aldrich (St Quentin Fallavier, France) unless otherwise stated.

2.2.2 Animals

Male Wistar rats (Harlan Laboratories, Inc., Horst, The Netherlands) at arrival weighing 100-125 g were housed in individual cylindrical cages in a temperature controlled room (22 ± 1°C) under a 12 hour light/dark cycle (lights off at 9 am) and were given ad libitum access to water and laboratory rat chow (Teklad Global 14%, N°2014, Harlan, Gannat, France) during the habituation phase. Body weight of rats was registered daily during the 11 days of dietary intervention at the end of the light phase before the feeding period.

All studies were carried out according to the guidelines of the French National Care Committee for Animal Care and the European Convention of Vertebrate Animals Used for Experimentation, under European Council Directive 86/609/EEC dated November, 1986.

2.2.3 Surgical procedures for selective hepatic vein deafferentation using capsaicin

To carry out the deafferentation on the hepatic branch of the vagus nerve, rats (Capsaicin, n=12) were anesthetised with an i.p. injection of 4 ml/kg xylazine mixed with 13 ml/kg ketamine (CEVA SANTE ANIMALE, Centravet, Plancoët, France) and 4 ml/kg morphine hydrochloride (CDM-Lavoisier, Paris, France). A 164 mM capsaicin solution was obtained by dissolving capsaicin in a vehicle made of Tween 80 (10 %), DMSO (10 %) and 0.9 % NaCl (80 %). Laparotomy was performed and with the help of cotton swaps the capsaicin solution was topically applied on the isolated hepato-portal zone of the vein (Figure 2.2) in order to destroy a specific subpopulation of vagal afferent sensory c-fibres via excitotoxicity (Patterson et al. 2003; Fujita et al. 2007). Adjacent tissues were covered with gauze and plastic wrap so as to prevent any contact with the capsaicin-soaked swabs. Cotton swabs were removed after 5 min, and the portal vein was rinsed thoroughly with normal saline. Control rats (Sham, n=12) underwent the same surgery procedure but with application of

only the vehicle solution. In each group 2 rats died during surgery, the remaining 10 rats per group could be used for the study.

2.2.4 Histological verification of selective deafferentation

2.2.4.1 Sampling of portal veins

At the end of the entire experiment, animals were anaesthetised with a lethal i.p. injection of 4 ml/kg sodium pentobarbital (Centravet, France). They were transcardially perfused with 500 ml 0.9 % saline supplemented with heparin (Pharmacie Ridoux, Paris, France) via a 26G needle placed in the left cardiac ventricle prior to 1000 ml of 4 % formaldehyde in 0.1 M phosphate buffer. The abdominal cavity was opened, a sample of the portal vein at the level of the treatment site was removed prior to fixing the tissue in 4 % formaldehyde during 24 hours. Subsequently the portal vein sample was placed for 48 hours in a 25 % sucrose solution in order to exude and stored at 20 °C.

2.2.4.2 Immunohistochemical analysis of CGRP reactivity

Transversally opened whole mount sections of the portal veins were brought to room temperature and passed through three 5-min rinses of phosphate-buffered saline (PBS; 0.1 M, pH 7.4). Sections were then placed for 2 h at room temperature in a blocking solution containing 2 % normal donkey serum (NDS), 0.1 % Triton, diluted in PBS, followed by three more rinses with PBS. Sections were then incubated for 48 h at 4 °C with a mouse monoclonal anti-CGRP antibody (kindly provided by MJ Prud'homme, NOPA, group RCC, INRA Jouy en Josas, France) diluted in PBS (with 2 % NDS, 0.25 % Triton) to a final concentration of 1:2000. Sections were subsequently rinsed in PBS (3 times) before being reacted for additional 24 h with a donkey-anti mouse IgG (HL) conjugated to Texas-Red (Jackson ImmunoResearch Europe Ltd., Suffolk, UK) diluted to 1:200. Following a final series of washes with PBS, sections were mounted on object slides, and cover slipped with a mounting medium (Vectashield) for fluorescence microscopy.

The immunoreactive sections were then digitally photographed with a digital camera fitted on a bright-field microscope (Zeiss AxioImager Z1, Le Pecq, France). Deafferentation was considered successful when no CGRP immunoreactivity could be detected.

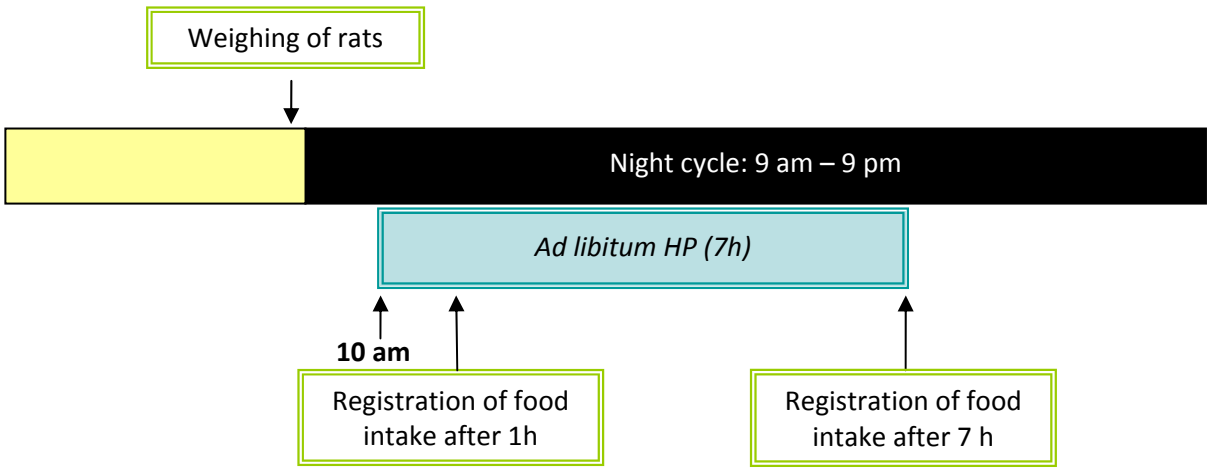


Figure 2.3 Daily feeding pattern of rats fed a high protein diet (HP).

Animals were weight at the end of the light phase. HP diet was presented ad libitum at 10 am for 7 hours. Food intake was registered 1h and 7h after presentation. Food was removed after 7h.

2.2.5 Diets and feeding procedures

The two diets used were modified versions of the AIN-93M diet; normal protein diet contained 14 % of energy from protein (NP) and high protein diet contained 55 % of energy from protein (HP) (For exact composition see Annex 6.1). All diets were moistened to minimise spillage in a powder to water ratio of 1:2 for HP and 1:1 for NP diet.

After 12 days post-surgery recovering phase, animals were fed the NP diet during 10 days in order to get habituate to the feeding cycle and form of diet presentation. Thereafter rats received the HP diet during 11 days. The last day of the NP diet was defined as day 0 and the first day of the HP diet day 1. Food was presented ad libitum one hour after beginning of the dark phase. Short-term food intake was determined by the difference in food cup weight 1 hour after food cup presentation and long-term food intake 7 hours after food cup presentation (Figure 2.3). Measurements were corrected for spillage, the amount of water added and evaporation. The food cup was removed after the second measurement.

2.2.6 Statistical analysis

Body weight and food intake data are expressed as means ± standard error of mean (SEM). Body weight data were evaluated by student t-test comparing initial and final body weight. Food intake data were analysed using analysis of variance for repeated measures using R (version 2.8.1) (Team 2009) with day as the repeated measure variable and diet as main effect. For all experiments differences were considered significant at $p \leq 0.05$.

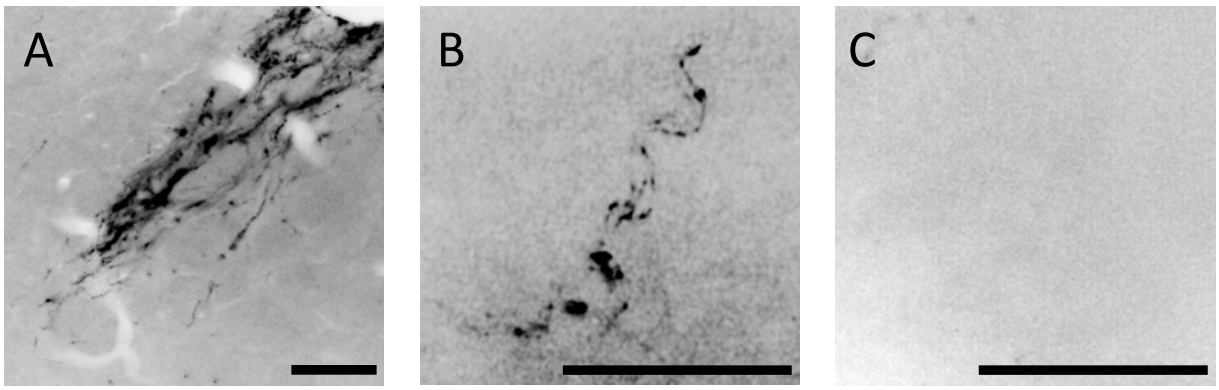


Figure 2.4 Verification of deafferentation of the hepatic portal vein area.

Immunohistochemical labelling of CGRP-reactive fibres. A) Nucleus of the solitary tract of a sham rat (scale bar 100 µm). B) Hepatic portal vein area of a sham rat (scale bar 10 µm). C) Hepatic portal vein area of a capsaicin treated rat (scale bar 10 µm).

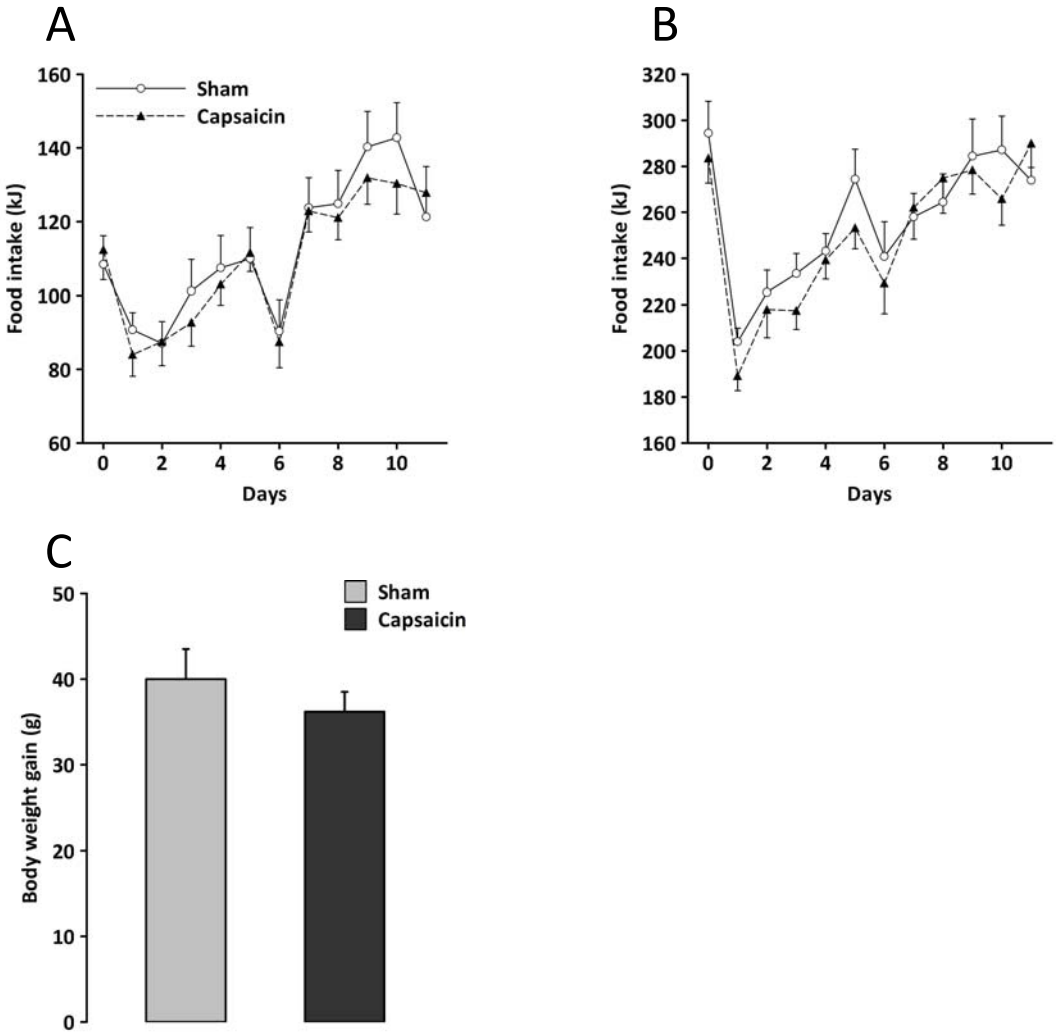


Figure 2.5 Effect of topical capsaicin application on food intake and body weight gain.

A) Mean ± SEM food intake over 12 days of intervention measured 1 hour after food cup presentation (short-term food intake). B) Mean ± SEM food intake over 12 days of intervention measured 7 hour after food cup presentation (daily food intake). C) Mean ± SEM body weight gain during 12 days of dietary intervention.

2.3 Results

2.3.1 Hepatic portal vein deafferentation

Immunohistochemical analysis revealed a network of CGRP-reactive fibres in portal veins as well as in the tractus solitaris taken from control animals. However, 33 days following the topical application of a capsaicin solution, CGRP reactivity was completely abolished (Figure 2.4) on capsaicin treated portal veins, thus confirming that the deafferentation was successful. In each group, 10 rats survived the surgical procedure and could be used for the study.

2.3.2 Daily energy intake and body weight

Rats recovered from capsaicin or vehicle treatment and restarted to gain weight quickly. The last day of the pre-feeding period (until day 0), the groups did not differ in daily intake of the NP diet and body weight (day 0: Sham: 240g ± 6g and Capsaicin: 235g ± 4g, p=0.2146). Transition from the NP to the HP diet induced an immediate depression in intake during day 1 and a gradual adaptation to the diet over the subsequent days. Moreover, as early as day 1 and during the 10 subsequent days of consuming the HP diet, capsaicin treated rats ate as much as sham rats after both 1h of food cup presentation (p=0.6890) or 7 hours of food cups presentations (p=0.5150) (Figure 2.5 A, B). At the end of the experiment the body weights were not different between the sham group and the capsaicin treated rats (280g ± 8g and 271g ± 6g, respectively, p=0.1766) as well as body weight gain during the 12 days of dietary intervention did not differ significantly (p=0.1777) (Figure 2.5 C).

2.4 Discussion

2.4.1 Deafferentation of the portal vein by a capsaicin solution

Rats treated with capsaicin solution expressed no CGRP at the level of the portal vein. Capsaicin binds to the VR1 which was demonstrated to be present on vagal afferent neurones innervating the GI tract (Patterson et al. 2003). Applying capsaicin on these unmyelinated c-fibres of the vagus nerve at the level of the hepatic portal vein leads to over activation of the VR1 which in turn results in a massive influx of calcium and sodium. This influx causes death of the neurones (Raybould 1991; Schwartz and Moran 1998). Efferent fibres, not expressing the VR1 are on the other hand believed to be not affected by the capsaicin treatment. After surgical total vagotomy where the entire vagal branch is disconnected, side effects such as destruction of gastrointestinal peristaltic or regulation of energy homeostasis have been observed. In comparison, the chemical method used here specifically disrupting only ascending signals, allows to avoid parts of these side effects.

2.4.2 The effect of hepatic portal vein deafferentation on food intake suppression induced by a high protein diet

The present study was undertaken in order to determine the possible role for hepatic afferent vagal mediation in the detection and satiating effect of protein in rats. Neither at the beginning of the dietary intervention period, nor during the entire period of feeding the high protein diet body weight of the animals differed. Suppression of the hepatic vagal afferent pathways did not reverse food intake depression induced by a high protein diet and also no difference in body weight gain during the dietary intervention was observed. The results therefore suggested that multiple and redundant mechanisms, either dependent or independent of vagal mediation, are likely to be involved in the signalling and response to ingested protein.

In agreement with previous studies, obtained from healthy rats, animals in both the capsaicin treated and the sham operated group decreased their food intake on the first day when switched from a normal protein to a high protein diet and compensated for this decrease in the following days without reaching the original level (Jean et al. 2001; Bensaid et al. 2003; L'Heureux-Bouron et al. 2003).

As for the high protein diet induced hypophagia compared to a normal protein diet, the vagus nerve, and more precisely sensory c-fibres of hepatic afferents, has been proposed to act as a key component. Indeed, in other studies rats with denervated portal veins showed an abolishing of food intake depression on diet rich in protein (Mithieux et al. 2005). The present results may be interpreted as inferring this hypothesis since c-fibre excitotoxicity-induced suppression by capsaicin did not alter food intake under HP dietary conditions. Indeed no significant changes in 1 hour or total food intake were recorded in capsaicin treated rats versus control rats. However, our group already demonstrated the absence of effects of subdiaphragmatic vagotomy on high protein diet consumption (L'Heureux-Bouron et al. 2003), what might lead to the assumption that the vagus nerve is not involved in HP diet induced hypophagia. On the other hand, there are pieces of evidence that protein hydrolysate as well as total milk protein infused duodenally increased vagal afferent discharge in rats (Darcel et al. 2005b; Tome et al. 2009). Additionally to its effect on vagal afferent activity, amino acids as well as protein were shown to stimulate the NTS which integrates vagal signals (Phifer and Berthoud 1998; Emond et al. 2001; Faipoux et al. 2008). On top of this, anorexigenic signals mediated by the vagus nerve are suggested to be predominantly involved in short-term regulation of feeding, such as ingestion rate and meal termination, determining meal size but have to be relayed by long-term effectors in order to prevent new meals to occur earlier (Moran et al. 2001). For these various reasons it can be assumed that the vagus nerve is sufficient for protein signalling to central brain structures, but it is unlikely that the vagus nerve alone can generate hypophagia; thus vagal afferent fibres are probably not critical for high protein induced food intake depression.

It is therefore highly probable that compensatory, vagal afferent independent mechanisms are involved in the maintenance of high protein diet induced food intake suppression. Such compensatory mechanisms have been described either in vagotomy (Broberger et al. 2001) or capsaicin induced c-fibre suppression (van de Wall et al. 2005b). The action of capsaicin is limited to unmyelinated sensory c-fibres of the vagus nerve (Raybould 1991). High protein diet induced satiety signals could therefore also be transmitted by hepatic afferents that are not sensitive to capsaicin as it was already shown for other satiety signals (Lutz et al. 1998; van de Wall et al. 2005a). Those mechanisms may explain why, in contrast with our predictions, we did not record significant changes in food

consumption even during the first days of the dietary intervention. Peripheral signals reach the brain not only via the vagus nerve, but also via humoral, gut peptide dependent pathways. Numerous publications have demonstrated the presence of intestinal chemoreceptors which are able to detect luminal nutrients such as glucose, fatty acids and amino acids (Schwartz and Moran 1998; Bezencon et al. 2007; Choi et al. 2007a) on enterocytes and vagal afferent endings as well as the presence of vagal glucose or amino acid sensors in the hepato-portal system (Tanaka et al. 1990; Saitou et al. 1993; Niijima and Meguid 1995; Torii and Niijima 2001). One example for these receptors is PEPT1, a G protein-coupled receptor found on enterocytes (Matsumura et al. 2005). Mediated by this kind of receptor, intestinal dietary proteins, or peptides from their digestion, are known to be able to induce a local release of CCK by enteroendocrine cells (Cordier-Bussat et al. 1997; Nishi et al. 2001; Foltz et al. 2008). According to several studies the CCK receptor is found not only on the vagus nerve, but on various locations throughout the GI tract and the brain (Moran et al. 1990; Wank et al. 1992; Moran and Kinzig 2004). The CCK pathway is consequently one possible compensatory mechanism in animals being deprieved of vagal afferent c-fibres. An increase in plasma protein and circulating amino acids acting as signal molecules can only be found in animals that are fed a high protein meal, but not after adaptation to a high protein diet (Morens et al. 2000; Morens et al. 2001) which explains the sharp decrease of food intake on the first day and the gradual increase on the subsequent days.

2.5 Conclusion and perspectives

To conclude, this study shows that vagal capsaicin sensitive afferent fibres are not critical for high protein-induced hypophagia to occur, but that different and redundant mechanisms are involved in the satiating effect of protein and amino acids. Subsequently a difference in body weight gain cannot be expected.

One can also question the role of the relief of the afferent fibres of the vagus nerve in case of ingestion of a high protein diet. Missing vagal input, regulation of other digestive processes such as enzyme release or gastrointestinal peristaltic might be interfered. This might in turn result in malabsorption of ingested nutrients and consequently lead to a similar depression in body weight gain as found for sham rats decreasing their food intake due to the high protein diet.

At last, the vagus nerve plays a role in directly or indirectly recording the presence of protein in the intestine and in controlling food intake, for example through CCK1-receptors, which are able to substitute a defect in vagal mediation. Further studies may help to understand the role of protein in meal size control and its implication in food intake control and body weigh regulation.

2.6 Article: Protein, amino acids, vagus nerve signaling, and the brain. D Tomé, J Schwarz, N Darcel, G Fromentin

Am J Clin Nutr. 2009 Sep; 90(3): 838S-843S. Epub 2009 Jul 29. PMID 19640948

The American Journal of Clinical Nutrition

Protein, amino acids, vagus nerve signaling, and the brain[1–4]

Daniel Tomé, Jessica Schwarz, Nicolas Darcel, and Gilles Fromentin

ABSTRACT

Dietary protein and amino acids, including glutamate, generate signals involved in the control of gastric and intestinal motility, pancreatic secretion, and food intake. They include postprandial meal-induced visceral and metabolic signals and associated nutrients (eg, amino acids and glucose), gut neuropeptides, and hormonal signals. Protein reduces gastric motility and stimulates pancreatic secretions. Protein and amino acids are also more potent than carbohydrate and fat in inducing short-term satiety in animals and humans. High-protein diets lead to activation of the noradrenergic-adrenergic neuronal pathway in the brainstem nucleus of the solitary tract and in melanocortin neurons of the hypothalamic arcuate nucleus. Moreover, some evidence indicates that circulating concentrations of certain amino acids could influence food intake. Leucine modulates the activity of energy and nutrient sensor pathways controlled by AMP-activated protein kinase and mammalian target of rapamycin in the hypothalamus. At the brain level, 2 afferent pathways are involved in protein and amino acid monitoring: the indirect neural (mainly vagus-mediated) and the direct humoral pathways. The neural pathways transfer preabsorptive and visceral information through the vagus nerve that innervates part of the orosensory zone (stomach, duodenum, and liver). Localized in the brainstem, the nucleus of the solitary tract is the main projection site of the vagus nerve and integrates sensory information of oropharyngeal, intestinal, and visceral origins. Ingestion of protein also activates satiety pathways in the arcuate nucleus, which is characterized by an up-regulation of the melanocortin pathway (α-melanocyte-stimulating, hormone-containing neurons) and a down-regulation of the neuropeptide Y pathway. *Am J Clin Nutr* 2009;90(suppl):838S–43S.

INTRODUCTION

Dietary protein and amino acids, including glutamate, are believed to generate signals involved in the control of gastric and intestinal motility, pancreatic secretion, and food intake. According to current understanding, multiple and redundant signals associated with nutrients, gut neuropeptides, and hormones activate specific areas in the brain either indirectly through vagus-mediated pathways or directly after their release into the peripheral blood (**Figure 1**). However, the precise mechanisms involved in these signaling processes for protein and amino acids from gut to brain are not completely understood.

PROTEIN AND AMINO ACIDS GENERATE SIGNALS ACTING ON GUT PHYSIOLOGY AND FOOD INTAKE

Protein is known to reduce gastric motility and to stimulate pancreatic secretion by a mechanism mediated through cholecystokinin. This leads to an increase in the proportion of pancreatic enzymes as proteases, presumably to increase protein digestion. It has been shown, for example, that the [106–169] fragment of milk κ-casein dose dependently stimulates pancreatic secretion by a cholecystokinin-dependent mechanism (1). It is also established that protein and amino acids are more potent than carbohydrate and fat in inducing short-term satiety in animals and humans. The quantity of protein or amino acids in the meal or diet is determinant. Increasing dietary protein usually reduces energy intake in animals and humans. This effect is not due to a conditioned taste aversion.

There is a control of protein ingestion and, when given the opportunity, animals usually select a relatively constant amount of protein and percentage of protein energy in their diet. Despite the low palatability of a high-protein diet, adult rats allowed to self-select macronutrients increase their protein intake to 35–50% energy, reduce total energy intake, and do not show aversive behavior. Interestingly, it seems that the spontaneous amount of protein ingestion does not correspond to the minimal percentage of protein energy (10–12% in the adult rat) required for nitrogen balance, but is usually much higher, and varies with a multiplicity of factors, including age, physiologic state, and the type of food. This suggests that different, complex, and redundant mechanisms are involved in the control of protein ingestion (2–4).

Over the long term, the ingestion of a high-protein diet most frequently promotes satiety, facilitates weight loss, and improves body composition. A high-protein diet is usually associated with a decrease in food intake in rats and human (5–8). When rats previously adapted to a diet providing normal amounts of protein are offered a high-protein diet, they immediately reduce total food intake, and then progressively increase intake on succeeding days, although notably not to the amount before presentation of the high-protein diet (9). A decrease in food intake has also been observed in rhesus monkeys fed a high-protein diet (10). This initial decrease in the intake of the high-protein diet has been suggested to arise from the palatability, the time required for metabolic adaptation, or the satiating effect of the macronutrient.

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[2] Presented at the "100th Anniversary Symposium of Umami Discovery: The Roles of Glutamate in Taste, Gastrointestinal Function, Metabolism, and Physiology," held in Tokyo, Japan, September 10–13, 2008.

[3] Supported by AgroParisTech, INRA, and the EEC Marie Curie Research Program NuSisCO.

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First published online July 29, 2009; doi: 10.3945/ajcn.2009.27462W.

Am J Clin Nutr 2009;90(suppl):838S–43S. Printed in USA. © 2009 American Society for Nutrition

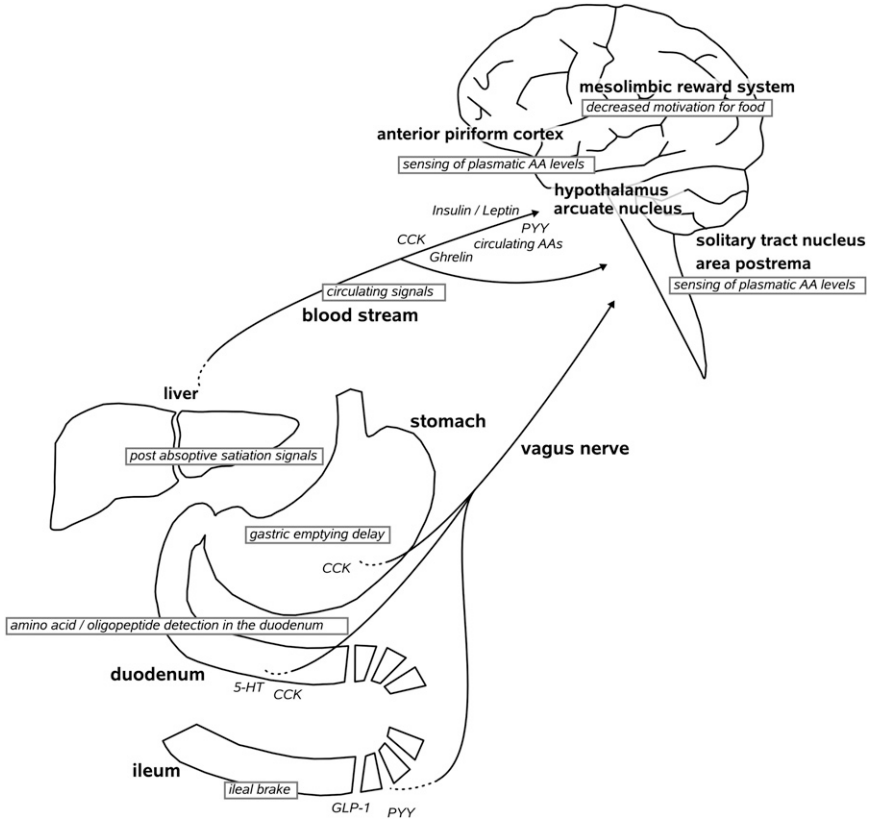


FIGURE 1. Mechanisms responsible for the protein-induced suppression of food intake. Dietary protein is detected in the gastrointestinal tract. Signals are generated in the stomach, duodenum, and ileum via the release of gut hormones that activate peripheral nerves (particularly the vagus nerve). These gut hormones are also carried by the bloodstream and act directly on brain centers such as the area postrema and hypothalamus to suppress food intake. Postabsorptive anorectic signals are also generated by the liver. Finally, protein detection may modulate the reward system and reduce the motivation for food. PYY, peptide YY; CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; 5-HT, 5-hydroxytryptamine; AA, amino acid.

Despite previous hypotheses, it is unlikely that a high-protein diet induces a conditioned taste aversion (ie, makes the animal sick) (11).

It is currently established that under most conditions protein is more satiating than the isoenergetic ingestion of carbohydrate or fat in animals and humans (12–14) and that a high-protein diet most frequently improves weight loss and body composition (5, 15). A high-protein preload was shown to increase the delay between the preload and the test meal compared with a high-fat or a high-carbohydrate preload, respectively (13, 16). A high-protein preload was also shown to lower the ad libitum intake of a test meal in comparison with a high-carbohydrate preload and was independent of the interval between the preload and the test meal (30 or 120 min) (14). In contrast, a high-protein breakfast compared with a high-carbohydrate breakfast did not modify the intake of a test meal served 3 h later (17).

PROTEIN AND AMINO ACIDS GENERATE PRE- AND POSTABSORPTIVE SIGNALS

Proteins and amino acids are very likely to generate preabsorptive signals while still in the digestive tract. Chemoreceptors located in the small intestine detect luminal nutrients (carbohydrates, peptides, amino acids, fatty acids, triacylglycerols) and trigger the release of gut hormones from mucosal enteroendocrine cells in response. These hormones act on gut vagal receptors or are released into the blood and reach the central nervous system. Absorbed amino acids can also induce metabolic signal produced in the periphery or directly in some specific brain area.

Dietary proteins and amino acids induce the release of the anorexigenic gut hormones cholecystokinin, glucagon-like peptide 1 (GLP-1), and peptide YY (PYY); the involvement of ghrelin, however, remains uncertain (18, 19). Cholecystokinin is released in response to the presence of protein and fat in the duodenum and has a well-established peripheral role in digestion, causing gallbladder contraction and the secretion of pancreatic enzymes. It is also the first gut hormone shown to influence food intake. In the periphery, cholecystokinin directly modulates exocrine pancreatic secretion and possibly gastric motility. In addition, the proximity of vagal afferent axons and cholecystokinin-secreting cells in the gut supports the idea that cholecystokinin released from enteroendocrine cells acts on local vagal sensory fibers. By that route, cholecystokinin could act on gastric motility through a vago-vagal loop and also stimulate satiety through low-affinity vagal cholecystokinin receptors that signal the brain. Indeed, cholecystokinin is known to inhibit food intake, mediating its anorexigenic effect mostly by cholecystokinin type 1 receptors on the vagus nerve, which in turn project into the brainstem (20–22). GLP-1 is also an important inhibitor of food intake that seems to be involved in protein and amino acid signaling (18, 23, 24). The vagus nerve is important in mediating the anorectic effects of GLP-1, because vagotomy abolishes its effect on food intake.

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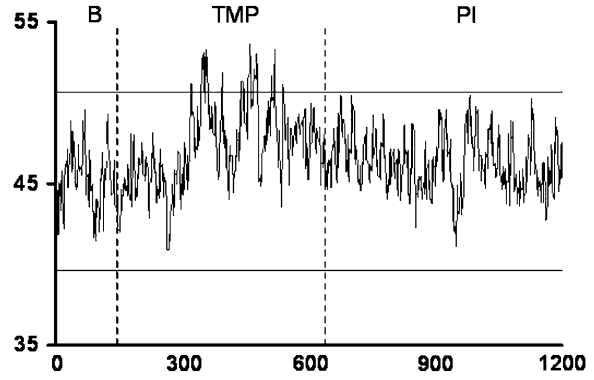


FIGURE 2. Mean activity (spikes/s) of 2 vagal afferent multiunits before (B; basal period), during (TMP; total milk protein), and after (PI; postinfusion period) the duodenal infusion of total milk protein (powder in distilled water, 0.15 kcal/mL, 300 mOsm/L). Horizontal lines represent the superior and inferior limits of the CI for significant changes in unit activity for $P < 0.001$. Vertical dashed lines correspond to the protein infusion period. Surgery and neurophysiologic recordings of gut vagal afferent nerve activity were performed with the use of conventional methods (36). Adapted from reference 35.

Furthermore, PYY release is stimulated by dietary protein and amino acids in rodents, but it probably acts directly on the hypothalamic arcuate nucleus (ARC) to induce satiety (25).

Metabolic signals are also suspected to be involved in protein and amino acid signaling. It has been suggested that elevated concentrations of blood or plasma amino acids are directly detected by specific areas of the hypothalamus. Intracerebroventricular administration of leucine or an increase in dietary leucine reduces food intake and body weight and improves glucose and cholesterol metabolism in rats and mice (26, 27). This effect appears to be specific to leucine, because leucine alone has the same effect on food intake as a mixture of amino acids (28). Other metabolic signals, including an increase in energy expenditure and the production of glucose through gluconeogenesis, have been also hypothesized to mediate the effects of dietary protein and amino acids on food intake. It also appears that, in humans, protein stimulates diet-induced thermogenesis to a greater extent than other macronutrients (29). This is due for a part to the utilization of 3≈P bonds to incorporate each amino acid into protein. The de novo synthesis of glucose in the liver from gluconeogenic precursors, including amino acids, is stimulated by a high-protein diet in the fed state (30). This process could be involved in the satiating effect of protein through a modulation of glucose homeostasis and glucose signaling to the brain. The main gluconeogenic organ is the liver, and liver gluconeogenesis is probably the main site of glucose production from amino acids, whereas intestinal gluconeogenesis, previously hypothesized as an alternative mechanism, remains to be confirmed (30–34).

VAGUS-MEDIATED ACTIVATION OF NEURONS IN THE NUCLEUS OF THE SOLITARY TRACT BY PROTEIN AND AMINO ACIDS

The vagus nerve responds to luminal and hepato-portal signals, including nutrient and non-nutrient substances. Protein, peptides and amino acids including glutamate are believed to elicit a visceral vagus-mediated activation of neurons in the nucleus of the solitary tract (NTS). Protein and amino acids infused into the duodenum activate vagal afferents in the rat, mainly through

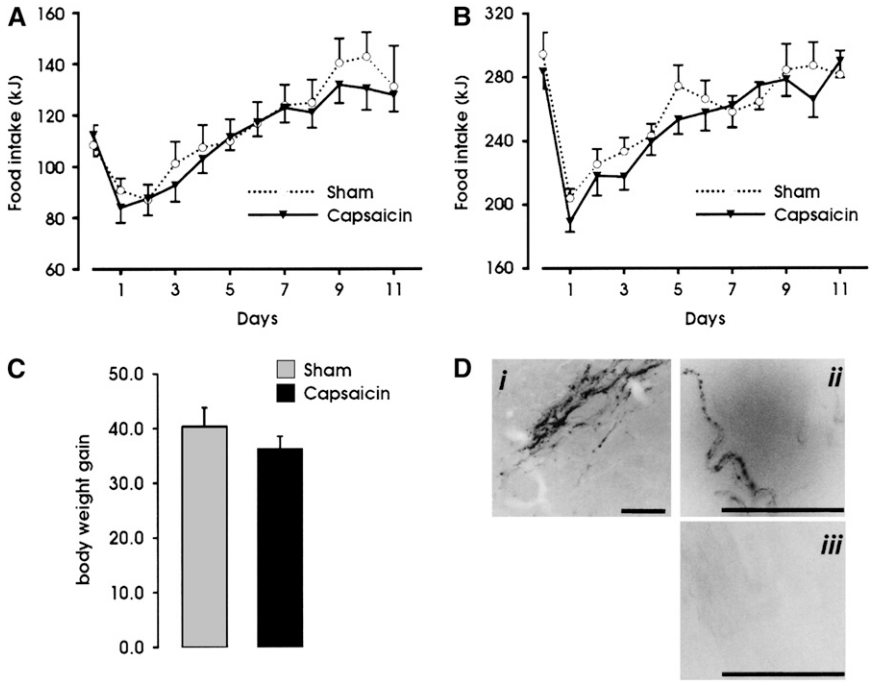


FIGURE 3. Hepatic portal vein vagal deafferentation does not reverse food intake depression induced by protein diet in the rat. Topical application of a 1% capsaicin solution on the isolated hepatoportal vein (n = 10) was performed. Sham rats received application of a saline solution (n = 10). A: Mean (±SEM) food intake measured 1 h after food cup presentation in rats. B: Mean (±SEM) food intake measured 7 h after food cup presentation in rats. C: Mean (±SEM) body weight gain (in g). D: Verification of deafferentation of the hepatic portal vein area: immunohistological detection of CGRP (calcitonin gene-related peptide)-positive fibers in (i) a sham rat within the solitary tract (scale bar, 100 μm), (ii) hepatic portal vein area of a sham rat (scale bar, 10 μm), and (iii) hepatic portal vein area of a capsaicin-treated rat (scale bar, 10 μm).

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a cholecystokinin-dependent mechanism (**Figure 2**) (35). First, protein and amino acids in the intestinal lumen induce the secretion of gut neuropeptides (cholecystokinin, GLP-1), which act on vagus nerve receptors. Nutrients are also sensed by hepatoportal sensors (amino acids, glucose).

Amino acid sensors in the duodeno-intestinal and hepatoportal regions have been shown to increase or decrease the activity of hepatic vagal afferent fibers, depending on the amino acid (37–40). Signals are passed along hepatic vagal afferent fibers to food intake-regulating areas of the brain. Individual amino acids appear either to excite or inhibit vagal hepatic afferents (41–43). Excitatory amino acids include L-alanine, L-arginine, L-leucine, L-lysine, L-serine, L-tryptophan, L-valine, and monosodium glutamate. Inhibitory amino acids include L-cysteine, L-glycine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, and L-threonine. Glutamate sensors are also found in the oral cavity, the intestinal wall, and the gastric wall. The infusion of monosodium glutamate into the stomach, duodenum, and portal vein increases afferent activity in the vagal gastric, celiac, and hepatic nerves, suggesting the existence of glutamate sensors in the gastric wall, intestinal wall, and hepatoportal region (44). Their activation induces reflex activation of efferent discharges in the vagal gastric and pancreatic nerve.

The involvement of vagal afferent pathways in protein sensing and signaling to the brain is supported by the finding that intraduodenal protein activates vagal afferent fibers and that high-protein feeding induces c-Fos expression in neurons within the NTS (20–49). The decrease in food intake observed after a high-protein load (compared with a normal-protein load) results from the activation of the noradrenergic neurons in the NTS which are related to cholecystokinin-induced anorexia (46). In contrast, neurons expressing GLP-1 in the NTS are not activated, which is consistent with the fact that protein-induced satiety is not associated with aversive behavior and conditioned taste aversion (11, 46).

BLOOD-MEDIATED ACTIVATION OF NEURONS IN THE ARC BY PROTEIN AND AMINO ACIDS

However, subdiaphragmatic vagotomy (47) and hepatic portal vein vagal deafferentation (**Figure 3**) do not block the reduction in food intake in rats induced by the ingestion of a high-protein diet. Hence, nonvagal signals are also involved in the behavioral responses to dietary amino acids and protein. Information from peripheral tissues and organs is centralized in the hypothalamus; the hypothalamus is a key brain region in food intake regulation and energy homeostasis (48, 49). Protein ingestion activates ARC satiety pathways, which include an up-regulation of melanocortin [α-melanocyte-stimulating hormone (α-MSH) containing] neurons and a down-regulation of neuropeptide Y (NPY) neurons. When a high-protein meal is ingested, eg, the number of ARC neurons expressing both c-Fos (a marker of neuronal activation) and α-MSH increases (46). At the same time, the number of neurons expressing c-Fos that lack α-MSH declines (46). Some of the latter ARC neurons could be NPY neurons.

The signals that modulate these pathways remain unclear. Amino acid-sensitive neurons have been detected in the lateral hypothalamus. A critical role for PYY neurons in protein-mediated satiety and body-weight regulation has also been proposed (25). This effect is mediated through the activity of the AMP-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR) (50). High-protein diets influence AMPK and mTOR in neurons in the ARC and the hypothalamic paraventricular nucleus. AMPK and mTOR may have overlapping and reciprocal functions (26, 51). The activation of mTOR and the suppression of AMPK phosphorylation seem to modulate hypothalamic neuropeptides, with a decrease in orexigenic neuropeptides such as NPY and agouti-related peptide, and an increase in the expression of the anorexigenic peptide α-MSH (28, 50).

CONCLUSIONS

Complex pathways are involved in protein and amino acid signaling to the brain. The signals to the brain associated with the ingestion of amino acids and other energy-providing nutrients originate from the visceral and metabolic processes and involve both indirect (mainly vagus mediated) and direct (plasma concentrations of nutrients and hormones) pathways. Gut hormones (cholecystokinin, GLP-1, and PYY) presently are major signaling candidates, acting either indirectly by activation of the vagus pathway (cholecystokinin, GLP-1) or directly at the level of the hypothalamus (PYY). Amino acids are also probably directly involved in signaling the vagus pathway in the hepatoportal area and the ARC in the hypothalamus. Other signals related to amino acid metabolism include gluconeogenesis and a possible increase in energy expenditure. (Other articles in this supplement to the Journal include references 52–80.)

The authors' responsibilities were as follows—DT: wrote the original draft of the manuscript, and participated in the protocols reported in Figures 2 and 3; ND and JS: contributed to the preparation and editing of the manuscript, and performed the experiments summarized in Figure 2; and GF: contributed to the preparation and editing of the manuscript, and participated in the protocols reported in Figures 2 and 3. The presenting author's (DT) travel expenses associated with participation in the symposium and an honorarium were paid by the conference sponsor, the International Glutamate Technical Committee, a nongovernmental organization funded by industrial producers and users of glutamate in food. None of the authors had a conflict of interest.

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3 Central cartography of macronutrients' internal sensibility

3.1 Integration of signals from intestinal nutrient sensing in the dorsal vagal complex

During ingestion nutrients are detected on various levels of the GI tract (Schwartz et al. 2000). This detection is converted into signals which are transmitted to the dorsal vagal complex in the caudal brainstem either via vagal afferents or via transportation of signalling molecules in the blood stream. The main recipient for vagal-transmitted signals is hereby the NTS while the AP, deprived in BBB, is mainly activated by blood-born signals (Tome 2004).

One of the first signals occurring when the food bolus reaches the stomach is gastric distension. It had been shown that activation of mechanoreceptors even during sham feeding increases NTS activity significantly and independently from nutrients (Emond and Weingarten 1995; Mathis et al. 1998; Phifer and Berthoud 1998; Hayes and Covasa 2006). Prior to their uptake, nutrients are then sensed in the upper intestinal tract by specific receptors and transporters. The activation of those leads on the one hand to activation of sensory vagal afferents and on the other hand to a release of gut peptides which in turn can either reach the brain via the blood stream or also bind to their receptors which can be found on the vagus nerve (Dockray 2003; Tome 2004, 2007).

Although the sensing and signal generation is macronutrient specific, it is still not known if this specificity also occurs at the level of signal integration in the dorsal vagal complex. Concerning the NTS, a topic organisation of digestive projections has been suggested following retrograde labelling studies, raising considerations of a physiological meaning of this subdivision, such as the site of detection of the ingested macronutrients (Figure 1.7) (Norgren and Smith 1988; Altschuler et al. 1989; Méi 1998; Zhang and Ashwell 2001). For other sensory processes, functional mapping was already demonstrated, for instance for audition (Malmierca et al. 2008; Luo et al. 2009), olfaction (Johnson and Leon 2007) and tactual sensing (Petersen 2003).

We were therefore interested in the specificity of central encoding of nutrient signalling in general and protein signalling in particular. In order to investigate if NTS activity

is specific, we compared 3-dimensional activity maps of the NTS of mice gavaged with either protein or carbohydrate and were able to demonstrate that indeed neurone populations activated by these nutrients do not occupy exactly the same positions within the NTS.

3.2 Article: Three-dimensional Macronutrient-associated Fos Expression Patterns in the Mouse Brainstem

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PLoS One. 2010 Feb 1;5(2):e8974.PMID: 20126542

OPEN ACCESS Freely available online

PLoS one

Three-Dimensional Macronutrient-Associated Fos Expression Patterns in the Mouse Brainstem

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Abstract

Background: The caudal brainstem plays an important role in short-term satiation and in the control of meal termination. Meal-related stimuli sensed by the gastrointestinal (GI) tract are transmitted to the area postrema (AP) via the bloodstream, or to the nucleus tractus solitarii (NTS) via the vagus nerve. Little is known about the encoding of macronutrient-specific signals in the caudal brainstem. We hypothesized that sucrose and casein peptone activate spatially distinct sub-populations of NTS neurons and thus characterized the latter using statistical three-dimensional modeling.

Methodology/Principal Findings: Using immunolabeling of the proto-oncogene Fos as a marker of neuronal activity, in combination with a statistical three-dimensional modeling approach, we have shown that NTS neurons activated by sucrose or peptone gavage occupy distinct, although partially overlapping, positions. Specifically, when compared to their homologues in peptone-treated mice, three-dimensional models calculated from neuronal density maps following sucrose gavage showed that Fos-positive neurons occupy a more lateral position at the rostral end of the NTS, and a more dorsal position at the caudal end.

Conclusion/Significance: To our knowledge, this is the first time that subpopulations of NTS neurons have be distinguished according to the spatial organization of their functional response. Such neuronal activity patterns may be of particular relevance to understanding the mechanisms that support the central encoding of signals related to the presence of macronutrients in the GI tract during digestion. Finally, this finding also illustrates the usefulness of statistical three-dimensional modeling to functional neuroanatomical studies.

Citation: Schwarz J, Burguet J, Rampin O, Fromentin G, Andrey P, et al. (2010) Three-Dimensional Macronutrient-Associated Fos Expression Patterns in the Mouse Brainstem. PLoS ONE 5(2): e8974. doi:10.1371/journal.pone.0008974

Editor: Carlo Polidori, University of Camerino, Italy

Received November 6, 2009; **Accepted** January 6, 2010; **Published** February 1, 2010

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Funding: The research that led to these results received funding from the European Commission's Sixth Framework Programme (FP6) under NuSISCO Contract No. MEST-CT-20058-020494. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The central nervous system (CNS) controls food intake integrating a broad variety of both environmental and internal inputs [1]. The caudal brainstem, which is the main recipient of information from the GI tract, is a key region in this control. Particularly during digestion, a complex signaling cascade informs brainstem centers of the nature and quantity of macronutrients entering the organism [1,2]. Both non-specific mechanical and macronutrient-specific meal-related stimuli are sensed in the GI tract, where they induce the release of gut peptides. Gut peptides and absorbed nutrients act either on the sensory vagal afferents that innervate the gut and the hepato-portal area, or directly in the brain through the bloodstream [3–8]. Within the central nervous system, vagal afferents synapse in the *nucleus tractus solitarii* (NTS) located in the caudal brainstem. In addition, the NTS receives input from the area postrema (AP), which displays elevated permeability of the blood-brain barrier and is sensitive to the blood-borne signals linked to ingested macronutrients [9].

Nutrient-specific behavioral responses have been observed, suggesting that the brain receives and integrates nutrient-specific signals [10]. Very little is known about the central encoding modalities of macronutrient-specific detection during digestion [11,12]. This information would be of considerable value to a clearer understanding of the physiological mechanisms that control food intake and its dysfunctions. All macronutrients exert different impacts on food intake control, especially regarding the degree of satiety. There is evidence to suggest that different macronutrients generate specific signals during their absorption in the gastrointestinal tract. For instance, cholecystokinin (CCK) is released in response to the presence of dietary fat and protein in the gut lumen, while serotonin (5-HT) levels rise in response to the presence of carbohydrates and lipids [7,10,13–15]. Furthermore, by quantifying the proto-oncogene Fos as a marker of neuronal activity, it has been shown that different macronutrients are associated with different levels of activation in the NTS and AP [12]. This effect is positively related to their potency to inhibit food intake [11]. According to Phifer et al., activation of the caudal

brainstem in correlation with macronutrient detection is dependent on the type of nutrient rather than its caloric value [11].

Very few studies have investigated the central encoding modalities of macronutrient-associated signals, and more precisely whether patterns of neuronal activity within the brainstem are specific to macronutrients. We therefore assessed the effects of different macronutrients on Fos expression and investigated whether specific activation patterns could be detected. Following the administration of sucrose or casein peptone, we quantified Fos expression throughout the rostrocaudal extent of the NTS. We compared: a) the total number of Fos-expressing neurons in the brainstem, AP and NTS, b) their distribution along the rostrocaudal axis, and c) the spatial distribution of NTS neurons using three-dimensional reconstructions [16] and statistical mapping of neuronal population densities [17]. Our findings indicate that macronutrients in the gastrointestinal tract elicit distinct patterns of neuronal activity in the caudal brainstem.

Materials and Methods

Animals

Male C57BL/6J mice (Harlan Laboratories, Inc., Horst, The Netherlands) aged 6 to 8 weeks at the time of the experiment were housed in groups of 4 to 6 at 22±1°C under a 12-hour light/dark cycle (lights off at 20.00 h). The mice were left for one week to recover from transport and become habituated to their housing conditions. All experiments were carried out according to the guidelines of the French National Committee for Animal Care and the European Convention of Vertebrate Animals Used for Experimentation, under European Council Directive 86/609/EEC dated November, 1986.

Macronutrient Administration

For one week prior to the experiment, mice were gavaged daily with increasing volumes of water in order to accustom them to the experimental procedure. On the day of the experiment, following an 18-hour fasting period, and at the beginning of the light phase (08.00 h), mice were gavaged with an iso-caloric (1.2 kcal), iso-volumetric (700 µl) and iso-osmotic load, using a 20G/30 mm-long feeding needle (Fine Science Tools GmbH, Heidelberg, Germany). Animals in the treatment group received either carbohydrate (0.4 g/ml D(+)-Sucrose; AnalaR NORMAPUR®, VWR International, Strasbourg, France) or protein (0.4 g/ml Peptone from casein, enzymatic digest; Fluka, Sigma-Aldrich, St Quentin Fallavier, France) diluted in water. Two control groups were used, one receiving 700 µl of water as a control for gastric distension and the other not being subjected to any stimulation. Three mice were processed in each experimental group.

Brain Fixation and Collection

Ninety minutes after administration of the loads, the mice received a lethal i.p. injection of sodium pentobarbital (4 ml/kg CEVA SANTE ANIMALE, Centravet, Plancoët, France) and were subsequently transcardially perfused via a 26G needle placed in the left cardiac ventricle with 50 ml of 0.9% saline (NaCl, VWR International) supplemented with 2% $NaNO_2$ (pH 7.5, 3 min, Sigma-Aldrich), followed by 100 ml of 4% paraformaldehyde (Merck KGaA, VWR International). Their brains were removed and transferred to a 4% formaldehyde fixative solution for 4–6 hours, and then placed overnight at 4°C in 0.1 M phosphate buffer containing 15% sucrose. The brains were then frozen in isopentane (Sigma-Aldrich) at −35+/−3°C, embedded in Tissue-Tek® (Tissue-Tek® O.C.T. Compound, Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) and stored at −20°C until sectioning. Approximately 100 coronal 20 µm-thick brainstem sections, covering the entire rostrocaudal extent of the NTS, were cut using a cryostat (CM3000, Leica, Germany) at −19°C. Brain positions were localized using a stereotaxic atlas [18]. Odd- and even-numbered sections were mounted on two separate sets of gelatin-coated glass slides and stored at −80°C until further processing.

Fos Immunohistochemistry

Immunostaining of the Fos protein was performed on the odd-numbered sections at room temperature as follows: a) rinsing in 0.01 M sodium phosphate buffer containing 0.9% NaCl (PBS, 10 min, pH 7.4), b) incubation in 0.5% Triton X-100 (Sigma-Aldrich) and 2% BSA (Sigma-Aldrich) in PBS for 1 h, c) overnight incubation with Fos primary antibody (Anti-c-Fos (Ab-5) (4–17) Rabbit pAB, Calbiochem, VWR International) at a dilution of 1:5000 in PBS supplemented with 0.5% Triton X-100 and 2% BSA, d) 3 rinses (10 min each) in 0.1% skimmed milk (Régilait, St-Martin-Belle-Roche, France) in PBS, e) 3 h incubation with the biotinylated secondary antibody (biotinylated anti-rabbit IgG (H+L), Vector Laboratories, AbCys S.A., Paris, France) diluted 1:200 in 0.5% Triton X-100 and 2% BSA in PBS, f) 3 rinses in PBS, g) 30 min incubation in 30% H_2O_2 (Merck KGaA, VWR International) in PBS h) 3 rinses in PBS, i) 30 min treatment with avidin-biotin complex (Vectastain Elite ABC kit, Vector Laboratories), j) 2 washes in PBS, k) 1 rinse in Trizma solution (pH = 7.6, Trizma® HCl and Trizma® Base, Sigma-Aldrich), l) revelation of peroxidase activity using 0.5% diaminobenzidine terahydrochloride (DAB, Sigma-Aldrich) with 50 ppm of 30% H_2O_2 for 8–10 min, m) DAB reaction stopped in Trizma solution, n) 3 rinses in water (10 min each), o) dehydration in ethanol (VWR International) rinses, immersion in graded toluene (VWR International) and cover slipping using DePeX mounting medium (VWR International). A Fos-positive control and a negative control, obtained by omitting the primary antibody on a sample of two sections from each animal, were processed during each experiment to check for antibody specificity.

Luxol Fast Blue/Neutral Red Histochemistry

Luxol fast blue/neutral red histochemistry was carried out on even-numbered sections as follows: a) 3 min rinse in water and 3 min in 95% ethanol, b) 4 h incubation at 58°C in 1% Luxol fast blue solution (1% Solvent Blue 38, 95% ethanol, 0.5% glacial acetic acid, Sigma-Aldrich), c) rinse in water (3 dips), d) differentiation in 0.1% Li_2CO_3 (Sigma-Aldrich) for 2–3 min, e) wash in water (3 dips), f) counterstaining for 10 min in 0.5% neutral red (pH 4.5, Neutral Red dye (Sigma-Aldrich) in water), g) 2 rinses in water, h) dehydration by graded ethanol rinses, immersion in graded toluene and cover-slipping with DePeX mounting medium (VWR International).

Image Digitization

For image acquisition *Mosaic* software (Explora Nova, La Rochelle, France) was used. This application, installed on a PC equipped with a digitizer board (Matrox Meteor2/MC4, Dorval, QC, Canada), used a Ludl-MAC5000 controller to drive a motorized stage (Marzhauser, Wetzlar, Germany) fitted to a microscope (AxioPlan2 Imaging, Carl Zeiss, Germany) equipped with a 3CCD camera (Hitachi HV-C20A, Tokyo, Japan). This setting enabled the acquisition of large surface areas as mosaics of images at a resolution appropriate to the identification of structures of interest. Two image stacks were acquired for each animal. Odd-numbered sections (Fos immunostaining) were digitized using a x20 lens (pixel resolution: 0.41 µm) to enable the identification of Fos-positive neurons (Figure 1A). Even-

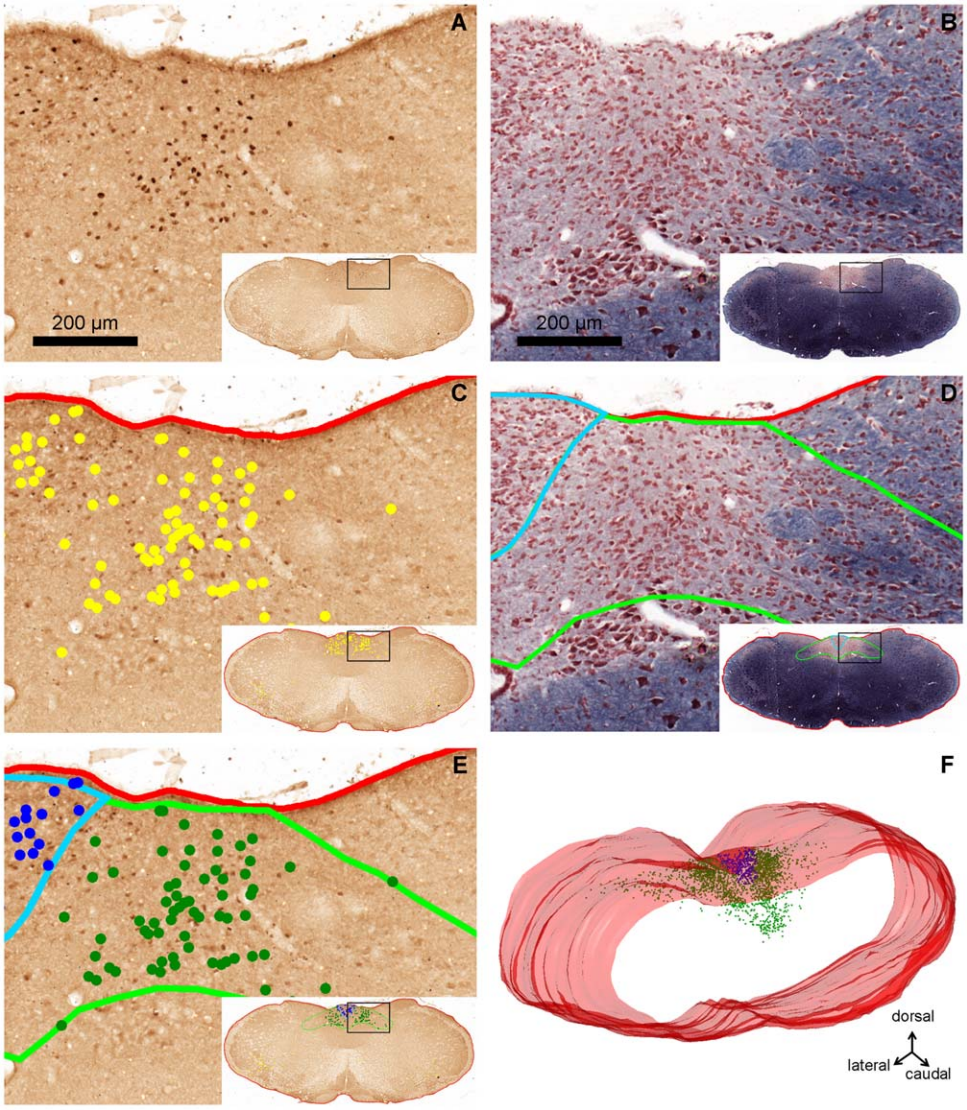


Figure 1. Reconstruction of a three-dimensional model using serial sections of mouse brainstem. A, C, E, coronal section immunolabeled for nuclear Fos protein. B, D, adjacent section with Luxol fast blue/neutral red counterstaining. A, brown spots are evidence for Fos-positive neurons in the dorsomedial part of the brainstem. Insert: whole brainstem section, the black rectangle indicating the enlarged picture. B, myelinated fibers labeled in blue and neuronal cell bodies labeled in red. C, section identical to A, the red line delineating the brainstem contour and yellow spots labeling Fos-positive neurons. D, section identical to B, with delineated brainstem (red line), NTS (light green) and the AP contour (light blue). E, section identical to A, NTS and AP contours from D are appropriately superimposed on C, allowing the assignment of labeled neurons (C) to the NTS (dark green) or AP (dark blue). F, the stack of brainstem contours (red) and labeled neurons (dark blue, dark green) generate a three-dimensional model, showing the caudal part of the brain.
doi:10.1371/journal.pone.0008974.g001

numbered sections (Luxol fast blue staining) were digitized using an x10 lens (pixel resolution: 0.82 µm), which was sufficient to identify the NTS and AP regions (Figure 1B).

Three-Dimensional Model Construction

In all mice, the same rostrocaudal region of the brainstem (from Bregma −8.3 mm to Bregma −6.2 mm according to a stereotaxic atlas [18]) was sampled in coronal sections. Three-dimensional models of the brainstem and cell populations were generated using *Free-D*, a reconstruction and modeling software [16] (Figure 1C–F). For this purpose, in each mouse, structures of interest identified on the digitized images were manually segmented: the brainstem contour and Fos positive cells on Fos-labeled sections (Figure 1C), the brainstem contour and those of the NTS and AP on Luxol-stained sections (Figure 1D). In a second step, the brainstem contours of both series of sections were registered using affine transformations: brainstem contours from Luxol-stained images were registered on a section-by-section basis onto the contours of Fos-immunolabeled sections. The same transformations were applied to NTS and AP contours in order to correctly superimpose them on the Fos images (Figure 1E). Finally, consecutive images of the Fos series were manually registered to yield a three-dimensional model of the brainstem encompassing the two scatters of points corresponding to NTS- and AP-Fos-positive cells (Figure 1F).

Spatial Normalization of Three-Dimensional Models

Statistical representations of Fos-positive neuron positions were built according to the following steps: a) spatial normalization of the

individual three-dimensional models, b) computation of one statistical NTS neuron density map per experimental group, c) extraction of isodensity surfaces. All steps have been described elsewhere [17,19] and are only briefly summarized here. Spatial normalization requires a reference structure which, in the present experiments, was the brainstem envelope. This was performed as follows: brainstem models were registered and averaged [19,20]. Polynomial deformations, mapping each individual model onto the average model, were then computed and propagated to NTS Fos-positive cell positions. This procedure generated spatially normalized three-dimensional models which could be superimposed to visualize the three-dimensional organization of NTS Fos-positive cells (see Figure S1). Morphological variability was assessed by computing the point-to-point root mean square distance between individual brainstem envelopes and the averaged model.

Data Analysis

Estimated total number of Fos-positive neurons. The numbers of Fos-positive neurons were determined in the regions of interest. The Abercrombie correction factor [21] was applied to correct for duplicate counting. The numbers of Fos-positive neurons in the three experimental groups were compared using the Kruskal-Wallis rank sum test in the R statistical software package (version 2.8.1) [22]. In brain regions where a significant difference was found, pair-wise comparisons were performed using the Wilcoxon rank sum test.

Rostrocaudal distribution of Fos-positive neurons. Fos-positive cells in each mouse were sorted according to their coordinates along the rostrocaudal axis, and the probability density function was assessed using Gaussian kernel density estimations [23]. For each animal, the individual probability density curve of rostrocaudal Fos-positive neuron positions was multiplied by the estimated total number of Fos-positive neurons and the resulting curves were averaged within each experimental group to yield an estimate of the rostrocaudal distribution of neurons. For raw data and density estimation curves, see Figure S2.

Three-dimensional distribution of Fos-positive neurons in the NTS. In each group, the local density of Fos-positive neurons was statistically estimated at each node of the grid from all NTS neuron sets of the group [17]. To achieve this, a three-dimensional grid overlapping all normalized NTS Fos-positive neuron positions over all groups was defined. Therefore, each group was processed separately as described below. The local density of Fos-positive neurons was then estimated statistically at each node of the grid from all NTS neuron sets for each group. The resulting three-dimensional grid of local density estimates constituted the density map of a group. To enable visual analysis and interpretation, three-dimensional isodensity surfaces were computed from these density maps. The isodensity threshold was chosen so that the corresponding iso-surface encompassed 90% of Fos-positive neurons in the group.

Results

Total Number of Fos-Positive Neurons

No Fos-positive neurons were observed on brainstem sections from control mice that did not receive intragastric loads (data not shown). By contrast, Fos-positive neurons were present in sections from all other experimental groups, as illustrated in Figure 2 which shows the number of Fos-positive neurons in the brainstem of mice after water, peptone or sucrose gavage. When considering the whole brainstem (Figure 2A), no difference was found between the three experimental groups. In the NTS (Figure 2B), the water group displayed a significantly smaller number of Fos-positive neurons than the two others, while no difference was found between the peptone and sucrose groups. In the AP (Figure 2C), the number of Fos-positive neurons was nutrient-dependent and all pair-wise comparisons reached statistical significance (water $<$ peptone $<$ sucrose). Outside the NTS and AP (Figure 2D), no differences were observed between groups.

Rostrocaudal Distribution of Fos-Positive Neurons

Figure 3 represents the rostrocaudal distribution of Fos-positive neurons in the whole brainstem (Figure 3A), the NTS (Figure 3B) and the AP (Figure 3C). Figure 3D shows their distribution outside these latter structures, where Fos immunoreactive neurons displayed a relatively uniform distribution with no marked differences between the water, sucrose and peptone groups. In the whole brainstem (Figure 3A), the distribution of Fos-positive neurons following water gavage was similar, with a maximum at around Bregma -8 mm and a slow decrease in the rostral direction. In the NTS (Figure 3B) and AP (Figure 3C), water gavage induced a weak Fos response. Thus, in the brainstem, Fos immunoreactive neurons in response to water gavage were mostly localized outside the NTS and AP. The picture was different concerning the responses to sucrose and peptone, which in the brainstem (Figure 3A) induced a peak of Fos immunoreactive neurons centered around Bregma – 7.5 mm. This peak was due to Fos immunoreactive neurons in the NTS and AP, with a major contribution from the NTS (see ordinate scales in 3B and 3C). Within the NTS, more Fos immunoreactive neurons were located in the NTS section located at the level of the AP (Bregma -7.2 mm to -7.8 mm).

Three-Dimensional Distribution of Fos Immunoreactive Neurons in the NTS

Figure 4A represents the average brainstem model calculated after spatial registration of the individual three-dimensional brainstem models obtained from eight mice (n = 3 for water- and sucrose-loaded groups, n = 2 for the peptone-loaded group). Note that in one peptone loaded mouse, the brainstem model was incomplete and therefore this animal was excluded from further analyses. The average three-dimensional model includes a color code expressing variability which was greater in the lateral parts of the brainstem than in the dorsal and ventral parts.

Superimposition of all eight spatially normalized NTS cell populations (Figure 4B) did not enable the identification of differences between neuronal sets activated by the three treatments. In contrast, a representation of these neuronal sets by isodensity surfaces computed from statistical density maps (Figure 4C) allowed a qualitative visual analysis. The surface corresponding to the water-loaded group encompassed the two other surfaces (see also Film S1).

Figures 4D to 4I focus on data from the peptone and sucrose groups. Superimposition of the populations of Fos-positive neurons in these groups (Figures 4D and 4E), showed that, as a whole, they had similar rostrocaudal, mediolateral and dorsoventral extensions. However, the respective isodensity surfaces were different (Figures 4F–4I). Caudally, peptone- and sucrose-induced Fos immunoreactive neurons reached the same position, while rostrally, the peptone group had a greater extension. Dorsoventrally, neurons from the peptone-treated group extended more ventrally while neurons from the sucrose-treated group occupied a more dorsal position. Finally, neurons from the peptone group occupied a narrower volume along the mediolateral axis. In order to assess the degree of overlap between the two populations, we determined their total volume and estimated the portion of this volume that was simultaneously occupied by the two groups. This intersection occupied 53% of the united volume.

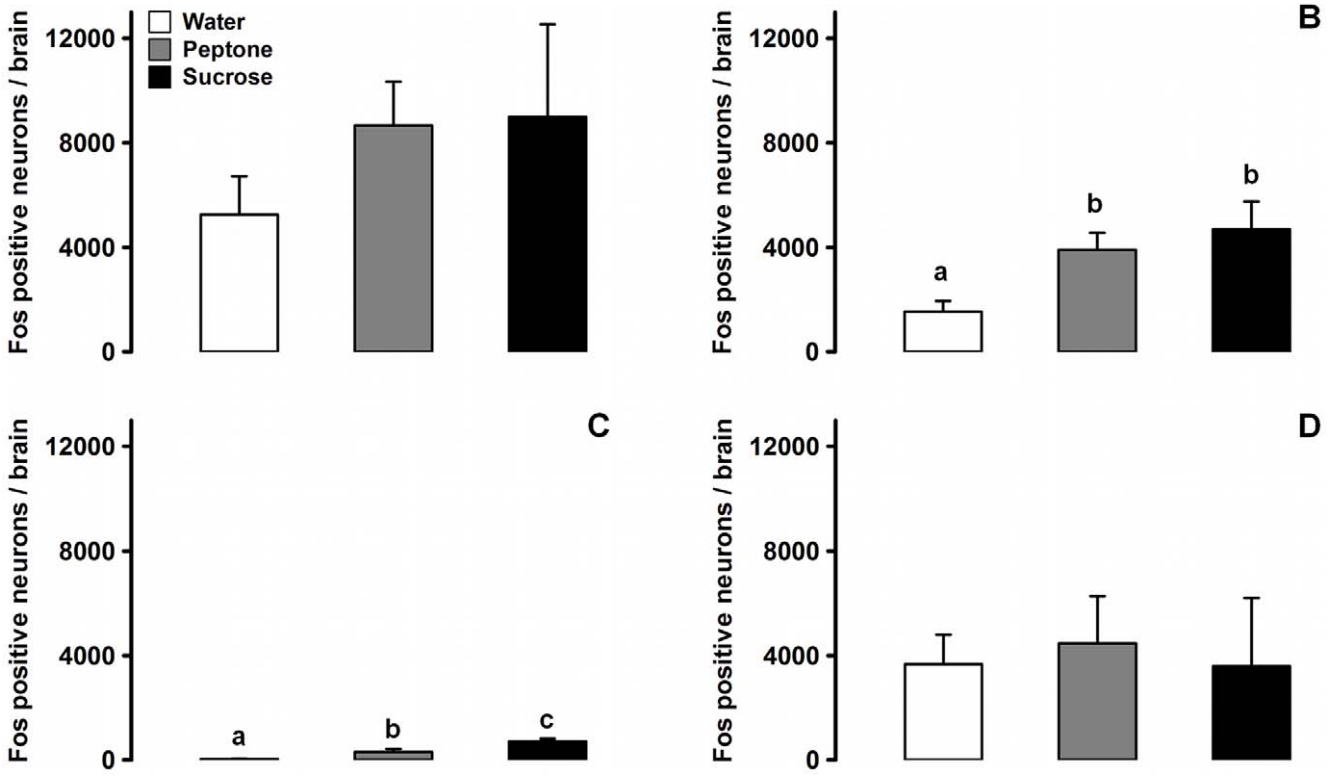


Figure 2. Number of Fos-positive neurons in the brainstem of mice following gavage with different loads. A, total number of Fos-positive neurons in the entire brainstem after gavage with casein peptone (grey bars), sucrose (black bars) or water (white bars). No differences were found between these groups ($p = 0.6703$). B, number of Fos-positive neurons in the NTS. The water group revealed a significantly lower number of labeled cells than the other two groups ($p<0.05$ for both comparisons). C, number of neurons in the AP. The number of Fos-positive cells was treatment-dependent ($p = 0.0273$). All pair-wise comparisons were statistically significant ($p<0.05$). D, number of neurons outside the NTS and AP. No statistical difference was found ($p = 0.7326$). Data are presented as mean ± SEM after Abercrombie correction. Bars labeled with different letters differ according to the Wilcoxon rank sum test, $p<0.05$.
doi:10.1371/journal.pone.0008974.g002

Discussion

This study shows that specific neuronal activity patterns are induced in the NTS by various macronutrients and that these different patterns can be revealed by three-dimensional density maps. These results are in favor of the processing of specific gastrointestinal information by subpopulations of NTS neurons. This conclusion represents an important contribution to the understanding of the mechanisms involved in macronutrient signaling to the brain.

The NTS is the point of convergence of peripheral information generated by the GI tract during digestion. It integrates nervous signals from vagal afferents and from central AP neurons [24]. It has been known for some time that in the rat, the NTS is organized in a viscerotropic manner [2]. In this scheme, NTS neurons receiving information from the upper GI tract (i.e. esophagus, stomach and duodenum) are located in the vicinity of the AP. For other sensory modalities such as audition, olfaction or touch, functional organizations have been revealed by three-dimensional analysis [25-28]. Functional activity maps may also exist for the NTS with respect to visceral sensitivity. Although it has been claimed that no functional mapping exists in the NTS [11], no spatial analyses have been performed in this region. We therefore undertook a three-dimensional study of neuronal activity following the administration of macronutrients. To take account of both experimental and inter-individual variability, a statistical strategy was adopted. We took advantage of a recently reported method for spatial normalization and statistical three-dimensional modeling of neuronal populations [17] in order to address the question of a possible functional segregation of NTS neurons.

Macronutrient-Elicited Fos Expression in the Brainstem Was Restricted to NTS and AP Neurons

In both the peptone and sucrose groups, the major contribution of total Fos immunoreactive neurons in the brainstem came from the NTS, with a minor contribution from the AP. The contingent of water-induced Fos-positive neurons was mainly located outside the NTS and AP, and was very limited in these two regions, suggesting that the Fos labeling induced by sucrose and peptone in the NTS and AP was stimulus-specific. Since no Fos immunoreactive neurons were observed when no load was applied, the Fos immunoreactivity observed outside the NTS and AP probably resulted from gastric distension during gavage. Taken together, these results are in line with the findings of previous reports [11,12,29] which also showed that macronutrients administered in the GI tract elicited specific Fos responses in the NTS and to a lesser extent in AP, and that gastric distension induced Fos expression at the same positions, independently of the macronutrient type.

Within the NTS, Macronutrients Elicited Specific Patterns of Neuronal Activity

A functional segregation of NTS neuron subpopulations could only be demonstrated after three-dimensional analysis, while in line

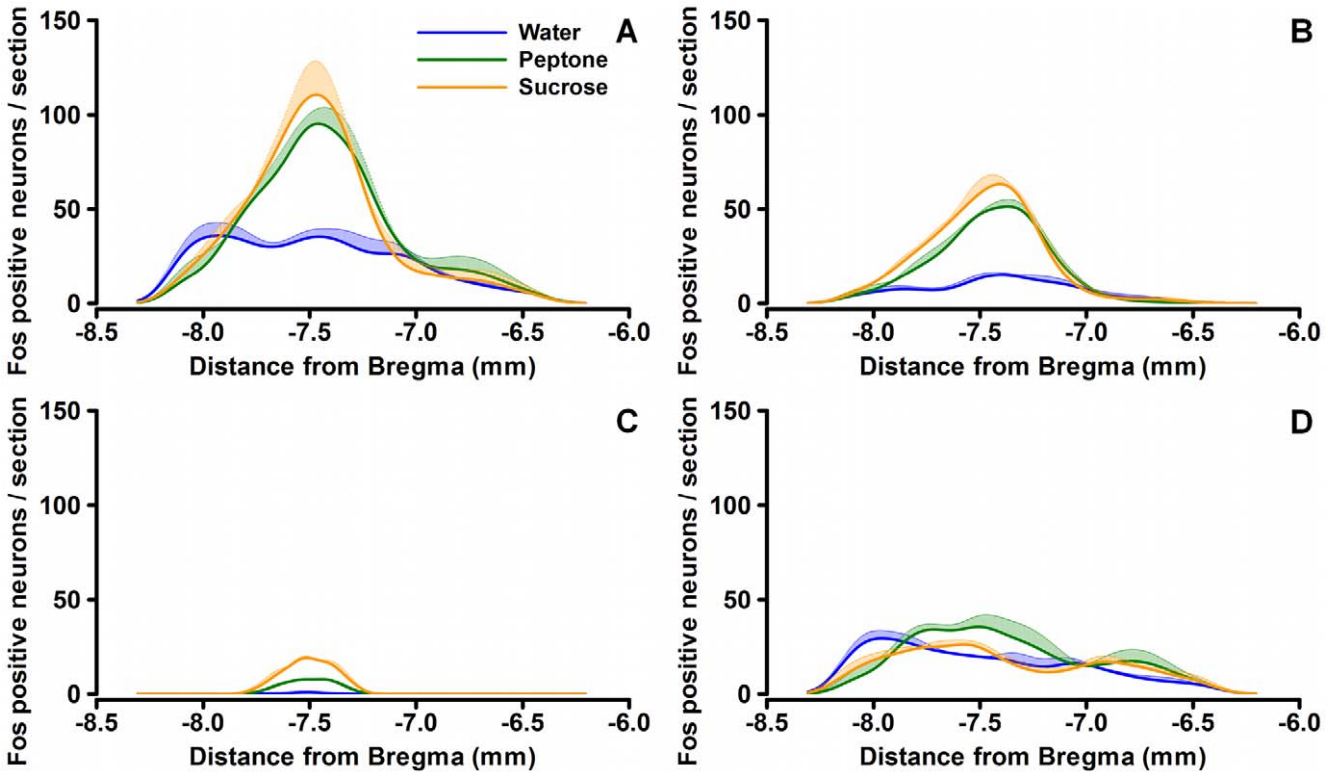


Figure 3. Distribution of Fos-positive neurons along the rostrocaudal brain axis. Smoothed histograms of the number of Fos-positive neurons in the entire brainstem (A), the NTS (B), the AP (C) and the entire brainstem without an object count from NTS and AP (D). Data are expressed as mean ± SEM. The X-axis represents the distance in millimeters from Bregma.
doi:10.1371/journal.pone.0008974.g003

with previous reports, analysis of the distribution of Fos-positive neurons either on coronal sections or along the rostrocaudal axis showed no difference. Individual three-dimensional models (Figure 1F) provided a clearer view of their spatial organization but were not appropriate for comparative analysis. Model normalization and fusion yielded representations of whole experimental groups (Figure 4D and 4E). These fusion models were the superposition of normalized populations. The larger the experimental group, the bigger was the apparent size of the population and the more confused the representation. Therefore, although synthetic, the representations in Figures 4D and 4E were difficult to analyze visually. Only a computation of density maps made it possible to draw some clearer conclusions. Using this approach, we revealed that two different macronutrients specifically activated two functional subpopulations of NTS neurons. Visual examination of the iso-surfaces extracted from density maps showed a subtle but clear segregation of these neuron sets in the mediolateral, rostrocaudal and dorsoventral directions. However, calculation of their overlap indicated that only one half of their total volume was shared by both subpopulations. Further work is necessary to design and implement statistical tests that will be applicable to three-dimensional data and allow us to confirm our visual conclusions.

Possible Physiological Bases

Both the rostrocaudal distribution and three-dimensional organization of Fos immunoreactivity showed that activated neurons in the NTS were located in the vicinity of the AP, a finding consistent with the viscerotropic organization previously reported [2]. The segregation between subpopulations of NTS neurons following peptone and sucrose gavage might result from anatomical differences in the gastrointestinal sites of signaling, thus having anatomical rather than functional origins. Moreover, the signaling sites of peptone and sucrose in the intestine are not known sufficiently accurately to suggest a correlation with the observed segregation in the NTS. Considering the greater ability of protein, rather than carbohydrate, to delay gastric emptying [30,31] one might propose that the observed differences in Fos-positive neuron positions were due to differences in gastrointestinal and absorptive kinetics. Indeed, these alterations certainly modulate the contributions of the signaling sites involved in gut-brain communication during digestion. Independently of the actual origin of these changes, it is clear that with respect to the greater potency of protein than carbohydrate in triggering control of ingestion, and considering the fact that the NTS is the key region in the satiation process, it is legitimate to hypothesize that the segregation of neuronal positions within the NTS reflects central satiation mapping.

Conclusion and Perspectives

We have thus shown that different macronutrients activate distinct subpopulations of NTS neurons. Because of its complexity, this segregation could only be unmasked using a statistical, three-dimensional approach. Its physiological meaning still requires further investigation, but it is likely to be relevant to understanding how the brain traduces different metabolic situations and their influence on the control of food intake.

The currently proposed conceptual framework of eating behavior studies states that a small subset of brain regions integrates internal and external sensory cues and generates hunger, satiety and satiation. How these brain areas integrate

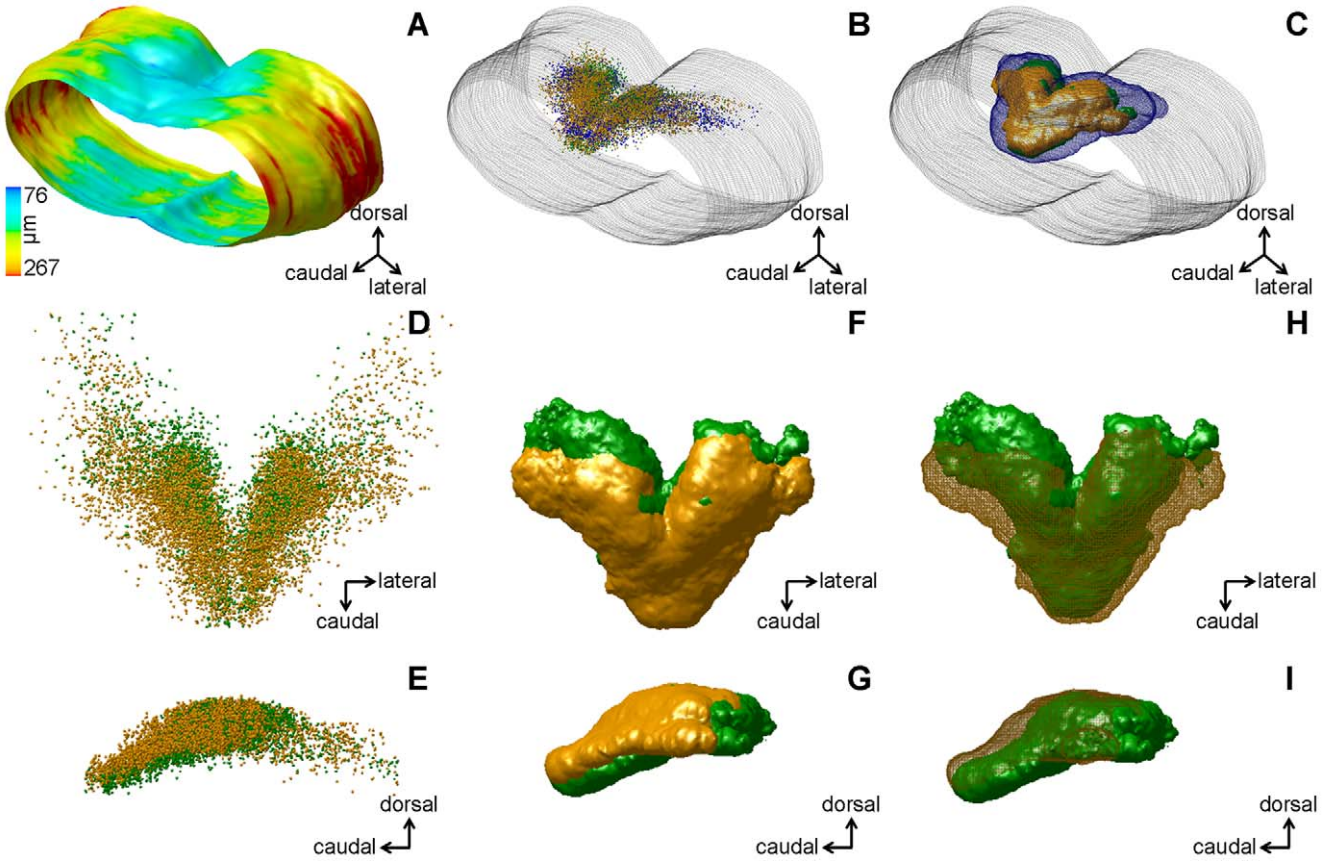


Figure 4. Merged normalized three-dimensional models and statistical distributions of cell populations. A, caudal view of the averaged brainstem envelope from 8 mice. The color code (bottom left) reflects the positional variability of each point of the model, from the least variable (blue) to the most variable (red). Variability is calculated as the root mean square distance (in µm) between a given point on the average model and homologous points on the individual models. Color coding for the following images: models for the water group are presented in blue, for the protein group in green and for the sucrose group in orange. B, all Fos-positive NTS neurons from the 8 mice displayed after normalizing their position according to the averaged brainstem, represented in grey mesh mode. Same orientation as A. C, superimposition of three isodensity surfaces, each corresponding to NTS Fos-positive neurons from one experimental group. Each iso-surface includes 90% of Fos-positive neurons from the experimental group. Same orientation as A. D, all NTS Fos-positive neurons in the peptone and sucrose groups are represented in a dorsal view, after spatial normalization. E, same as D, lateral view. F and G, dorsal and lateral views of isodensity surfaces for the peptone and sucrose groups. Thresholds were selected so that the isodensity surfaces encompass 90% of Fos-positive neurons in each group. H and I, same as F and G, respectively, but the isodensity surface of the sucrose group is displayed in mesh mode. Models in A, B and C, as well as in D, E, F, G, H, and I are displayed at the same magnification.
doi:10.1371/journal.pone.0008974.g004

feeding-related information remains largely unknown, mostly because these signals are multiple and signal continuously to the brain. Instead of focusing separately on each factor and its signaling to the brain (e.g., gastric volume or CCK), the method developed here is designed to gain insight into the neurobiology of eating using an integrated approach that focuses on a central representation of a complex internal situation rather than fine, but reduced, action/reaction effects.

Supporting Information

Figure S1 Three-dimensional models of the brainstem and Fos-expressing neurons. A, fusion of the average brainstem model with single brainstem models for mice gavaged with water. B, fusion of the average brainstem model with single brainstem models for mice gavaged with saccharose. C, fusion of the average brainstem model with single brainstem models for mice gavaged with peptone. D, average brainstem model with the total number of neurons marked in mice gavaged with peptone, E, average brainstem model with the total number of neurons marked in mice gavaged with saccharose, F, average brainstem model with the total number of neurons marked in mice gavaged with water.
Found at: doi:10.1371/journal.pone.0008974.s001 (1.69 MB TIF)

Figure S2 Raw data, means and density curves presenting the distribution of Fos-expressing neurons along the rostrocaudal axis.
Found at: doi:10.1371/journal.pone.0008974.s002 (0.93 MB TIF)

Film S1 Three-dimensional models of Fos-expressing neurons in the NTS for water (blue) peptone (green) and sucrose (orange)-loaded mice. Start of the film: view from caudal position, turning to a ventral view, then a rostral view, a dorsal view and then back to the caudal view. Second part: rotation around the dorso-ventral axis.
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Acknowledgments

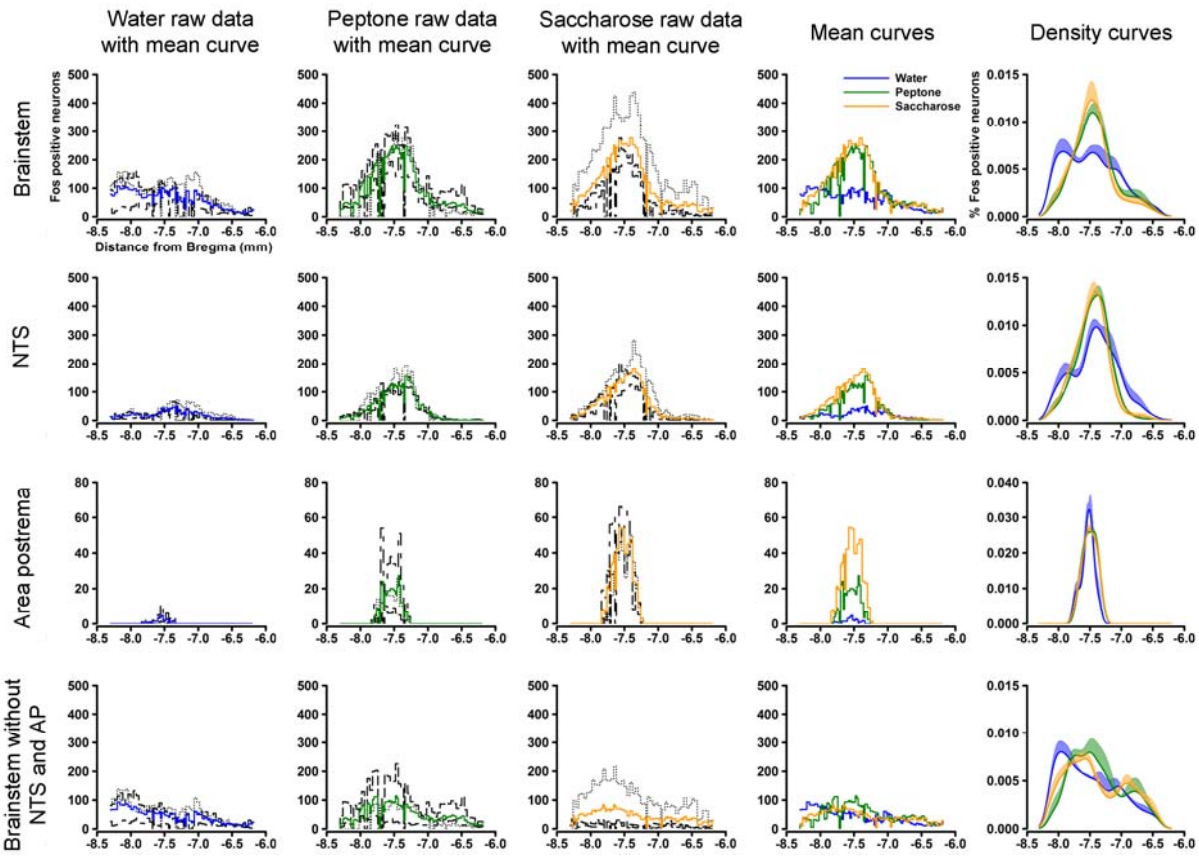
We would like to thank Christian Ouali and Anne-Ruth Reisiger for their assistance with image acquisition and data analysis.

Author Contributions

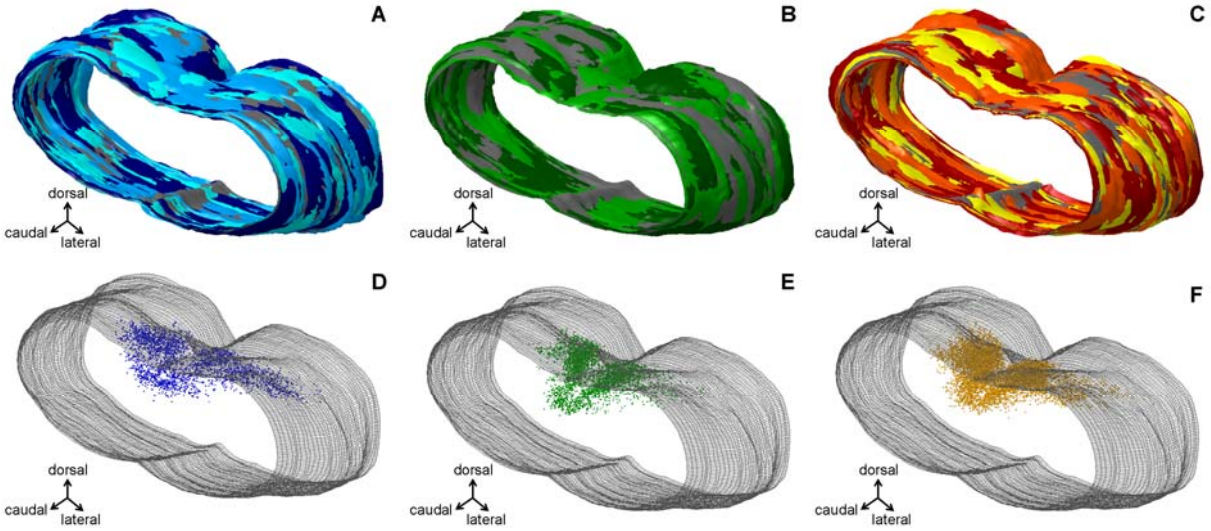
Conceived and designed the experiments: JS JB OR GF PA DT YM ND. Performed the experiments: JS. Analyzed the data: JS JB OR PA YM ND. Contributed reagents/materials/analysis tools: JB OR GF PA DT YM ND. Wrote the paper: JS JB OR GF PA DT YM ND.

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Supporting information 1. Raw data, means and density curves presenting the distribution of Fos-expressing neurons along the rostrocaudal axis.



Supporting information 2. Three-dimensional models of the brainstem and Fos-expressing neurons. A, Fusion of the average brainstem model with single brainstem models for mice gavaged with water. B, Fusion of the average brainstem model with single brainstem models for mice gavaged with sucrose. C, Fusion of the average brain stem model with single brain stem models for mice gavaged with peptone. D, Average brainstem model with the total number of neurons marked in mice mice gavaged with water. E, Average brainstem model with the total number of neurons marked in mice gavaged with saccharose. F, Average brainstem model with the total number of neurons marked in mice gavaged with peptone.

Supporting information 3. Three-dimensional models of Fos-expressing neurons in the NTS for water (blue) peptone (green) and sucrose (orange)-loaded mice. Start of the film: view from caudal position, turning to a ventral view, then a rostral view, a dorsal view and then back to the caudal view. Second part: rotation around the dorso-ventral axis. Found at doi: 10.1371/journal.pone.0008974.s.003 (6.07 MB MOV).

3.3 Additional methods: Calculation of density curves

Fos-positive cells in each mouse were sorted according to their coordinates along the rostro-caudal axis (Supplementary information 1 in article) and plotted as cell count per section once Abercrombie correction (0.76) and a correction factor for skipped sections (0.5) were applied. In order to examine the distribution Fos expressing neurones along this axis, kernel density estimations (Silverman 1986) using a Gaussian kernel function with the bandwidth of 10 µm (except for the AP, with a bandwidth of 5 µm) were acquired using R statistical software (R version 2.8.1) (Team 2009). We obtained density curves containing 358 equally distributed data points that could be seen as a smoothed and normalised histograms of rostro-caudal cell positions, describing the probability of a Fos expressing neurone being found at a specific intercept of the examined brain region (Supporting information 1 in article). For each animal, the individual probability density curve was then multiplied by the total number of Fos-positive neurones estimated after Abercrombie correction, before averaging these resulting curves within each experimental group obtaining weighted density curves for comparing the actual distribution of the c-Fos positive neurones (Figure 3 in article).

3.4 Discussion, conclusion and perspectives

During ingestion, nutrients such as protein and carbohydrates are sensed by specific receptors and transporters in the upper small intestine (Raybould 1999; Tome 2004). Signals generated in response to this sensing are subsequently transmitted to central brain structures, either by sensory vagal afferents or via the blood stream (Schwartz et al. 2000). We can now suggest that the nutrient specific signals derived in the GI tract also result in specific activation patterns within the brain areas in which they are processed, namely the caudal brainstem. This kind of functional mapping has already been demonstrated for other sensory processes for instance audition (Malmierca et al. 2008; Luo et al. 2009), olfaction (Johnson and Leon 2007) as well as tactual sensing (Petersen 2003).

The novel technique of analysing histological data used in this work allows identifying differences in the distribution of neurones activated by nutrients. These differences could not be detected with classical methods (Figure 2 in article). Fusion of the envelopes of the animals from the different experimental groups allowed us to generate an average brainstem model that was subsequently used to place the individual data in a comparable position. Isodensity surface models obtained from the pointed Fos expressing cells of several animals per group gave us the possibility to compare their position to each other and suggest that signalling in the NTS is macronutrient specific.

There are several hypotheses that could explain the suggested macronutrient specificity. Using retrograde labelling techniques, the NTS as part of the DVC has been demonstrated to possess horizontal viscerotropic organisation as the vagal projections it receives are derived from distinguished sites within the GI tract (Norgren and Smith 1988; Altschuler et al. 1989; Zhang and Ashwell 2001). The here identified activity maps might be linked to this topical organisation of the NTS and the site of detection in the intestine might therefore have an influence on the regionalisation of NTS activation. Protein and carbohydrate are known to possess differences in GI tract kinetics, such are certain types of protein known to delay gastric emptying in comparison to carbohydrates and fat (Boirie et al. 1997; Bowen et al. 2006; Karhunen et al. 2008). In addition to reaching the intestine earlier and have the possibility to descend further then protein, carbohydrates also may activate other, additional signalling pathways.

Berthoud et al. reported a higher number of Fos expressing neurones in more central sub-nuclei of the NTS after duodenal infusions compared to gastric distension and therefore concluded that the specific activation by nutrients might be possible due to the different location of second order neurones (Berthoud et al. 2001). Indeed it is likely that carbohydrates and protein lead to an activation of different neurone subsets within the NTS. It had been shown that within the NTS at least two types of neurones can be distinguished. Noradrenergic/ adrenergic neurones are suggested to be sensitive to CCK (Rinaman 2003), while another subset is activated by GLP-1 (Thiele et al. 1997; Rinaman 1999; Seeley et al. 2000). In order to investigate if these subsets of neurones are differently activated by the macronutrients, double labelling of NTS neurones would have to be carried out.

Carbohydrates and proteins stimulate the release of a number of gut peptides, namely CCK that is released in response to protein and lipids (Liddle et al. 1985; Glatzle et al. 2004; Darcel et al. 2005b; Foltz et al. 2008) and 5-HT released at the presence of carbohydrates (Raybould et al. 2003; Freeman et al. 2006). A different plasma profile of these gut peptides might be responsible for the macronutrient specificity. However, the stimulation of the NTS by gut peptides transported to the caudal brainstem via the blood stream is not the only possibility for their action. Receptors were also found on vagal afferents and along the vagus nerve and it can therefore been suggested that the vagus nerve itself is able to transmit specific signals (Havel 2001; Berthoud 2002, 2004a).

Still, the technique presented here also shows some limitations, for instance there are no statistics available yet. Therefore, instead of statistically analysing the spatial position of the neurone populations, it is only possible at this moment to compare the volume occupied by the isodensity surfaces and the percentage of their overlap. Another limitation of the here presented experiments is the fact that only one timepoint can be regarded at once, but using functional MRI might help to define the ideal timeframe of maximal brainstem activity.

The described findings also raise new questions, such as if the identified patterns can be linked to specific gut peptides. One possible candidate for this is PYY which was suggested to specifically activate the caudal medial and gelatinous parts of the NTS via vagal afferents, while the commissural part is not affected (Blevins et al. 2008). Another question that can be asked is if the activation patterns identified can be modulated by environmental factors like

special diets. For example a high fat diet has been shown to modulate the response of the NTS to the gut peptide CCK (Nefti et al. 2009). Answering these questions may help to obtain a better understanding of how signalling for the short-term regulation of food intake can be modulated and in which way this can be used in anti- obesity treatment.

4 Modulation of body composition and the effect of gut peptides by a high protein diet

4.1 Introduction

In the regulation of food intake and appetite two main central neuronal networks are believed to be involved. Neurones in the brainstem and within here in the AP and the NTS are receiving and integrating intestinal signals transmitted by the vagus nerve as well as blood derived hormonal signals which are implicated in the short-term regulation of food intake so as meal termination. The second important structure, the hypothalamus is known to be responsible for the long-term maintenance of energy balance and body weight. Still, both structures are reciprocally connected in order to keep up the balance of food intake and energy expenditure (Berthoud et al. 2001).

Several pieces of evidence have suggested that diet can alter these neural networks controlling food intake. For instance, in murine models, long-term exposure to a high fat diet has been shown to decrease the efficiency of neural pathways for controlling meal size. This causes an increase in food intake and as a consequence elevated body weight (Savastano and Covasa 2005; Paulino et al. 2008).

Dietary protein is likewise suggested to have multiple effects on short-term appetite as well as long-term regulation of energy balance and body weight. In rats a load of protein prior to a meal reduced food intake significantly (Bensaid et al. 2002). Feeding rats a HP diet for a period of two to four weeks led to a reduction in body weight gain and cumulative food intake (Jean et al. 2001; Pichon et al. 2006; Kinzig et al. 2007). As an important additional effect, some of these animals were shown to have less adipose tissue compared to their controls (Jean et al. 2001) and adipocyte size was found to be smaller (Pichon et al. 2006). After a 6 months dietary intervention period, in HP diet fed rats not only a diminution of body weight gain, adipose tissue and cumulative food intake occurred, NP fed control rats had developed liver steatosis while no hints for that were found in the HP animals (Lacroix et al. 2004). It is therefore suggested that feeding rats a HP diet has a positive effect not only on the total body weight, but also on the body composition and liver health. In addition to these effects, immunohistochemical studies, using Fos as a marker of neuronal activity, had

shown that feeding rats a HP diet affects the activity of various areas within the CNS. Namely a change in Fos expression in the NTS and in both the VMH and the amygdale cortex after ingestion of the diet was detected (Darcel et al. 2005a). Desensitization or hypersensitisation of the gut brain axis to anorexigenic signals is likely to be at the core of these alterations. As immunohistochemical experiments can only observe one timepoint, in the present study manganese enhanced magnetic resonance imaging (MEMRI) was used to investigate the long-term effect of feeding HP diet on neuronal activity in the hypothalamus *in vivo*.

Up to now, most of the studies investigating the effect of a high protein diet on body weight, body composition and food intake were carried out in rats. The rat as a model has indeed important limitations. It is therefore of great interest to develop mouse models for this kind of studies, what will make it possible to study existing mouse models for specific diseases or transgenic mice. In order to develop investigations in this direction, mice were used in the following experiments.

4.1.1 Aims of studies

In this study, we investigated the effect of long-term high-protein intake on body weight and body composition as well as on the central response to OXM. Diet can not only modulate body composition but also the neural networks controlling of food intake by desensitising to certain anorexigenic gut peptides. OXM is an anorexigenic peptide binding to the GLP-1 receptor which is widely distributed in the hypothalamus. No studies are available yet investigating if the effect of OXM can be influenced by a specific diet. Our hypothesis was therefore that the localisation and intensity of OXM-induced activity in specific brain areas may be dependent on the nature of the diet, and more precisely its protein content. We firstly investigated the effect of a HP diet on bodyweight, food intake and body composition and secondly assessed the effects of the HP diet on the central response to OXM by manganese-enhanced magnetic resonance imaging.

4.2 Material and Methods

4.2.1 Materials

All chemicals were purchased from Sigma-Aldrich Ltd. (Poole, Dorset, UK) and Oxyntomodulin was purchased from Phoenix Pharmaceuticals (Belmont, CA, USA), unless otherwise stated.

4.2.2 Animals

Male C57BL/6 mice (Harlan, UK) were housed in groups of four in individually ventilated cages at a 12 hours light-dark cycle (lights off at 7 pm) at controlled temperature (21-23 °C) and had *ad libitum* access to food and water unless otherwise stated. They were fed a standard chow (RM3 diet, Special Diet Services Ltd, Witham, Essex, UK) during at least one week of adaptation phase prior to dividing them into two groups. Animals were 10 weeks old at the start of the feeding intervention and had a body weight of 20-25 g. During the following six weeks of dietary intervention one group of mice was fed a normal protein diet (NP, 14 % of energy from protein, see Annex for exact composition), while a second group received a high protein diet (HP, 55 % of energy from protein, see Annex for exact composition). All mice that underwent body composition measurements were used for MEMRI studies after at least 5 days of recovery. Additional mice which did not undergo body composition MR scan were used for the MEMRI experiment in order to reach a sufficient number of subjects.

All animal procedures undertaken were approved by the British Home Office under the Animals (Scientific Procedures) Act 1986 (Project License No. 70/6402, Personal License No. 70/21492).

4.2.3 Effect of a HP diet on body weight and food intake

Food intake in groups of 4 animals and body weight of the mice were recorded twice a week during the first five weeks of the feeding period. After start of the body composition measurements, body weight and food intake were still recorded but did not go into final analysis to avoid bias from handling and fasting the animals for the MR measurements. Body weight is expressed as mean body weight (g) ± SEM and body weight gain as mean BWG (g) ±

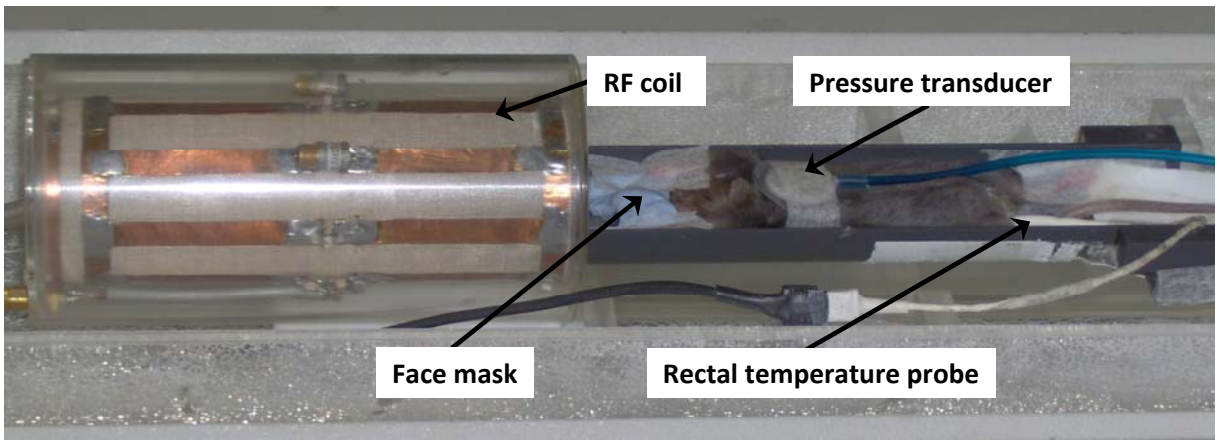


Figure 4.1 Set-up of mouse and radio frequency (RF) body coil.

The mouse is strapped down dorsally on the bed in a standardised position with its breathing rate and temperature monitored via the pressure transducer and the temperature probe. Anaesthesia is maintained through the face mask. The bed will be placed inside the RF coil which in turn will be positioned in the centre of the scanner magnet (Figure kindly provided by Jelena Anastasovska).

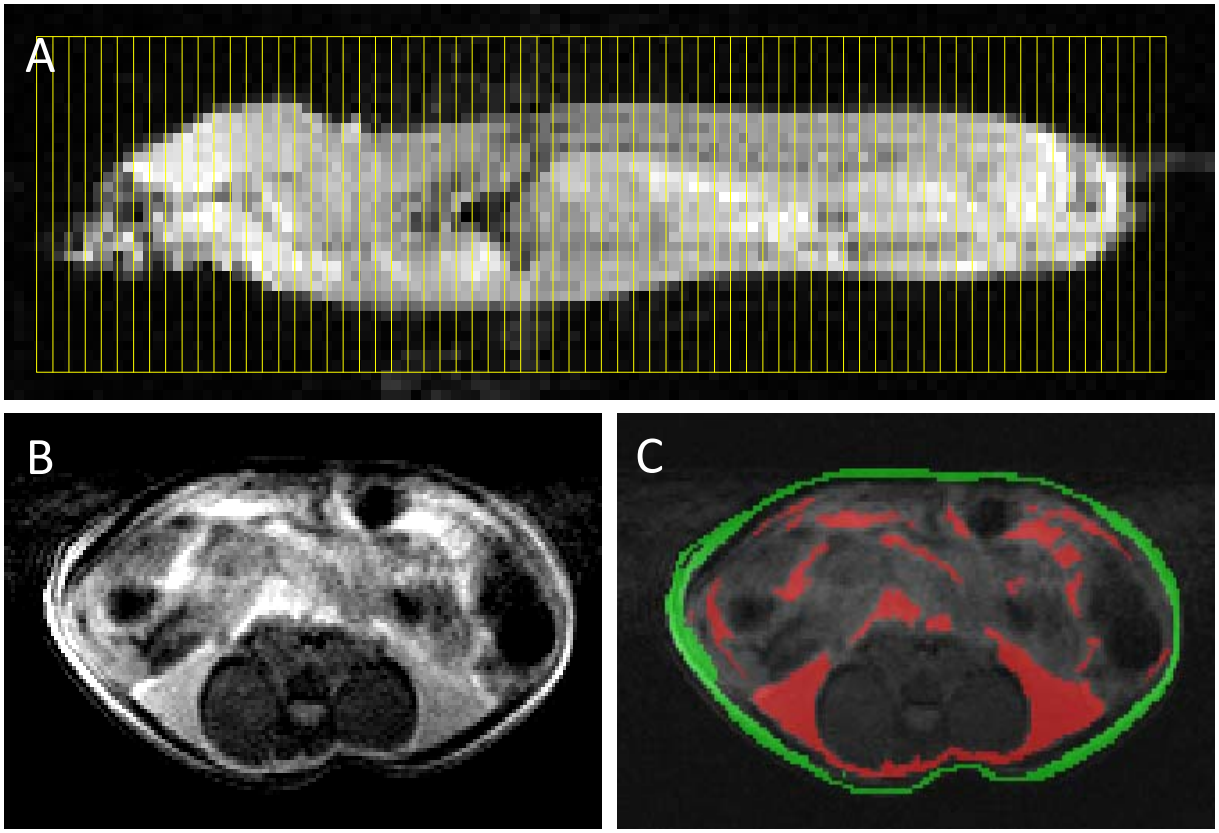


Figure 4.2 Planning of the axial MR images across the body.

A) Sagittal pilot image through the whole mouse body (neck left to tail right), with the location of the 50 slices that will be acquired. B) Example of a transverse slice acquired in the abdominal region and C) the same slice after fat segmentation (red: visceral adipose tissue, green: subcutaneous adipose tissue) (Figure kindly provided by Jelena Anastasovska).

SEM. Food intake and cumulative food intake is expressed as mean intake per mouse (kJ) ± SEM.

4.2.4 Effect of a HP diet on body adipose tissue composition

Body adipose tissue composition was measured in mice after feeding them a NP diet (n=10) or a HP diet (n=10) for 5 weeks. For examining the muscle mass and regional fat variations, magnetic resonance imaging (MRI) was carried out while ^{1}H magnetic resonance spectroscopy (MRS) allowed investigating lipid content of the whole body, liver and muscle. Both measurements took place during the same scan in a 4.7 T MR scanner (Varian DirectDrive console, Varian Inc, USA) running VnmrJ 2.3A software and using a 100 mm long 40 mm i.d. quadrature birdcage transmit-receive radiofrequency (RF) coil.

After a 16 hour fast, but with access to water, animals were anesthetised using a 1-2 % isoflurane-oxygen mixture applied by a face mask with a flow rate of 1.5-2l/min. During the scan they were maintained at around 1 % isoflurane according to their desired respiration rate of 60 to 80 breaths/min, monitored by a pressure transducer strapped on the chest of each mouse (Physiological monitoring, SA Instruments, Inc, NY). The mice were placed dorsally on a bed and strapped down in a standardised position with adhesive tape (Figure 4.1). Body temperature was monitored using a rectal temperature probe and maintained at 37.0 ± 0.5 °C via a heating system (SA Instruments, Inc, NY).

4.2.4.1 Whole body MRI

Once frequency and power were calibrated, a trial spectrum acquired and the line width measured, shimming was performed manually in order to obtain a homogeneous magnetic field. To determine the correct positioning of the mouse in the coil and to plan the scan, a rapidly acquired 3-plane gradient echo image was used (Figure 4.2 A). Thereafter we carried out a spin-echo multislice sequence (sems) acquiring a total of 45 to 50 axial images covering the entire body of the mouse, using the following parameters: Repetition time (TR) = 2.2 sec, echo time (TE) = 20 msec, field of view (FOV) = 45 mm x 45 mm, data matrix = 256 x 192, 2 averages, slice thickness = 2 mm.

After images were converted into a stack and saved into an appropriate file format using ImageJ (ImageJ 1.41o, Wayne Rasband, NIH, USA), analysis was performed with

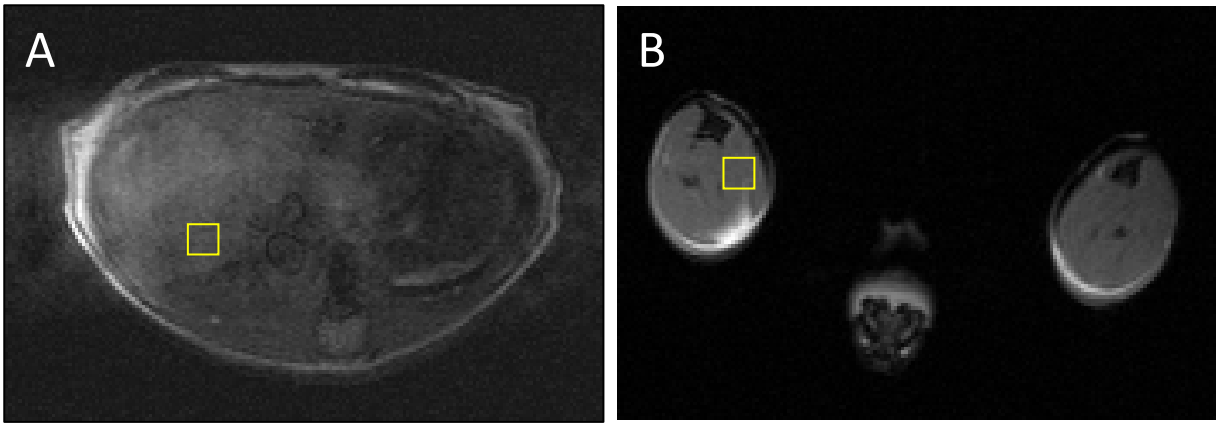


Figure 4.3 Localisation of voxel on MR images for ^{1}H MR scan.

A) Axial MRI slice through the liver, with a 2 x 2 x 2 mm^3 voxel placed on a specified section of the liver. B) Axial MRI slice through the lower legs and section of the tail, with a 2 x 2 x 2 mm^3 voxel placed on the left leg muscle. ^{1}H MR spectra will be acquired from these voxels (Figure kindly provided by Jelena Anastasovska).

SliceOmatic™ Version 4.2 (Tomovision®) segmenting visceral and subcutaneous adipose tissue deposits in order to obtain their volumes and mass. Adipose tissue appears at a brighter contrast than other tissues in this kind of anatomical T_1-weighted images (Figure 4.2 B). Pixels of this particular contrast were assigned as adipose tissue and tagged in a specific colour (Figure 4.2 C). A trained observed investigator carried out the analysis blinded to the animals' study groups.

The imaging parameters mentioned above and the number of pixels highlighted as a particular depot of adipose tissue were used to calculate the volume of tissue in that area, using the following equation:

Adipose tissue volume (mm^3) = (FOV / matrix size)^2 x number of pixels x slice thickness

Thereafter the obtained volume (mm^3) was converted into adipose tissue in g, using the density value of adipose tissue of 0.9 g/mm^3:

Adipose tissue mass (g) = Adipose tissue volume (mm3) x 0.9

Mass values were subsequently normalised by expression as a percentage of individual body weights:

% Adiposity = 100 x Adipose tissue mass (g) / body weight (g)

4.2.4.2 ^{1}H MRS of the whole body, liver and muscle

Following the whole body MRI, whole body ^{1}H MRS was conducted using a single-pulse sequence (spuls) with the following parameters: TR = 10 sec, pulse angle = 45°, 4 averages, spectral width (SW) = 20 kHz.

Thereafter ^{1}H MR Spectra of liver and muscle were acquired to determine intrahepatocellular lipid (IHCL) and intramyocellular lipid (IMCL) content. IHCL and IMCL have been shown to be a good indicator for body adiposity in both humans and rodents (Thomas et al. 2005; So et al. 2007). A 2 x 2 x 2 mm voxel was therefore placed in a specified position in the liver or in the left lower leg muscle using the axial MR images (Figure 4.3), following manual shimming on the voxel. A point resolved spectroscopy sequence (press)

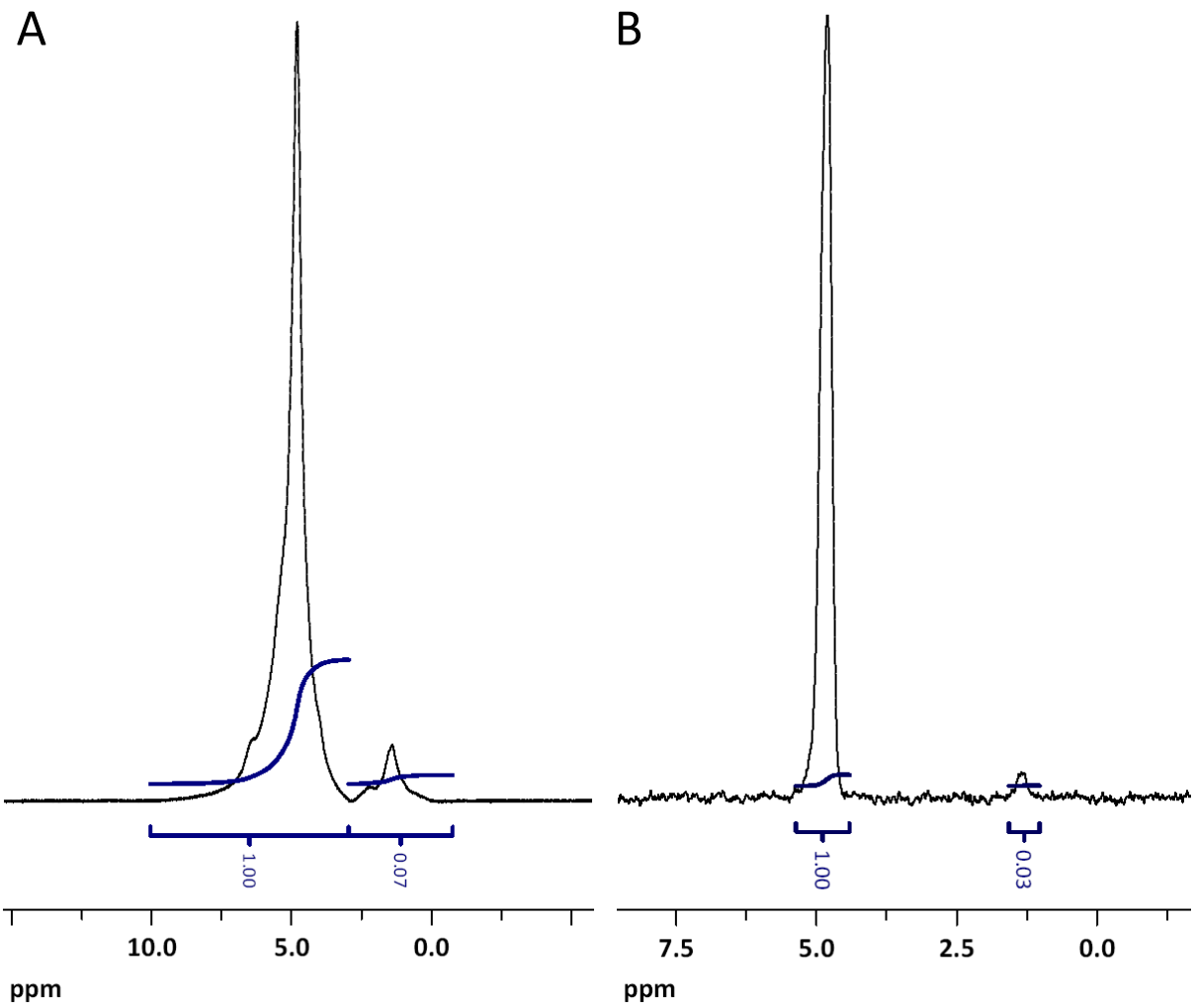


Figure 4.4 Example for typical referenced and analysed MR spectra.

A) Whole body ^{1}H MR spectrum of a mouse fed high protein diet acquired using a single-pulse sequence (spuls). B) ^{1}H MR spectrum from a voxel localised in the liver, acquired using a point resolved spectroscopy sequence (press). The chemical shift is expressed in parts per million (ppm). Both spectra underwent appropriate phase and baseline correction and integration of the lipid peak (at 1.4 ppm) was conducted in reference to the water peak (at 4.8 ppm) using MestRe-C.

with the following parameters was used: TR = 10 sec, TE = 9 msec, 64 averages, SW = 2.8 kHz.

A complete combined MRI and MRS scan lasted for about 45 minutes. After the scan the mice were recovered in a recovery box at 37 °C and monitored for at least 2 hours before placing them back in their cages.

MRS spectra were analysed using MestRe-C (MestRe-C 4.8.6, Santiago de Compostela University, Spain). Once Fourier transformation (FT) and application of an exponential line broadening of 1.5 Hz had been carried out, the spectra were manually phased and baseline corrected. Water (reference at 4.7 ppm) and lipid (at 1.2 ppm) peaks were integrated (Figure 4.4). The final lipid content values for IHCL and IMCL were determined as percentage of the water peak.

Whole body adiposity was calculated according to the following equation (Mystkowski et al. 2000):

% Adiposity = 100 x lipid integral / (lipid integral + water integral + (0.38 x water integral))

This equation takes the ratio lean mass to water of 0.38 into account. Lean mass was determined as followed:

Lean mass (g) = (100 - % Adiposity) x Body weight

4.2.5 Manganese Enhanced MRI of the appetite centres in the brain to measure alteration of the effect of oxyntomodulin by a high protein diet

In order to study if the effect of oxyntomodulin on neuronal activity in specific central appetite centres can be altered by a HP diet, manganese enhanced MRI (MEMRI) was carried out in a 9.4 T Unity Inova MRI scanner (Varian DirectDrive console, Varian Inc, USA) running VnmrJ 2.3A. In the study we injected animals fed for 6 weeks one of the two diets either 100 µl of a 1400 nmol/kg OXM solution or 100 µl saline as control. The OXM dose was

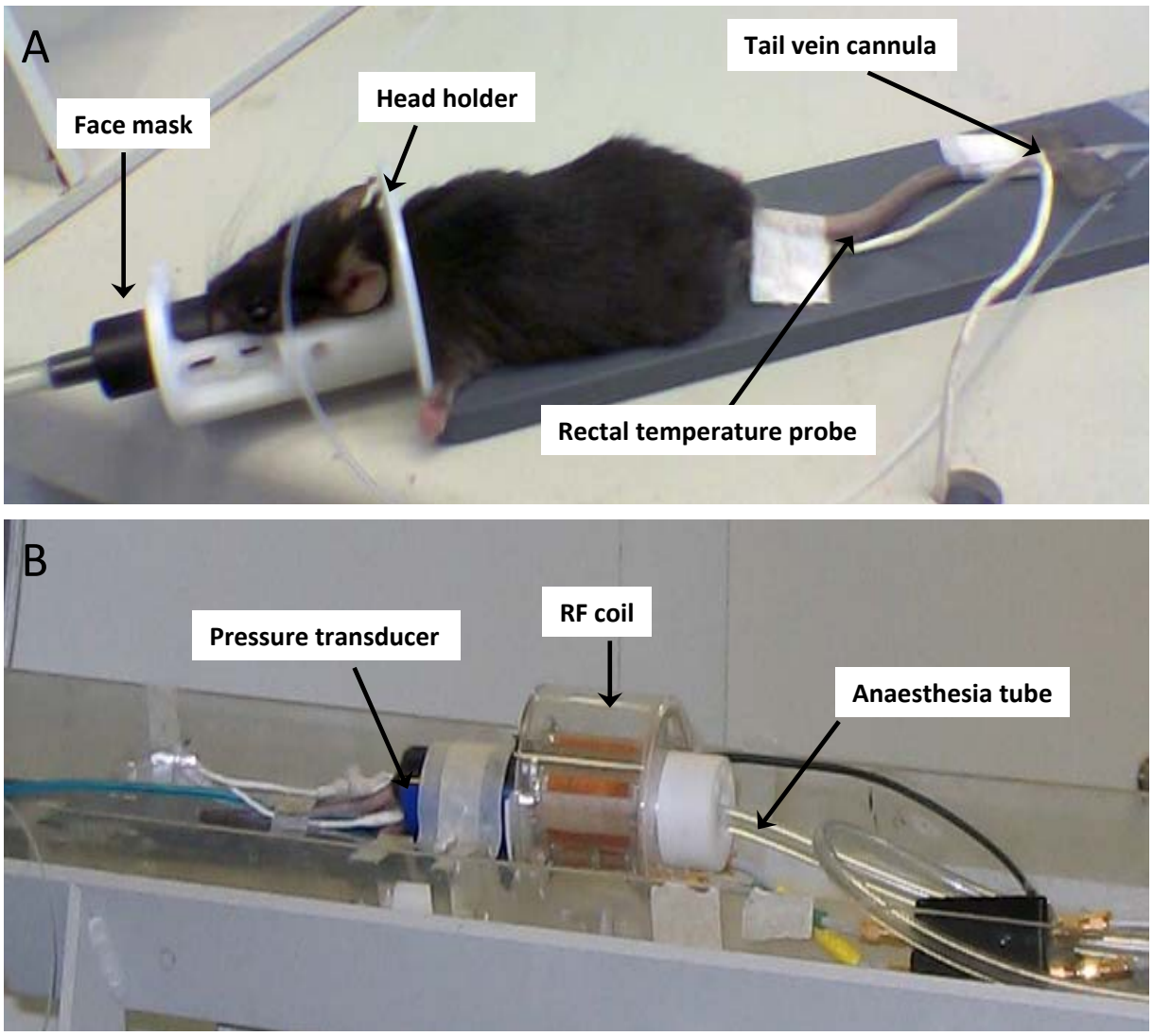


Figure 4.5 Set-up of mouse and radio frequency (RF) head coil.

A) The mouse is set-up ventrally on the bed with its head fixed in the head holder and with the tail vein cannula connected to an infusion line containing $MnCl_2$ solution. B) Mouse head holder placed inside the RF coil which will be positioned in the centre of the scanner magnet. Anaesthesia is maintained through the face mask. Breathing rate and temperature are monitored via the pressure transducer and the rectal temperature probe (Figure kindly provided by Jelena Anastasovska).

selected based on existing literature for administration of OXM to mice (Parkinson et al. 2009).

The OXM was prepared in sterile ddH_2O and stored in aliquots of 50 nmol/ 15 µl at -20 °C. The final solution was prepared in the morning of the scan.

Mice fed either a HP or a NP diet had their food removed 16 hours prior to the experiment but still had ad libitum access to water and were brought to MRI laboratory in the morning. They were anaesthetised with a 1-2 % isoflurane-oxygen mixture applied by a face mask with a flow rate of 1.5-2 L/min. During the scan mice received around 1 % isoflurane according to their respiration rate maintained at 90 to 100 breaths/min before Mn^{2+} infusion and 30 to 50 breath/minute during infusion. This was monitored by a pressure transducer strapped on the chest connected to the Physiological Monitoring Unit (SA Instruments, Inc, NY) as well as a rectal temperature probe which was used to maintain body temperature at 37.0 ± 0.5 °C via a heating system. A 1 ml syringe was prepared containing 63.2 mM $MnCl_2$ ($MnCl_2 \cdot 4H_2O$) and was connected to a 4-5 m of 0.38 µm i.d. tubing. Once a 27G butterfly needle was correctly placed in the tail vein of the mouse, the tubing from the $MnCl_2$ syringe was connected to the cannula avoiding any air bubbles. The syringe was placed in a syringe pump (PHD 2000, Harvard Apparatus, MA) and infusion rate and total volume were set once being calculated from the lean mass acquired during MRS according to the following equations:

Infusion rate (µl / hour) = 8.67 x lean mass

Total volume = 5.42 x lean mass

To be able to inject simultaneously 100 µl of either OXM or saline during the scan, a second syringe with 3 m of 0.38 µm i.d. tubing was prepared and filled with the injection solution. A 27G butterfly needle connected to the other end of the tubing was inserted i.p. and strapped on the mouse.

After preparation the mouse was placed ventrally on a bed being hold in place with the help of a tooth bar (Figure 4.5 A). The head went inside a 40 mm long 30 mm i.d. quadrature birdcage transmit-receive RF coil (Figure 4.5 B). A phantom, consisting of a glass tube

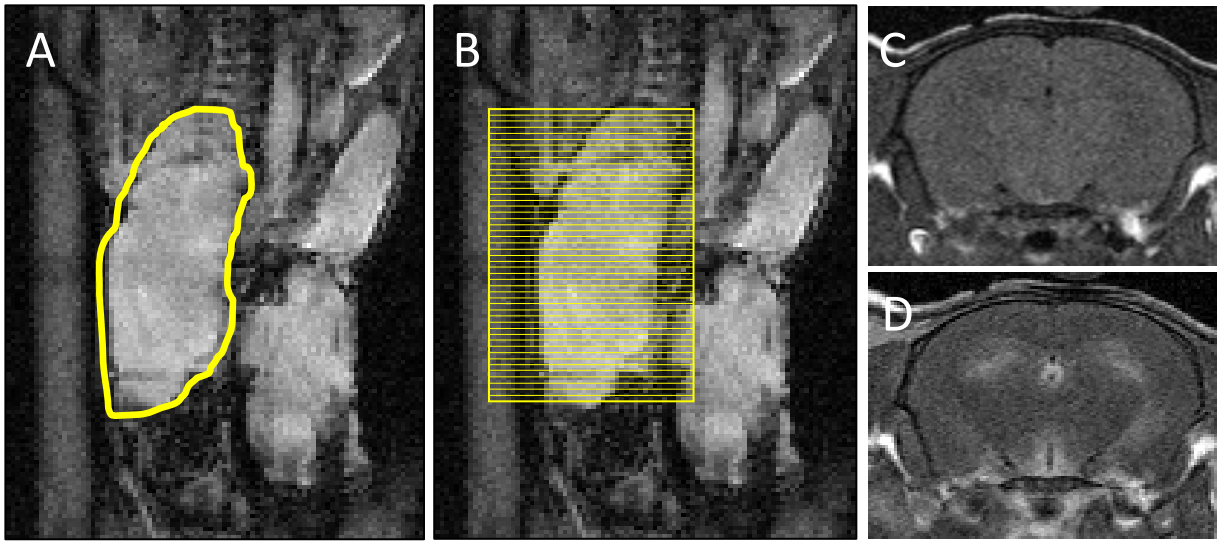


Figure 4.6 Planning of the axial MR images through the brain.

A) Sagittal pilot image through the mouse head (brain is outlined in yellow), showing the rostral ending of the brain at the bottom of the image. B) The same sagittal pilot image of the brain with the location of the 46 slices that will be acquired. C) Axial slice acquired in the region of the hypothalamus before and D) after start of the $MnCl_2$ infusion (Figure kindly provided by Jelena Anastasovska).

containing 0.9 % saline was placed into the head coil holder and scanned simultaneously with each animal. The phantom was subsequently used for normalisation of variations in signal intensity between different slices as it produces a homogenous signal.

Using the following scan parameters, fast spin-echo multislice sequence (fsems) was carried out; TR = 1800ms, TEeff = 5.6ms (echo spacing = 5.6ms, 6 echoes, k-space centre = 1), FOV = 25 x 25, matrix = 192 x 192, 2 averages.

After setting frequency and power, a spectrum was acquired and the line width measured. A rapidly acquired 3-plane gradient echo image was used to determine the correct positioning of the mouse in the coil and to position the slices for the fsems images (Figure 4.6 A, B). For shimming by FASTMAP (Gruetter 1993; Gruetter and Tkac 2000), a 4 x 4 x 4 mm voxel was placed in the centre of the brain. Another trial spectrum was acquired from this voxel, the line width measured and shimming started. This step was repeated once. Thereafter the scan was started and 46 axial images of 0.4 mm thickness (voxel size = 130 × 130 × 400 µm) were acquired per sequence lasting for 1 min 55.2 sec (Figure 4.6 C, D). The sequence was repeated 66 times, whereby after the third acquisition the $MnCl_2$ infusion was started and the i.p. injection carried out. The entire image acquisition lasted for 2 hours 6 min 43.2 sec.

4.2.5.1 Image analysis

Regions of interest (ROIs) were defined in order to compare the signal intensity (SI) profiles of the different treatment groups which in turn were dependent on the rate of accumulation of Mn^{2+} ions in the neurones of these regions. ROI definitions were made by comparing the obtained MR images to sections of the hypothalamus in the mouse brain atlas (Paxinos and Franklin 2001). The regions chosen for the analysis corresponded to the following hypothalamic nuclei: arcuate (ARC), supraoptic (SON), ventromedial (VMH) and the paraventricular (PVN) (Figure 4.7). All these nuclei are involved in the regulation of energy intake and have been implicated in the mediation of gut peptide effects in the CNS (Stanley et al. 2005). Prior to extraction of ROI data, the images were spatially normalised within and between the mice, which allows the same ROI to be used on every mouse. For this, time courses (dynamic MEMRI images) were motion corrected using SPM5 (The FIL Methods

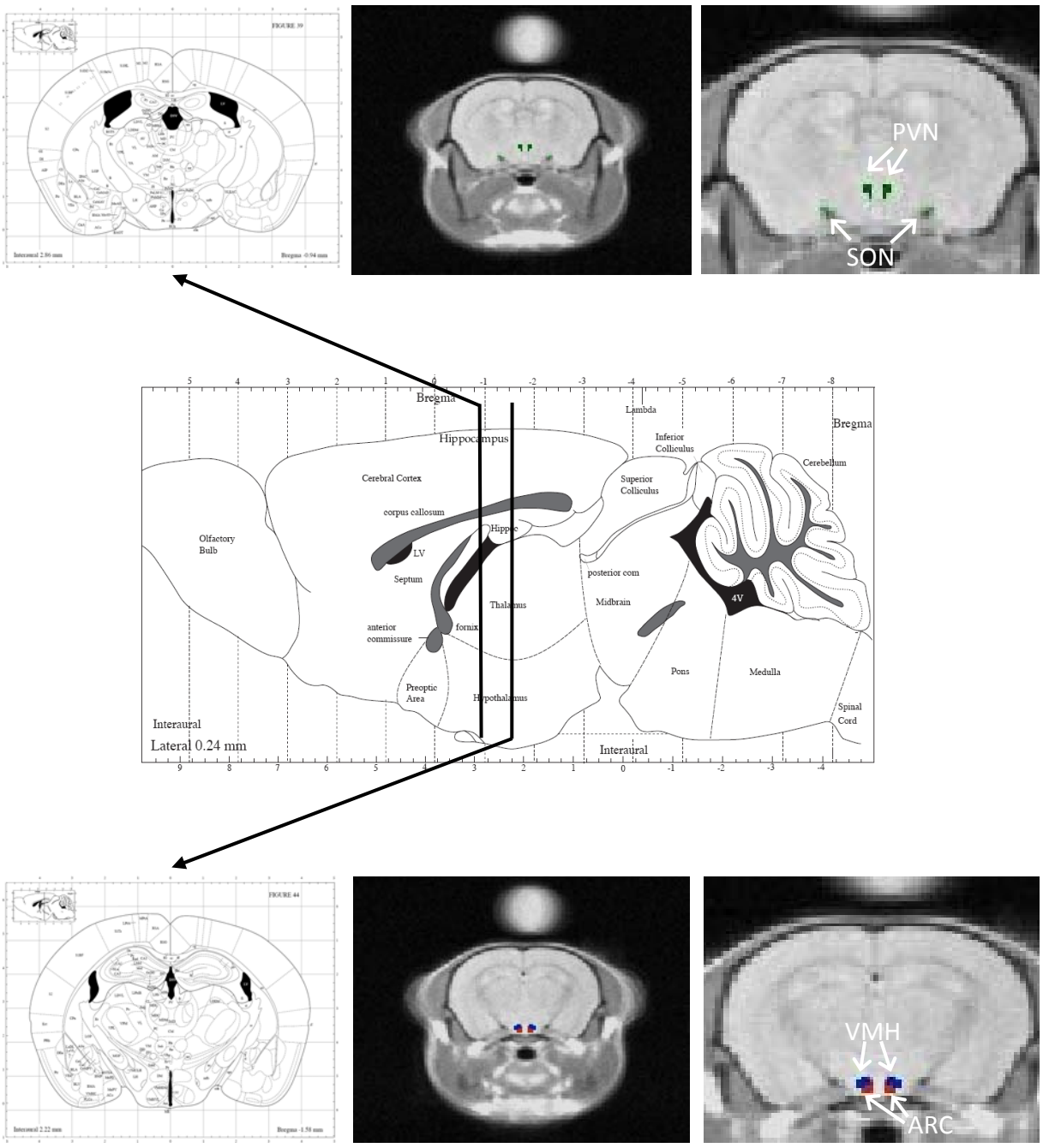


Figure 4.7 Localisation of regions of interests (ROIs) of appetite centres in the hypothalamus.

Upper part: drawings taken from the mouse brain atlas (Paxinos and Franklin 2001), images with ROIs and reference structures, zoom into ROIs: paraventricular hypothalamic nucleus (PVN, dark green), supra-optic nucleus (SON, light green). Middle part: Localisation of sections in the mouse brain shown above and below. Lower part: drawings taken from the mouse brain atlas, images with ROIs and reference structures, zoom into ROIs: arcuate hypothalamic nucleus (ARC, red), ventromedial hypothalamic nucleus (VMH, blue).

Group, 2005) and co-registered using a combination of tools from FSL (Smith et al. 2004) and AFNI (Cox 1996) to a standard mouse brain template (Dorr et al. 2008).

ROI SI data was extracted from the spatially normalised images using AFNI and exported to the statistical programming environment R (Team 2009). Here, to correct for inter-scan variance, the first three time points obtained before the start of the Mn^{2+} infusion and i.p. injection were used as baseline values, and the ROI data was converted to percentage change over baseline.

4.2.6 Statistical analysis

Statistical analysis was carried out using R statistical software (R version 2.8.1) (Team 2009). For all experiments values of $p<0.05$ were considered as statistically significant.

Data for food intake and body weight was analysed with a two-way ANOVA with the repeated measures method, whereby time being the repeated measure variable and diet being the main effect. Total BWG within a dietary group was compared using paired t-tests and between the groups using Welch Two Sample t-test. Food intake and cumulative food intake were calculated as food intake (kJ) per mouse and are expressed as mean ± SEM. It was analysed using two-way ANOVA with the repeated measures method, also with time as the repeated measure variable and diet as main effect.

In body composition measurements percentage of fat in the different compartments was compared between groups using the Wilcoxon rank sum test.

For data from MEMRI experiments group comparisons were made using linear mixed-effects modelling with the mouse experimented on as a random effect, and a model of Mn^{2+} uptake as an independent explanatory variable. This model was derived from a brain-only MELODIC analysis (Beckmann and Smith 2004) of separately acquired dynamic MEMRI data.

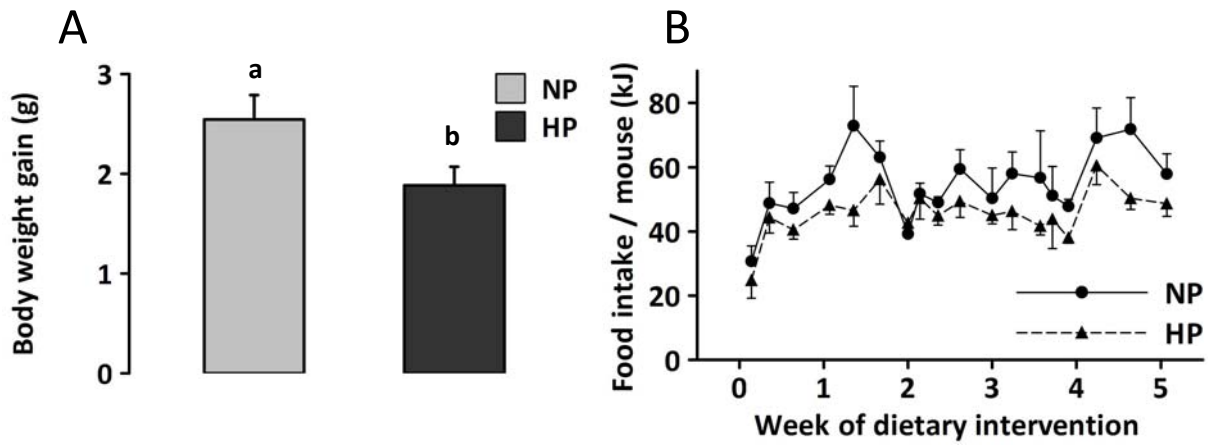


Figure 4.8 Effect of a high protein diet on body weight and food intake.

A) Body weight gain in normal protein (NP) and high protein (HP) fed mice over 5 weeks of dietary intervention. B) Food intake in 5 weeks of HP and NP fed mice in kJ. Data presented as mean ± SEM.

4.3 Results

4.3.1 Effect of a HP diet on body weight and food intake

Mice fed the NP diet had an initial body weight of 23.6 g ± 0.5 g and a final body weight of 25.7 g ± 0.7 g while mice fed a HP diet initially weight 23.1 g ± 0.4 g and had a body weight of 24.9 g ± 0.5 g after 5 weeks. Feeding mice the HP or the NP diet did not lead to significant differences in body weight (p=0.1922) over the 5 weeks of intervention. However, mice fed the NP diet gained significantly more weight in the 5 weeks of intervention (p=0.0170) than mice fed the NP diet (Figure 4.8 A).

There was a tendency for a difference in food intake between the two groups of mice (p=0.0784) (Figure 4.8 B). In average mice fed a normal protein diet ate 54.0 kJ ± 6.7 kJ per day and mice fed a high protein diet ate 45.2 kJ ± 4.6 kJ per day.

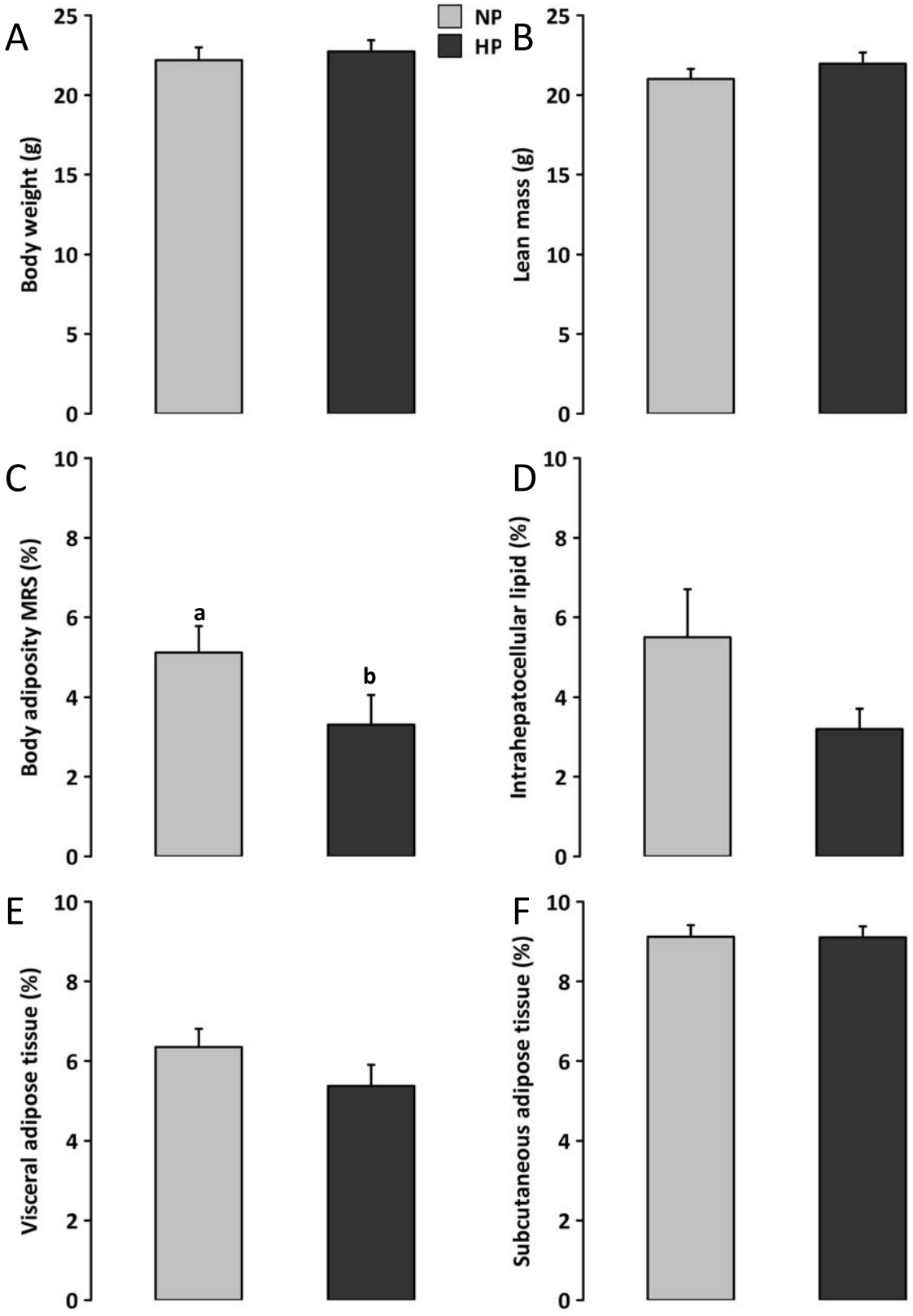


Figure 4.9 Body composition of mice fed a normal protein (NP) or a high protein (HP) diet.

A) Body weight at the day of the experiment. B) Lean mass. C) Percentage of adiposity in whole body measured by MRS. D) Percentage of intrahepatocellular lipids (lp). E) Visceral adipose tissue in percentage of body weight. F) Subcutaneous adipose tissue in percentage of body weight. Data presented as mean ± SEM, analysed by Wilcoxon rank sum test. n=10 for each treatment group.

4.3.2 Effect of a HP diet on body composition

Weight at the day of the MR scans, after an overnight fast, was 22.2 g ± 0.8g and 22.7 g ± 0.7 g for NP diet and HP diet fed mice respectively and did not differ significantly (*p*=0.2601) (Figure 4.9 A). Lean mass was calculated from weight and percentage of body adiposity and was found to be 21.0 g ± 0.6 g in mice fed a NP diet while lean mass of mice fed a HP diet was 22.0 g ± 0.7 g (*p*=0.1534) (Figure 4.9 B). MRS revealed a significant higher mean body adiposity of 5.1 % ± 0.7 % for mice fed a NP diet compared to 3.3 % ± 0.7 % for mice fed a HP diet (*p*=0.0506) (Figure 4.9 C).

In the liver the relative amount of intrahepatocellular lipid (IHCL) was 5.5 % ± 1.2 % for mice fed a NP diet and 3.2 % ± 0.5 % for mice fed a HP diet (Figure 4.9 D). There was a tendency for a significant difference between the two diets (*p*=0.0899). Intramyocellular lipid was not detectable in the muscle of mice from both groups.

Using MRI we found that visceral adipose tissue deposits were 6.4 % ± 0.5 % of the body weight for NP fed animals and 5.4 % ± 0.5 % for animals fed a HP diet. There was a tendency for a significant difference in visceral adipose tissue between the two groups (*p*=0.0560) (Figure 4.9 E). Regarding subcutaneous adipose tissue, they were 9.1 % ± 0.3 % of the body weight for NP fed animals and 9.1 % ± 0.3 % for animals fed a HP diet (*p*=0.4398) (Figure 4.9 F).

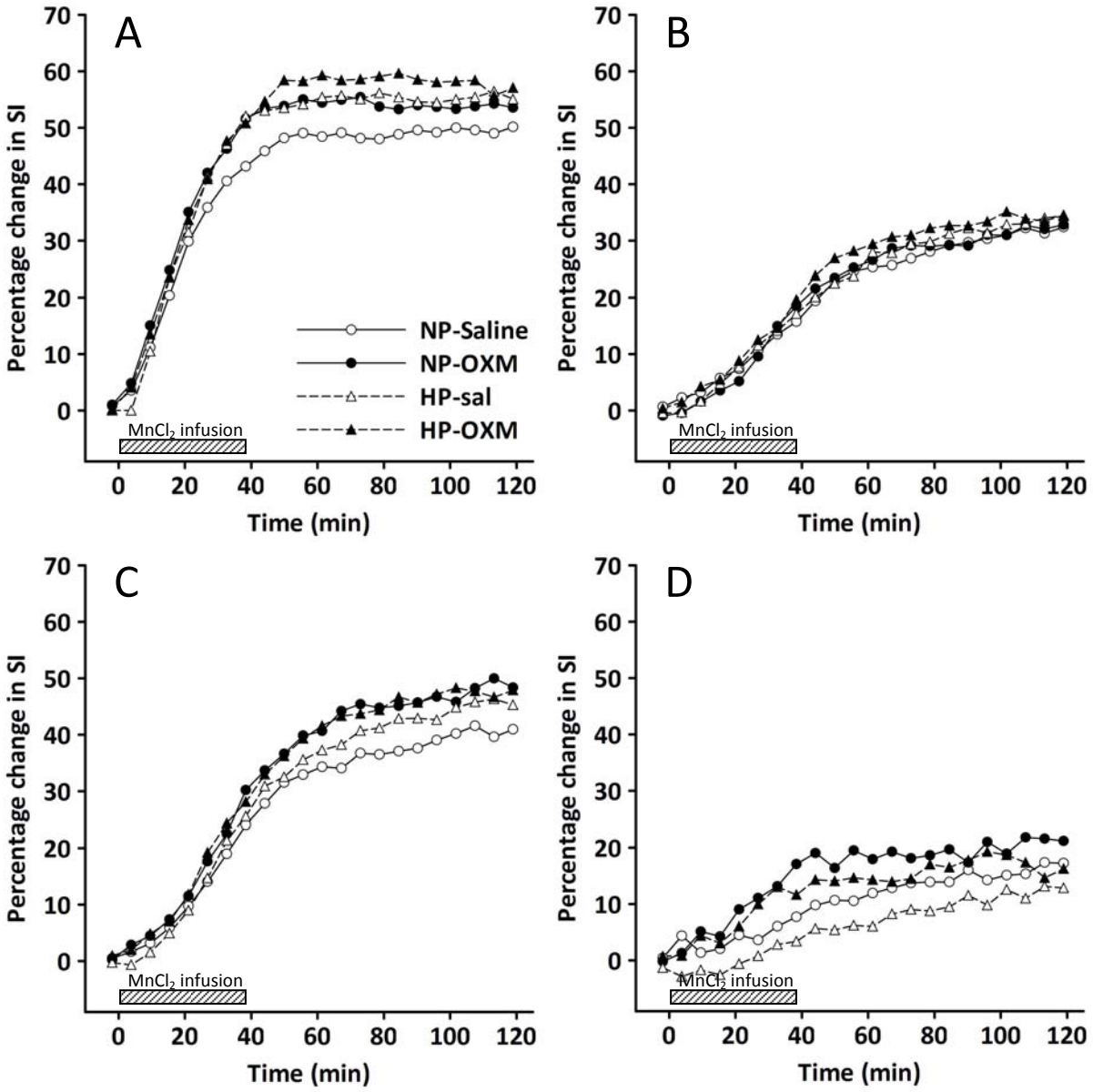


Figure 4.10 Time course of change in signal intensity (SI) as percentage of baseline following i.v. $MnCl_2$ infusion in NP and HP fed mice after injection of 100 µl OXM (1400 nmol/kg) or saline.

A) Arcuate hypothalamic nucleus (ARC), B) paraventricular hypothalamic nucleus (PVN), C) ventromedial hypothalamic nucleus (VMH), D) supraoptic nucleus (SON). The bar indicates the duration of the $MnCl_2$ infusion. Oxyntomodulin (OXM) or saline injection was given at 0 min. Data are presented as mean of three consecutive image acquisitions and were compared using LME. (n=8 for NP fed animals and n=7 for HP fed animals)

4.3.3 Alteration of the effect of oxyntomodulin by a high protein diet

Time courses of changes in SI displayed as percentage change from baseline were compared in the three hypothalamic nuclei, arcuate (ARC), paraventricular (PVN), ventromedial (VMH) and in the supraoptic nucleus (SON). Neither diet (NP vs. HP) nor treatment (saline vs. OXM) affected changes in SI in any of the studied brain regions (Figure 4.10). In Table 4.1 *p*-values of comparisons are summarised.

In the ARC, SI increased rapidly during the $MnCl_2$ infusion and reached 50-60 % over baseline value (Figure 4.10 A). SI in the VMH increased slower than in the ARC and reached a maximum of 40-50 % (Figure 4.10 B). In the PVN percentage of SI increased slower and later reached a plateau of only 30-40 % of change in SI (Figure 4.10 C) while in the SON, located the furthest from the ARC, SI increased the slowest and reached only 10-20 % change in SI (Figure 4.10 D).

Table 4.1 Comparison of changes in percentage SI over time by LME following administration of either OXM or saline to mice fed a HP or NP diet.

	Region of interest			
	ARC	PVN	VMH	SON
Injection (saline vs. OXM)	0.6662	0.6015	0.4983	0.0731
Diet (NP vs. HP)	0.6674	0.4691	0.9823	0.4472
Interaction injection X diet	0.7230	0.7436	0.6705	0.7321

Values are representing *p*-values obtained by LME from statistical comparison of the SI time courses of mice fed either a HP or a NP diet and got either an OXM (1400 nmol / kg) or saline injection.

4.4 Discussion

4.4.1 Effect of a HP diet on body weight and food intake

Mice fed either a high protein (55 % of energy from protein) or a normal protein (14 % of energy from protein) diet over a time period of 5 weeks significantly gained weight, but did not develop different body weight during the feeding period. The absolute weight gain from the day of the start of the intervention to the last day of the 5 weeks period in contrast was significantly different. Comparing the food intake of mice in both dietary groups, there was a tendency of a higher intake for animals being fed the NP diet, but no significant differences.

Protein is known to have a more satiating effect than other macronutrients and therefore reduces food intake (Jean et al. 2001; Weigle et al. 2003; Meckling and Sherfey 2007; Tome et al. 2009). Rats fed a HP diet were found to decrease their food intake compared to rats being fed a NP diet, especially on the first days of diet and also express a lower body weight gain then the control group (Jean et al. 2001; L'Heureux-Bouron et al. 2004; Lacroix et al. 2004; Pichon et al. 2006). Feeding rats a high protein and at the same time low carbohydrate diet resulted equally in a lower food intake and decreased body weight compared to the control group (Kinzig et al. 2007). However, these findings are very dependent on the amount of protein used for the studies. While for the above mentioned experiments the HP diet contained around 50 % of protein, Baum et al using a diet with 30 % protein found an effect on final body weight but not on food intake (Baum et al. 2006). This suggests, similar to our findings, that the effect of a high protein diet on the metabolism and body composition is not only due to a decrease in food intake, but also involves other, satiety-independent metabolic changes.

Yet, all these studies have been carried out in rats, and little knowledge is available on the effect of HP diet on mice. Investigating the effect of a high energy diet in mice by either increasing the amount of fat or fat and protein could not confirm any effect of the augmented protein on body weight, body composition or food intake (Corva and Medrano 2000). Some previous work has shown that mice fed a HP diet had a decreased daily energy intake and body weight compared to mice fed a NP diet (Batterham et al. 2006; Nefti 2009).

Though, the study by Nefti et al. used a different mouse strain and the intervention done in 4 weeks old mice lasted only two weeks (Nefti 2009).

4.4.2 Effect of a HP diet on body composition

During the 5 weeks dietary intervention period prior to the MR body scan, feeding the mice the HP diet did not alter their final body weight and lean mass compared to the animals fed the control diet. In contrast, body adiposity measured by MRS of HP fed mice was significantly lower than in NP fed mice. For the other investigated parameters, namely IHCL and visceral adipose tissue, there was a tendency for a lower amount in the HP fed mice.

Similar results were found in other rodent studies, for instance Jean et al showed that rats fed a HP diet were found to have 50 % less adipose tissue than the control animals (Jean et al. 2001). Batterham et al could confirm these results in mice fed a HP diet (Batterham et al. 2006). These findings are of great interest as it has been suggested that body fat distribution is a better predictor for cardiovascular diseases than obesity *per se* (Corva and Medrano 2000). No data is available yet to compare the effect of HP diet on IHCL in mice. In rats in contrast, in two groups fed either a HP or a NP diet during six months, only the NP fed animals developed liver steatosis revealed by dissection, while the livers of the HP ones did show no sign of it (Lacroix et al. 2004).

Our results therefore suggest that HP diet has an effect on energy metabolism. Studies in rats revealed adaptation of the energy metabolism to a diet with increased protein intake at the expense of carbohydrate. These conditions led to increased energy usage from amino acids as well as *de novo* liver glucose production. At the same time glycolysis and lipogenesis were decreased (Pichon et al. 2006; Stepien 2010).

4.4.3 Alteration of the effect of oxyntomodulin by a high protein diet

Comparing time courses of percentage change in SI over baseline values following $MnCl_2$ infusion, neither diet nor peptide injection lead to a significant difference in the hypothalamic nuclei ARC, VMH and PVN as well as in the SON.

The hypothalamus is known to be the key structure in the CNS controlling food intake and body weight (Schwartz et al. 2000). It was shown previously that feeding mice a high

protein diet altered MEMRI-associated signal in the PVN and the lateral hypothalamic area in comparison with a normal protein diet (Zeeni et al. 2009). In addition, the hypothalamus is currently considered as the main action site of OXM to inhibit food intake in both humans and rodents (Stanley et al. 2005). The hypothalamic nuclei studied in these experiments were chosen as they were shown to express the GLP-1 receptor with specific affinity to OXM (Wei and Mojsov 1995). Immunohistochemistry studies on Fos revealed a decrease in activity in the VMH of rats that were fed a HP diet compared to those fed a NP diet (Darcel et al. 2005a). However, in the present study feeding mice a HP diet for 6 weeks did not alter their brain activity in the hypothalamus nor their response to OXM.

The fact that we could not detect any difference in the change of SI in the ARC after injection of OXM and saline might be explained by an actual absence of activity in any of the neurones. On the other hand, the measured SI reflects the net changes in neuronal activity. The investigated hypothalamic structures consist of different subsets of neurones. For instance in the ARC, it is thus possible, that the food inhibitory subset of POMC/CART neurones was activated by OXM, but the signal was compensated by the inhibited activity of the orexigenic NPY/AgRP neurones which lead to a net compensation of the signal.

Although MEMRI studies have many advantages such as being able to measure brain activity time courses *in vivo*, the low regional resolution is a clear inconvenience. We can also not exclude that the similar time courses occurred due to diffusion into the brain and not an active, activity-dependent uptake. Carrying out immunohistochemistry studies and for instance co-labelling the nuclear protein Fos and the two different neurone populations in the ARC can help to understand if these neurones are actually activated by an injection of OXM. However, this technique has its limitations as well because only one timepoint per animal can be studied. It would therefore be interesting to carry out further MEMRI studies in combination with immunohistochemical labelling in order to examine if our findings occurred due to compensation or a missing effect of OXM in the investigated regions.

4.5 Conclusion and perspectives

Numerous publications demonstrated an effect of a HP diet on food intake, body weight gain and body composition in rats; however, in the present study we could only partly confirm effects on body weight gain and body composition in mice. This suggests that the changes detected in this study are independent from the satiating effect of protein. On the other hand metabolic changes are likely to take place which are not yet reflected in total body weight, but already in the amount of adiposity.

However, one could question if the results and diets used in rats can easily be transferred to mouse studies. Yet, mice, if given a choice in composing their meals, were shown to feed 15-20 % of their total energy intake from protein but also only around the same amount from carbohydrates (Smith et al. 2000). The rest of the energy mice in this study gained from fat (around 60-70 %) due to its higher palatability (Brain et al. 2000). For that reason it is arguable if even the NP diet used in this study is correct for the amount of protein, the fat might play a more crucial role in mice compared to the data available for rats. South et al showed that mice preferred feeding from a high fat diet obtaining 11 % of the energy from fat (South and Huang 2006). Even standard mouse chow also only contains 3-3.5 % energy from fat, it is to consider if the composition of our used diets is appropriate for mice. A diet more adapted to the needs and preferences of mice may thus be used in studying the effect of proteins in these rodents.

Regarding the investigation of HP altering the effect of OXM on hypothalamic appetite centres, there was not only no influence of the diet, but also a lack of an effect from the OXM injection. In future experiments the biological activity of the used peptide should be examined. Moreover it should be verified if OXM does indeed not affect neurones in the hypothalamic nuclei or if the lack of measureable difference is due to a compensation of activated and inhibited neurones in separate populations. For this reason, immunohistochemical studies should be carried out in parallel to answer the raised questions.

5 General conclusion and perspectives

My work consisted in studying some of the satiety mechanisms of central integration of dietary protein signalling along the gut-brain-axis and was carried out on three levels: first the investigation if hepatic vagal afferents are necessary for the high protein induced depression of food intake, second in the dorsal vagal complex short-term signalling of dietary protein present in the intestine during digestion and third the modulation of body composition and the effect of oxyntomodulin, a specific gut peptide strongly believed to play a major role in food intake depression induced by dietary protein.

Hepatic portal vein deafferentation did not alter the satiating effect of a HP diet in rats

It is still unknown in which way high protein induced decrease of food intake occurs. One possibility is the sensing of amino acids, lipids and glucose in the liver and signal transmission to the brain via the vagus nerve, which had been shown to be activated by protein hydrolysates and is thus sufficient for signal transmission. In order to investigate if it is also necessary, we studied if partial vagal denervation at the level of the hepatic portal vein is able to abolish the reduction in food intake induced by a high protein diet. Our results showed that even after denervation of the portal vein, rats decreased their 1h and daily food intake when switched to the high protein diet exactly as the sham operated controls. Also body weight gain did not differ between the groups.

These results suggest that in the case of disrupted vagal afferent signal transmission other, compensatory mechanisms are set on. Possible compensatory mechanisms might be, amongst others, the release of gut peptides such as CCK and GLP-1. These hormones are released from enteroendocrine cells in the intestine during protein ingestion and could help to bypass the missing vagus nerve by acting either directly on central structures of feeding control or bind to other, intact vagal afferents in different locations. Moreover, absorbed amino acids, also circulating in the blood in higher concentrations after ingestion of a high protein meal, can reach the brain and directly activate structures such as the ARC which misses BBB. Finally, metabolic signals, including an increase in energy expenditure and the production of glucose through gluconeogenesis, have been also hypothesized as signals for protein and amino acids.

On top of this, during a high protein diet the vagus nerve is possibly not only involved in satiety signal transmission, but also in important digestion processes. Ingestion mechanisms such as gastric and intestinal peristaltic might be modified due to missing vagal input at central structures. Resulting malabsorbtion and intestinal discomfort might lead to a similar feeding pattern in capsaicin compared to sham operated rats.

In order to further investigate the implication of the vagus nerve in high protein induced food intake depression, it would be of interest to compare the effect of the interruption of both afferent and efferent hepatic vagal fibres at the level of the portal vein to our findings. Moreover, it should be verified if portal vagal efferents are indeed not disrupted during the capsaicin treatment and surgery in order to exclude that our findings are a result of side effects rising from vagal efferent signal interruption.

Central cartography of macronutrients' internal sensibility

Prior to absorption and after enzymatic breakdown, all the macronutrients, protein, carbohydrate and fat, are sensed in the intestinal tract. This sensing appears macronutrient specific, so are for instance the peptide transporter PEPT1, specific GPCR and a large number of carrier proteins responsible for the detection and uptake of peptides and amino acids. The nutrients then lead to activation of different signalling systems, directly or via the release of gut peptides. Protein is known to be a strong stimulator for the release of CCK, but also of GLP-1 and PYY. Depending on the nature of the nutrients ingested, the plasma profile of these peptides can vary. On the one hand, both gut peptides and absorbed circulating nutrients then stimulate afferent fibres of the vagus nerve which transmit signals to the NTS. On the other hand, they directly stimulate central structures themselves after being transported to the brain via the blood stream. Still it remained unclear if the signal transmission and integration in the dorsal vagal complex is also nutrient specific. Classical methods looking only at the number of activated neurones within these structures were not able to answer this question satisfactory. The novel approach using three-dimensional mapping of the distribution of activated neurones enabled to visually distinguish NTS density maps of mice from different treatment groups. Stimulus specific activation maps have already been presented for other sensory processes such as audition, olfaction as well as tactual sensing. Our density maps from protein and carbohydrate gavaged animals were

shown to overlap to some extends but still do not occupy exactly the same region within the NTS. It can thus be suggested that intestinal signalling from the studied macronutrients protein and carbohydrate is specifically integrated in the NTS.

A hypothesis which could explain the macronutrient specificity is for instance the viscerotropic organisation of the NTS which was demonstrated by the use of retrograde labelling techniques. According to this theory vagal projections arriving in the NTS are derived from distinguished sites within the GI tract. Due to their different effect on gastric emptying, protein and carbohydrate may reach the intestine at different topographic levels with different intensities after gavaging. Not being detected at the same site fort he same duration in the small intestine, macronutrients could activate a different region in the NTS according to the shown topical organisation.

Moreover, as protein and carbohydrates were shown to lead to different plasma hormone profiles, these circulating gut peptides might also play a role in the nutrient specific activation. Receptors for these peptides are found on the one hand along the vagus nerve and, on the other hand, in brain feeding centres. A modified plasma profile might therefore result in different neuronal activity. In the NTS different subsets of neurones were shown to be present which then express for example GLP-1 or POMC (Vrang et al. 2003; Fan et al. 2004) and project to distinct hypothalamic subnuclei. To study if protein and carbohydrate lead to activation of these different neurone subsets, double labelling immunohistochemical studies on GLP-1 and POMC identifying the neurone populations would have to be carried out.

The idea of having nutrient specific satiety signalling in the NTS may help to develop a better understanding of short-term food intake regulation and meal size and can for instance also be used for studying in which way different diets can modulate protein signalling. These kinds of studies were already carried out using MRI, however, the resolution of this technique is very low and different subsets of neurone populations can not be distinguished. Using immunohistochemistry in combination with 3D activation maps will make it possible to exactly identify which type of neurones is activated but which stimulus and where these neurones are located. As soon as statistical tests that can be applied to the three-

dimensional density maps will be available, it will be possible to draw clearer and more objective conclusions from these findings.

Modulation of gut peptides by a high protein diet

Diets high in protein do not only have an effect on short-term satiety signalling, fed on long-term they were shown to influence body composition by decreasing body adiposity and promote weight loss or at least weight maintenance. Changes in body weight and food intake of rats in response to a high protein diet often have been demonstrated, in the here presented study these differences could not be found in mice. However, although having the same final body weight, mice fed a HP diet developed lower body adiposity compared to their control group and there is a tendency that they also express a lower lipid level in the liver. These findings might be explained by metabolic changes which occur when dietary carbohydrates are replaced by protein, rather than by the satiating effect of protein. When feeding animals a high protein diet at long-term, in the liver PEPCK, the first regulated enzyme in gluconeogenesis, is up regulated while genes involved in lipogenesis are down regulated. These metabolic changes induced by long-term exposure to a high protein diet might thus not be obviously visible by only regarding body weight and food intake. Lower body adiposity can increase the metabolic turnover and therefore initially lower food intake might be compensated by higher energy needs.

In order to study which kind of metabolic changes are actually occurring it would be of great interest comparing metabolites in plasma, urine or the liver of animals fed a normal and high protein diet. Especially the liver would be an organ that is important to look at, as there was a tendency for lower lipid contents in the high protein fed rats and the liver is, also regarding other studies, the organ where important changes in metabolism are expected. Additionally, in order to screen for other target genes in liver and gut metabolism which can be modified by a high protein diet that have not been detected yet, transcriptomic techniques could be used. Feeding animals a high protein diet for different time periods would make it possible to observe the metabolic changes over time on a transcriptome level. In order to investigate functional changes, the detected modifications in the transcriptome could be subsequently verified on a protein level.

Regarding the investigation of HP altering the effect of OXM on hypothalamic appetite centres, there was not only no influence of the diet, but also a lack of an effect from the OXM injection. A longer dietary intervention period, maybe with younger animals at the start, would be useful to be carried out in combination with the just mentioned genomic techniques investigating if there is a change in gene expression in distinct brain regions. In future experiments also the biological activity of the used peptide should be examined and it should be verified if OXM does indeed not affect neurones in the hypothalamic nuclei or if the lack of measureable difference is due to a compensation of activated and inhibited neurones in separate populations. For this reason, immunohistochemical studies using double-labelling of different neurone populations should be carried out in parallel to answer the raised questions.

The above presented techniques of three-dimensional reconstruction and activity mapping of immunohistochemical data are good means to further investigate the modulation of brain activity by a high protein diet. Regarding a diet induced change in the sensitivity of hypothalamic nuclei in response to a peptide hormone injection, after having identified the concerned subpopulations of neurones, these data could be reconstructed using Free-D in order to compare the spatial localisation of the populations to each other. In order to define the optimal time point to do so, time courses done with MRI could be used. It will therefore be possible to better localise not only the action site and impact on activity of the hormone, but also if this site undergoes structural changes induced by a high protein diet.

It can be concluded that sensing and signalling of dietary protein indeed plays a crucial role in the control of food intake and energy balance. Nevertheless many questions are still not solved and it is difficult to conclude from rodent studies to applications in obesity control in humans. The high percentage of protein used in these studies extends largely the current protein intake. Although an increase in protein in the daily diet would be desirable for the various reasons discussed here, other physiological problems have also to be taken into consideration and are currently discussed controversially. A life-long increased protein intake has been correlated with a negatively influence on kidney physiology as well as bone demineralisation. It is therefore probably more reasonable instead of promoting a permanent diet enriched in proteins to focus on separate, high protein meals. Still, it is

necessary to develop a long-term strategy in treatment of obesity in which the satiating effect of dietary protein will find its place.

6 Annex

6.1 Composition of the experimental diets[1]

	Normal protein diet (NP)	High protein diet (HP)
Metabolizable energy, *kJ/g*	14.6	14.6
	g/kg dry matter	
Total milk protein[2]	140	530
Cornstarch[3]	622.4	286.2
Sucrose[4]	100.3	46.5
Soybean oil[5]	40	40
AIN 93M mineral mix[6]	35	35
AIN 93V vitamin mix[6]	10	10
a-Cellulose[7]	50	50
Choline[6]	2.3	2.3

[1] All dietary components were purchased (sources given below) or prepared by the A.P.A.E. (Atelier de Preparation des Aliments Expérimentaux, French National Institute of Agronomic Research, INRA, Jouy en Josas, France).
[2] Nutrinov, Rennes, France.
[3] Cerestar, Haubourdin, France.
[4] Eurosucre, Paris, France.
[5] Bailly SA, Aulnay-sous-bois, France.
[6] ICN biochemicals, Cleveland, OH.
[7] Medias filtrants Durieux, Torcy, France.

6.2 Used R codes

6.2.1 Chapter 2

Body weight: gain:

Student t-test

t.test(x, y, alternative="greater", paired = FALSE)

Food intake:

Two-way ANOVA with repeated measures

model=lme(intake ~ day + diet, random=~1|rat, na.action=na.omit)

6.2.2 Chapter 3

Number of Fos-positive neurons:

Kruskal-Wallis rank sum test

kruskal.test(list(pep, sac, h2o))

Wilcoxon rank sum test

wilcox.test(sac,pep, alternative="greater")

Calculation of density curves using a Gaussian kernel function with a bandwidth of 10 µm:

a<-scan("C:/… / … .txt", sep=",")

write(round(density(a,bw=10)$y,5),"C:/… /… .txt",sep=",", ncolumns=1)

6.2.3 Chapter 4

Food intake and body weight gain:

Two-way ANOVA with repeated measures

model=lme(intake ~ day + diet, random=~1|cage, na.action=na.omit)

Body and liver adiposity:

Wilcoxon rank sum test

wilcox.test(HP, NP, alternative="greater")

MEMRI time courses:

*SI=IBBBmodel+diet*injection, random=1/mouse*

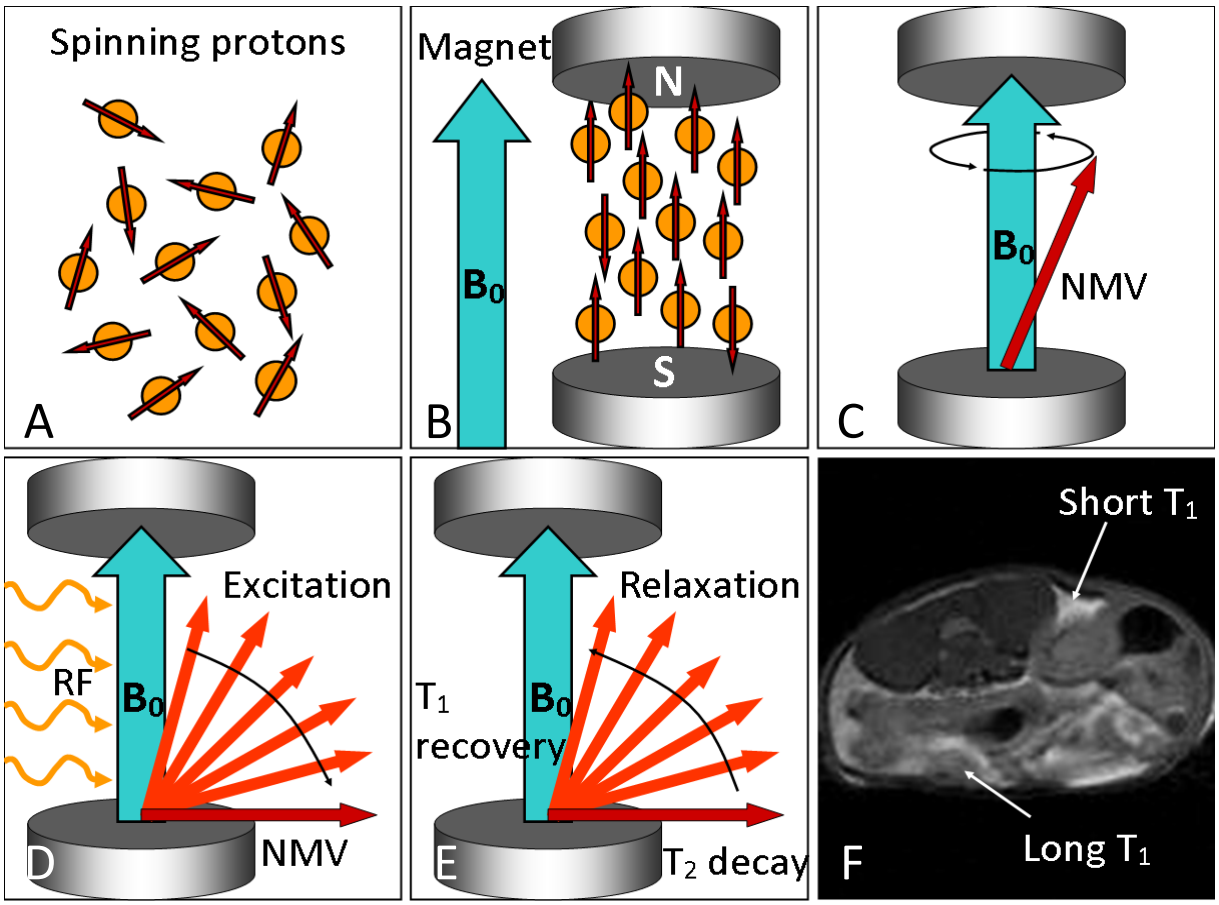


Figure 6.1 Principle of the magnetic resonance technique.

A) Randomly spinning protons. B) Protons aligning with the external static magnetic field B_0. C) Net magnetisation vector (NMV) from combination of aligned spins precessing around B_0. D) Excitation of NMV by application of a radio frequency (RF) pulse at 90 °. E) Relaxation of NMV after stopping the RF pulse with T_1 recovery to the longitudinal plane and T_2 decay along transversal plane. F) Example for long and short T_1 recovery resulting in low and high signal intensity respectively (Adapted from Pautler 2004).

6.3 Principle of the magnetic resonance technique

Some elementary particles as well as nuclei in organic structures are charged due to an odd mass number. If they additionally have a spin they are inducing a magnetic field. In Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectroscopy (MRS) are two sub forms of the Nuclear Magnetic Resonance technique these kinds of nuclei are sensitive to. They are appropriate for solids and in both signal from hydrogen (^1H) is studied because of its proportionally large presence in organic tissue as well as its large magnetic moment.

In the scanner, an external static magnetic field (B_0) is applied to the sample what makes all spins, prior randomly orientated (Figure 6.1 A), aligning with the magnetic field lines of B_0 (Figure 6.1 B) Combining all the spins aligned with B_0, they result in a net magnetisation vector (NMV) precessing around B_0 with a specific frequency which falls into the radiofrequency (Figure 6.1C) (Pautler 2004). Subsequently applying a RF pulse at 90 ° to the NMV, the vector absorbs the energy and its precessing direction is changed from the longitudinal to the transversal plane as long as the RF pulse lasts (Figure 6.1 D). This process is known as excitation. As soon as the RF pulse is stopped, the NMV recovers and flips back to the longitudinal plane where it again precesses around B_0 (Figure 6.1 E). This process is also called relaxation, whereas the recovery process to the longitudinal plane is known as spin-lattice relaxation (T_1) and the decay along the transversal plane is called spin-spin relaxation (T_2). T_1 recovery appears due to nuclei giving up energy to the surrounding environment, while T_2 decay is due to nuclei exchanging energy with other nuclei.

A receiver coil placed in the transverse plane can detect the NMV by a voltage induced in the coil when the coherent NMV cuts across this plane. This detectable voltage is the MR signal. The time between the RF pulses is called repetition time (TR) while the time from the RF pulse to the detection of the signal is known as the echo time (TE). Varying these two parameters allows adjusting the contrast for the desired purpose.

Tissue differentiation is possible due to their inhomogeneous compositions and therefore altered relaxation rates. Adipose tissue for instance, largely consisting of hydrogen and carbon, causes fast NMV recovery and consequently a short T_1 and high signal intensity (SI). Water in contrast, comprised of oxygen bond hydrogen recovers slower because of its high molecular mobility and results in long T_1 and lower SI (Figure 6.1 F).

6.3.1 ^{1}H MR Spectroscopy

In ^{1}H MR Spectroscopy the molecular composition of a tissue is explored by measuring the chemical shift of the molecules versus a reference molecule. The chemical shift can be detected as the change in the resonance frequency of the nuclei within the molecules and is expressed as parts per million (ppm), a value providing information about the molecular group carrying the ^{1}H nuclei (Mehta et al. 2008). We performed single voxel spectroscopy where 3 RF pulses select the voxel of interest, located at the intersection of the 3 orthogonal planes. The recorded echo only results from the voxel submitted to the 3 RF pulses. Protons within a lipid molecule appear on a spectrum as a peak, of which the height and width of depends on the relative amount of the lipid. Quantification of hepatic fat requires integration of the area under the peak from the two dominant peaks within the spectrum, the water peak at 4.8 ppm and the lipid peak at 1.4 -1.5 ppm.

6.3.2 Brain activity measured by Manganese enhanced MRI (MEMRI)

In order to study brain activity *in vivo*, an advanced approach of MRI had to be applied. Mn^{2+}, which is used as a contrast agent in manganese enhanced MRI, can entre the brain at the level of the hypothalamus which is close to the ME without disruption of the BBB and is taken up into neurones in the CNS via voltage-gated Ca^{2+} channels (Kuo et al. 2007). These channels are opened during depolarisation by an action potential and the accumulation of Mn^{2+} is therefore a good marker of neuronal activity.

Due to their paramagnetic nature, manganese ions have the ability to shorten T_1 relaxation time what therefore results in increased signal intensity (SI) in T_1-weighted MRI acquisitions (Kuo et al. 2005; Kuo et al. 2006). Previously it has been shown that MEMRI is a good method to study differences in neuronal activity of appetite centres within the hypothalamus without compromising the BBB as Mn^{2+} ions can entre at specific permeable regions (Kuo et al. 2006). By comparing the activity dependent uptake of Mn^{2+}, neuronal activity can be studied *in vivo* as at the same time neuronal activity was proven not to be affected by the presence of Mn^{2+} ions (Chaudhri et al. 2006).

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