

The neuroanatomical expression profile of novel membrane proteins.

The effect of macronutrients on gene expression.

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Abstract:

Worldwide obesity is an increasing problem. Apart from the fact that obesity greatly impairs the health, quality and length of life for the affected individuals, it is also has the potential to become a major socioeconomic problem in a near future. However preventive actions require an understanding of the cause. Before the psychological influence on eating can be evaluated a profound understanding of the biological regulatory system and how this interacts with the food consumed is required. On the assumption that food consumption is regulated by interplay between food and genes, the food itself may influence the genes that regulate consumption, hence change the expression levels of the genes regulating food intake.

To evaluate the interplay between food and gene expression, the project contained several parts, reflecting different aspects of the area of research. The feeding studies had in common that they were initial trials in a larger project. The results of these will be evaluated and used in combination with further studies.

The mice typed for food preference illustrate the complexity of the feeding regulatory system by pointing out the differences between individuals even in a relatively small group of animals. Mice in general like food high in fat and here the animals that showed a preference for sugar also showed a significant increase in their intake of chow. Since chow consists mainly of carbohydrates the results might indicate a preference not for sucrose in particular but for carbohydrates in general. The effect this may have on other studies is still unclear as further studies are needed to determine whether the difference may be the result of an innate genetic difference.

Leucine has been previously shown to reduce the total caloric intake. When given in combination with palatable food the addition of Leucine primarily reduced the intake of chow. From a dietary perspective this would translate to a preference to sweets and fast food at the expense of food with more nutritious content.

The RT-PCR analysis's gives clues to how the energy regulatory circuitry responds to the intake of selected macronutrients. When it comes to gene expression there is a significant effect of macronutrients on the gene expression levels. The common theme for many of the genes tested seems to be down regulation of satiety signals, as if to support over feeding on palatable diets and in many cases sucrose in particular.

The intake of macronutrients such as sugar or fat has been showed to have an effect on the feeding regulatory circuitry, demonstrated by the change in gene expression levels. The response to said macronutrients is site specific which is clearly shown both by RT-PCR analysis of samples from different parts of the brain, such as the brainstem or hypothalamus, and by immunohistochemistry of selected areas. The immunohistochemistry also confirms that the novel Oxytocin receptor-antagonist, who is injected IP, actually passes over the blood-brain barrier and has an actual affect on the regions of interest. The areas affected by the antagonist can be visualized and identified through the staining of active sites.

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INTRODUCTION

Along with basic needs like breathing, sleeping and drinking, the body also needs to maintain its energy homeostasis. This is done by feeding and though the process is governed by a number of complex regulatory systems, our eating behaviour is highly influenced by a multitude of social and psychological patterns. Eating disorders are a growing problem and whether the problem is eating too much or too little, the issue needs to be addressed. The increasing problem of obesity that is spreading across the world has severe consequences including high blood pressure, heart disease, type 2 diabetes, hyperinsulinemia, stroke, reproductive problems and complications of pregnancy.¹ If not dealt with this growing health problem will also become a major socioeconomic problem in a near future. Preventive actions require an understanding of the cause and, before the psychological influence on eating can be evaluated a profound understanding of the biological regulatory system and how this interacts with the food consumed is required.

Background

The current view on how the hypothalamus and various peptides collaborate in the neural regulation of food intake is still a work in progress. The lesion studies performed by Heathersington and Ranson showed bilateral electrolytic lesions in the ventromedial hypothalamus of rats to cause hyperphagia and clearly identified the hypothalamus as a part of the system that regulates energy homeostasis. Subsequent studies, where electrical and chemical stimulation of the lateral and ventromedial areas of hypothalamus were performed, further characterized the regulatory properties of the hypothalamus. Stimulation of the lateral area dramatically increased food intake and conversely, ventromedial hypothalamic stimulation abruptly inhibited food motivated behaviour.² The role of peptides as satiety factors became apparent following a study where administration of cholecystokinin (CCK) to rats before feeding caused a dose-dependent decrease in meal size.³ More recent studies indicate that genes do not only control what we eat but conversely, that what we eat affects the expression of our genes. Woods et al showed that consumption of a diet high in fat alter the homeostatic regulation of energy balance.⁴ Mercer and Holder showed how sweet foods can cause a release of β -endorphins in the hypothalamus hence activating the opioid system.⁵ β -endorphin is a cleavage product of pro-opiomelanocortin (POMC) and has a high affinity for the μ -subtype of opioid receptors, which are the same type of receptors favoured by morphine.⁶ The overlap between the systems regulating feeding and reward motivated behaviour have been showed by Cota et al and the connection between addiction to drugs and feeding was further explored by Yeomans and Wright.^{7,8} Their study showed an opioid receptor antagonist caused a reduction in consumption of foods preferred by the subjects.

On the assumption that food consumption is regulated by interplay between food and genes, the palatable food in itself may have an influence on the genes that regulate consumption and food high in fat or sugar hence will change the expression levels of genes regulating food intake. As indicated by Welch and Levine the palatability of food seem to have an effect, where the food is high in sucrose or fat.⁹ Increased levels of

hypothalamic Dynorphin and proDynorphin mRNA levels suggest a palatable induced over consumption of calories.

Project outline

The outline of the project was to study the effects of macronutrients on gene expression. To explore the different aspects of the area of research studies of eating behaviour were performed in combination with studies of gene expression and of Oxytocin activity. The studies are aimed at the effects of palatable diets in general and of the effect on satiety related genes in particular.

The feeding studies were designed to examine the effect of Leucine on the intake of palatable foods and to test food preferences within a group of animals. Studies, on rodents, performed by Cota et al have showed central administration of Leucine to decrease food intake and hence bodyweight.¹⁰ This feeding study was the first of several planned to evaluate the effect of Leucine on the intake of palatable foods. The verification of food preferences within the group was a first step toward deciding to which extent food preferences affects consumption and whether the differences in food preference are due to variation in innate gene expression.

Many neuronal peptides, whether related to the regulation of energy homeostasis or not, are produced in response to stress. When the need for the peptide disappears the amount of peptide is down regulated to its previous level. On the other hand when the stressor persists and the concentration of peptide in the system remains at a constantly elevated level this will instead lead to a decrease in the number of receptors associated with the peptide. This since the higher concentration will make the peptide more easily detected and thereby lessen the need for as many receptors. Mice fed palatable diets ad libitum consume a larger amount of food both in terms of volume and calories and with this in mind it would be logical to expect peptides related to satiety to be up regulated in the animals feed the palatable diets.

To explore the effect of macronutrients on gene expression, rodents (rats and mice) were subjected to feeding studies where they were offered either normal laboratory chow or more palatable alternatives (diets high in fat or sugar). The ensuing responses (up or down regulation) in the anorexigenic set of genes tested (appendix 1) were then compared by detecting mRNA levels in hypothalamus and brainstem. Since the taste preferences of different animals vary, as in humans, a feeding study was conducted to divide animals into groups based on preference for fat or sugar and to see whether this preference corresponds to a difference in the total caloric intake.

The rodents were treated with different combinations of chow, intralipid, saccharin or sucrose solution and at the end of each study the animals were sacrificed by decapitation and dissected. The tissue of the hypothalamus (whole or subdivided) and the brainstem were subsequently treated to extract RNA. The detection of gene expression levels were made by Real Time Polymerase Chain Reaction (RT-PCR) on complementary DNA (cDNA) synthesized from the extracted RNA.

Areas of the brain affected by a selected peptide can be detected and visualized by immuno- or in situ treatment of sections. As Oxytocin was one of the satiety related peptides studied further analysis was made in regards to its activity in the paraventricular nucleus (PVN). The activity of Oxytocin in the PVN at the initiation and termination of food intake was detected by double staining. The effect of a novel Oxytocin-receptor antagonist on the feeding related circuitry in the PVN was confirmed by immunohistochemistry.

Neuronal peptides

The melanocortin system plays a major part in the neuronal control of energy homeostasis by integrating long-term adipostatic signals from leptin and insulin, primarily received by the hypothalamus, with acute signals regulating hunger and satiety primarily received by the brainstem. The system is also unique from a regulatory point of view in that its components express both agonists and antagonists of melanocortin receptors.¹¹

The melanocortin receptor (MC4R) is inhibited by endogenous antagonist agouti signalling protein and activated by endogenous agonist melanocyte-stimulating hormone (α - and β - MSH). Since the receptor is activated by the α - and β - MSH, which regulate energy homeostasis by reducing food intake and increasing energy expenditure, an up regulation of the expression levels would be expected when the animals were fed an energy dense diet ad libitum. The up regulation of the receptor of the MSH could also be a sign that the expression of the peptide is being suppressed. Suppression of the MSH expression would lower the available concentration of peptide hence creating a need for a higher receptor density.

Cocaine-Amphetamine regulated transcript (CART) is expressed at the same neurons as Proopiomelanocortin (POMC) and results in a strong anorectic effect when stimulated. POMC is a precursor to the melanocyte-stimulating hormones (α - and β - MSH) and β -endorphin. Since the two peptides have an antagonistic effect, where one (α -and β -MSH) inhibits and the other (β -endorphin) stimulates food intake, the expression levels when fed ad libitum for this short duration of time should show no significant difference as there is no negative energy state nor weight gain to compensate for.

In addition to the two forms of MSH, POMC is also the precursor to adrenocorticotrophic hormone (ACTH).¹² The release of ACTH is stimulated by the corticotropin releasing hormone (CRH) which is produced in the paraventricular nucleus of the hypothalamus.¹³

Some studies show Oxytocin to have a dampening effect on the development of tolerance to various addictive drugs (opiates, cocaine and alcohol) and to reduce the withdrawal symptoms.¹⁴ Oxytocin also plays a part in the interaction (bonding, love, sexual arousal) between mates.¹⁵ Some learning and memory functions are impaired by centrally administered Oxytocin. More importantly Oxytocin has a role in the control of homeostasis and has satiety related effects by limiting over feeding on agents that are harmful or toxic, as with salt and sugar that can disrupt the body homeostasis in high enough blood concentrations. The release of oxytocin also occurs as a response to severe

distension of the stomach, which is important to keep in mind when animals are fed palatable diets ad libitum, hence are given the opportunity to feed unlimited on food with a taste appealing to them.

The obesity gene (FTO) is highly expressed in the feeding-related nuclei of the brainstem and hypothalamus. The expression levels of FTO are highly influenced by feeding and fasting. In addition to this, a strong negative correlation between the expression levels of FTO and an orexigenic galanin-like peptide have been observed by Fredriksson et al supporting the role of FTO as a participant in the regulation of energy homeostasis.¹⁶ As seen in the results of this study a significant up-regulation of the gene expression can be observed in the hypothalamus of rats and mice after a short period of food deprivation. Variations in the FTO have been strongly associated with changes in body mass index and obesity. Recent data shows that the gene codes for an enzyme that can act directly on DNA to modify it, which might indicate that FTO has a role in controlling the expression of other genes.¹⁷

The orphan sodium- and chloride-dependent neurotransmitter transporters (SLC6A15 and SLC6A17) are part of a subfamily of neurotransmitters responsible for the release, re-uptake and recycling of neurotransmitters at synapses. Many of the transporters derive energy from the co-transport of Na^+ and Cl^- to facilitate transport of neurotransmitters into the cell against their concentration gradient.¹⁸ However the specific function of many of these transporters are still unknown and hence their relation to the transport of hunger and/or satiety regulation peptides is still being explored.

CCK is a peptide hormone that acts as a hunger suppressant within the central nervous system and causes the release of pancreatic exocrine enzymes within the gastrointestinal system¹⁹. There has also been documented hunger reducing effects when CCK is administered to rats²⁰.

Arginine vasopressin (AVP) is a peptide hormone that regulates the body's retention of water. When the body is dehydrated AVP cause the kidneys to concentrate the urine ridding the body of salt but not water.¹²

Real Time Polymerase chain reaction

RT-PCR²¹ is used to amplify short DNA sequences (100-600 bases). It is highly sensitive and allows for detection of very small samples since it amplifies just the fragment of interest, in this case the messenger RNA (mRNA). As previously mentioned RNA is isolated from the tissue samples and since RNA is a very unstable molecule it could never withstand the high temperature of the PCR. To be able to measure gene expression reverse transcriptase is therefore used to convert mRNA into cDNA for further analysis.

In RT-PCR measurements are made regularly during the PCR progress, rather than at the end. Reactions are therefore characterized by the point when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

As in ordinary PCR, RT-PCR entails the use of a pair of primers that are complementary to a defined sequence on each of the two strands. These primers are extended by a DNA polymerase so that a copy is made of the designated sequence. After making this copy, the same primers can be used again, not only to make another copy of the input DNA strand but also of the short copy made in the first round of synthesis which in every cycle leads to logarithmic amplification.

The detection of samples, in RT-PCR is made by integrating the fluorescent probe SYBR-Green²² to each new strand being built. The binding of SYBR-green to DNA is highly specific and results in emission of light of a known wavelength. The intensity of the light is proportional to the amount of DNA and is used to make an estimate of the quantity.

RT-PCR has the advantage of simultaneous measurement of gene expression in many different samples, high reproducibility and fast analysis. However the actual concentration of mRNA in the isolated RNA samples can vary significantly. To counter effect this imbalance, a set of more stable housekeeping genes is used to calculate normalization factors to make data from the different samples comparable to each other.²³

MATERIAL AND METHODS

MATERIAL

Chemicals

Agarose D-I Medium EFO, Laboratorios Conda, Cat#8022, Lot#101146
c-Fos (4)-G, Goat Polyclonal IgG, Santa Cruz Biotechnology, Sc-52-G, Lot#L1406
Chloroform, Merck KGaA, Cas#76-66-3
DNA polymerase, Biotools, Ref#10.047
DNase I recombinant RNase-free, Roche, Ref#04716728001, Lot#13413900
EDTA, WMR International, Batch#0503476, Prov#20 244.294
Ethanol 99.7%, Solveco Chemicals AB
Glycerol min. 99%, Sigma Aldrich chemie Gmbh, Cas:56-81-5, Batch#045K0116
Intralipid[®] Infusionsvätska, Fresenius Kabi, Batch#UM17066
Isopropanol, Solveco Chemicals AB, Batch#6084230
L-Leucine 98%, Sigma-Aldrich chemie Gmbh, Batch#067KI738
Primers (appendix 1)
QuantiFast SYBR[®]Green PCR Kit, Qiagen GmbH, Cat#204052, Lot#130157172
RevertAid[™]H Minus First Strand cDNA Synthesis Kit, Fermentas Life Sciences, Cat#K1631, Lot#00017991
RNAlater, Ambion, Cat#AM7021, Lot#0704006
RNeasy[®] Plus Microkit, Qiagen GmbH, Cat#74034, Lot#130161174
Sodium Chloride, Merck KGaA, Cat#106404.100, Lot#9-472EF34C
Saccharose C₁₂H₂₂O₁₁, VMR International, Ref#27483.363, Lot#071170050
Triton[®] X-100, Sigma Aldrich chemie Gmbh, Cas:9002-93-1, Lot#064K0070
Trizma[®] base, Sigma-Aldrich chemie Gmbh, Lot#69H5420
Trizma[®] Hydrochloride, Sigma-Aldrich chemie Gmbh, Lot#1305345
TRIzol[®] Reagent, Invitrogen, Cat#15596-018, Lot#50175114
Tween 1%, Invitrogen, Cat#1219580
Water, MilliQ and DEPC
Vectastrain[®] ABC Kit, Vectorlaboratories Inc.,
 Biotinylated Antirabbit IgG (H+L) BA-1000, Affinity purified, Lot#T0730
 Anti-Guinea Pig IgG (H+L) BA-7000, Lot#T0323
 Anti-Goat IgG (H+L) BA-5000, Lot#T0530

Equipment

Pipettes

Biohit, 0.5-10, 10-100, 100-1000 µl
Biohit Proline, 5-100 µl, SN 5075812

Tips for pipettes

Ordinary 10, 100, 1000 µl
Filter 10, 100, 1000 µl

Tubes with lid 1.5, 2 ml, Eppendorf

PCR Tubes, Axygen Scientific

Instruments

Galaxy Mini Centrifuge, VWR International, SN 06101213

Biofuge Fresco, Heraeus

MiniSpin, Eppendorf, SN 0036283

RT-PCR, Bio-Rad

PCR, VWR International, SN 1200-10894

Autoclave

Vortex Genie 2, G-560E, Scientific Industries, SN 2-67401

Vibratome Leica VT 1000S, Kat#047235613, SN 1812/10.2004

Nanodrop® ND-1000 spectrometer, NanoDrop Technologies

Software

Microsoft Windows XP Professional (version 5.1.2600)

Microsoft Office 2003 (version 11)

Statistical software MINITAB by Minitab Inc. (release 14.20)

MyiQ system software by Bio-Rad (version 1.0.410)

Minitab 14 Statistical Software, Minitab Inc.

METHODS

Experimental and calculations

Determination of food preference

Mice were housed in a light:dark cycle of 12 hours (LD 12:12) in a temperature- and humidity-controlled environment (with lights on at 7:00). The mice had a five day period during which they were offered daily the sucrose- and intralipid solution for an hour in addition to their normal diet of chow and water. This was followed by two days of normal chow and water. During the study they were offered either the 10 % sucrose or 5 % intralipid solution, ad libitum, in addition to chow and water and the intake of sucrose, intralipid and chow was monitored on a daily basis.

The effect of Leucine on the intake of palatable food

Mice were housed in LD 12:12 (lights on at 7:00) in a temperature- and humidity-controlled environment. During the study they were offered a 10% Sucrose solution with or without the addition of Leucine (1.5%) ad libitum for 24 h a day. In addition to the sucrose solution they had constant access to chow and water. They were non native to the sucrose solution and their intake of sucrose or chow was monitored on a daily basis.

RT-PCR analysis of gene expression in Hypothalamus from Mice

Mice were housed in LD 12:12 (lights on at 7:00) in a temperature- and humidity-controlled environment. They had constant access to water and chow pellets. 48 hours prior to decapitation, they were randomly divided into three groups (n=8) and offered ad libitum 10% sucrose or 5% Intralipid solution in addition to the regular diet and water; the control group's diet was not enriched with sweet or fat tastants. Following the 2-day exposure to their special diets, the animals were sacrificed (11:00-13:00) by decapitation, their brains were excised and the hypothalamus and brainstem were placed in the RNAlater solution at room temperature for 2 h and then at -20°C until further processing.

Extraction of RNA from tissue

Extraction of RNA from brainstem was made by using TRIzol[®].

Day 1

Eppendorf tubes were filled with of RNAlater (~500 µl) and weighed. Following dissection the tubes were weighed again to determine the amount of tissue. The samples were then incubated at room temperature for 2 h and frozen at -80°C.

Day 2

The samples were defrosted and put on ice shortly before they were to be used again. As the samples defrosted new tubes were filled with TRIzol[®] (200 µl). The tissue was then transferred to the new tubes, without transferring any of the RNAlater, and gently pushed into the TRIzol[®] to make sure it was completely covered. The tubes were then placed on ice again. Homogenization was made by using an ultra sonic probe, and continuous sonification. Heating of the samples was avoided by restricting each sonification

treatment to a maximum of 30 seconds and keeping the samples on ice between treatments to avoid cross contamination of the samples. The probe was washed with EtOH and water between samples. Following homogenization additional TRIzol[®] (300 µl) was added to the samples, which were incubated at room temperature for 5 min and frozen at -80°C.

Day 3

As before the samples were defrosted and put on ice shortly before they were to be used again. Chloroform (100 µl per 500 µl TRIzol[®]) was added to each of the tubes, which were then gently turned for 15 sec and incubated at room temperature for a total of 2-3 min. The samples were centrifuged at 4°C on 13000 rpm for 20 min to separate the homogenate into a solid and liquid phase. The liquid phase, containing the RNA, was then transferred to new tubes and Isopropanol (250 µl per 500 µl TRIzol[®]) was added to produce floccules of RNA. Again the samples were centrifuged at 4 °C on 13000 rpm for 20 min following which the liquid phases were removed from the samples. The RNA was washed twice. First by adding 70 % room tempered Ethanol (500 µl per 500 µl TRIzol[®]) to each tube centrifuging at 4°C, >13000 rpm in 10 min, and then by repeating the same procedure but with 80 % ice cold Ethanol. The Ethanol was then removed from the tubes by using a fine pipette. The pellets were then set to dry by placing the tubes in the fume hood with the lids open for a maximum of 30 min. After which they were resuspended in 40 µl 1xDNase buffer each.

Day 4

To get rid of any remaining DNA or proteins 2 µl of DNase I was added to each sample. The samples were then incubated at 37°C for 2.5 h following which the enzyme was inactivated by heat treatment at 75°C for 15 min. The samples were then left on ice to cool off and any droplets from condensation was spun down with the tabletop centrifuge.

PCR of isolated RNA

The purity of the isolated RNA was confirmed by PCR amplification and gel electrophoresis.

The proper amount of master mix (recipe below) to make was calculated by adding the number of samples including positive- and negative control plus 2 extra samples. The ingredients, templates and tubes were placed and kept on ice all through the procedure. The ingredients were added to the mix in the order listed in the recipe, vortexed and spun down. 9.5 µl of master mix was dispensed into each PCR-tube and 0.5 µl of template was added to all except the negative control.

PCR Mastermix

Number of wells	1
Total volume	10 μ l
MQ-water	6.75 μ l
10xBuffert	1.00 μ l
MgCl (50mM)	0.30 μ l
Tween (W)	0.25 μ l
dNTP	0.10 μ l
Primer mix	1.00 μ l
Tag Pol	0.10 μ l
5 ng/ μ l template	0.50 μ l

Gel electrophoresis

The parts used to cast the gel were washed in ethanol and left to dry while preparing the gel. For the 2% agarose solution dry agarose (4 g) was placed in a glass bottle, 200 ml 1 x TAE was added. The solution was brought to a boil and then carefully simmered until clear. When the gel solution was done Ethidium Bromide (4 μ l) was added and the gel was casted. After 30 to 40 min the gel was ready for the samples to be added. Each well was loaded with sample (4 μ l) and 6 x DNA dye (1 μ l) except the 100 bp ladder which was complete with dye. The gel was then placed in the tray, covered with 1 x TAE and run at 130 V for 30 min. Following which the gel was placed on a UV lit light board and photographed.

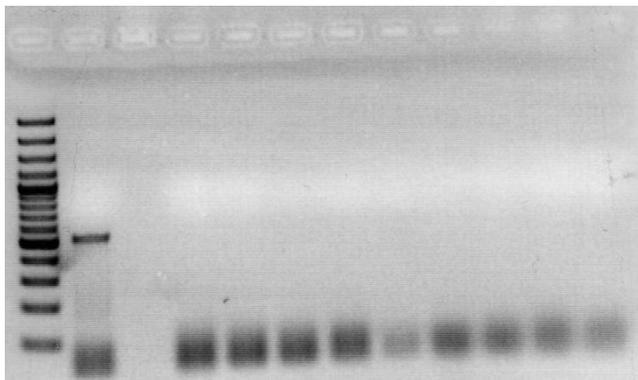


Figure 1. Picture of electrophoresis gel showing the result of a successful DNase treatment in which all the samples failed to produce bands. From left to right was the ladder, positive control, a blank, the eight samples and the negative control.

RNA concentration

When the purity of all RNA samples had been confirmed their concentrations was measured using the nanodrop. Concentrations of samples in ng/ μ g were used to calculate the amount needed to produce the diluted samples containing the amount of RNA (5 μ g) needed for the cDNA synthesis.

$$\text{Ex. Sample 1 had a concentration of } 3507,7 \frac{\text{ng}}{\mu\text{l}} = 3,5 \frac{\mu\text{g}}{\mu\text{l}}$$
$$\frac{5 \mu\text{g}}{3,5 \frac{\mu\text{g}}{\mu\text{l}}} = 1,4 \mu\text{l of sample and } 10,6 \mu\text{l of water to make } 12 \mu\text{l}.$$

Synthesis of cDNA

8 μ l of Master mix (recipe below) was added to each 5 μ g/12 μ l sample of RNA solution and they were incubated at 37°C for 1 h following which the enzyme was inactivated by heat treatment at 95°C for 15 min. The samples were then left on ice to cool off and any droplets from condensation were spun down with the tabletop centrifuge and the samples were subjected to PCR and electrophoresis (as described for RNA isolation).

cDNA Master mix

Total volume	8 μ l
5 x FS Buffer	4,0 μ l
0,1 M DDT	2,0 μ l
20 mM dNTP	0,5 μ l
Random Hexamers (diluted 1/6,25 with MQ)	0,5 μ l
Reverse Transcriptase	1,0 μ l

Synthesised cDNA was diluted to 100 ng/ μ l by adding 30 μ l of MQ water.

PCR of synthesized cDNA

The presence of cDNA in the samples was confirmed by PCR amplification and gel electrophoresis. The procedure for both PCR and electrophoresis was identical to that described for isolation of RNA.

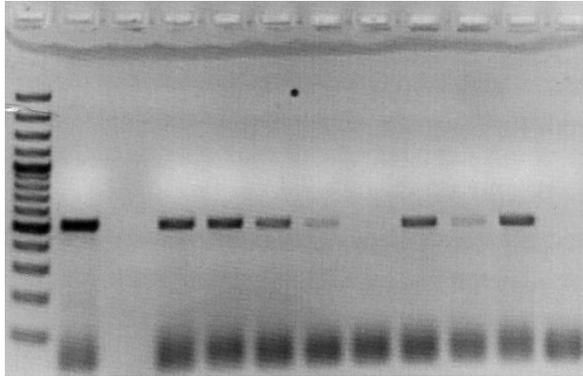


Figure 2. Picture of electrophoresis gel showing the result of the cDNA synthesis. From left to right was the ladder, the positive control, a blank, the eight samples and the negative control. The fifth sample produced no band indicating that the cDNA synthesis needs to be repeated for that sample. The rest of the samples showed clear visible bands corresponding to the positive control.

RT-PCR

Real Time PCR reactions were performed with a 20 μ l reaction mix composed of 5 μ l cDNA solution and 15 μ l Master mix containing a SYBR green probe for detection.

The 5 ng/ μ l solution of cDNA was prepared by diluting appropriate amount of a 100 ng/ μ l stock solution of cDNA (prepared as described above). Diluted solution (template) is stored at -20°C. Template is defrosted at room temperature, mixed properly, spun down carefully and kept on ice.

RT-PCR Mastermix

Number of wells	1
Total volume	20 μ l
MQ-water	9.52 μ l
10xBuffert	2.00 μ l
dNTP	0.20 μ l
MgCl	1.60 μ l
Primer up	0.05 μ l
Primer down	0.05 μ l
DMSO	1.00 μ l
SYBR green	0.50 μ l
Tag Pol	0.08 μ l
5 ng/ μ l template	5.00 μ l

5 µl of the template was added to each well and all samples were tested in duplicates with water (MQ) used as negative control (as shown in schematics below).

	1	2	3	4	5	6	7	8	9	10	11	12
A	1a	1a	2a	2a	3a	3a	4a	4a	5a	5a	6a	6a
B	7a	7a	8a	8a	9a	9a	10a	10a	11a	11a	12a	12a
C	13a	13a	14a	14a	15a	15a	16a	16a	17a	17a	18a	18a
D	19a	19a	20a	20a	21a	21a	22a	22a	23a	23a	NEG	
E	1b	1b	2b	2b	3b	3b	4b	4b	5b	5b	6b	6b
F	7b	7b	8b	8b	9b	9b	10b	10b	11b	11b	12b	12b
G	13b	13b	14b	14b	15b	15b	16b	16b	17b	17b	18b	18b
H	19b	19b	20b	20b	21b	21b	22b	22b	23b	23b	NEG	

Figure 3. Example of plate set up for RT-PCR with each sample represented by a number (1-23) and two different primers named a and b. The plate has eight by twelve wells and depending on the number of samples several primers may be tested at once.

The panels were centrifuged at 400 rpm for about 30 sec before put in the RT-PCR machine.

Primer test

Before new primers were used to run RT-PCR on panels their optimum working temperature must be determined. This was done by conducting a primer test which were preformed with a 20 µl reaction mix composed of 5µl genomic DNA (gDNA) solution and 15 µl Master Mix (same as for RT-PCR analysis above) containing a SYBR green probe for detection.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Primer 1	neg	Primer 2	neg	Primer 3	neg	Primer 4	neg	Primer 4	neg		
B	Primer 1	neg	Primer 2	neg	Primer 3	neg	Primer 4	neg	Primer 4	neg		
C	Primer 1	neg	Primer 2	neg	Primer 3	neg	Primer 4	neg	Primer 4	neg		
D	Primer 1	neg	Primer 2	neg	Primer 3	neg	Primer 4	neg	Primer 4	neg		
E	Primer 1	neg	Primer 2	neg	Primer 3	neg	Primer 4	neg	Primer 4	neg		
F	Primer 1	neg	Primer 2	neg	Primer 3	neg	Primer 4	neg	Primer 4	neg		
G	Primer 1	neg	Primer 2	neg	Primer 3	neg	Primer 4	neg	Primer 4	neg		
H	Primer 1	neg	Primer 2	neg	Primer 3	neg	Primer 4	neg	Primer 4	neg		

Figure 4. Example of plate set up for primer test on RT-PCR. Each primer requires three by eight wells and up to four primers can be tested at once.

The analysis was similar to the one for RT-PCR above but with the difference that each row on the plate is set to run at a different temperature. This allows for analysis of the CT-values and melting curves to determine the optimal working temperature for up to four primers at once.

RT-PCR analysis of gene expression in arcuate nucleus from rat

Rats were housed in LD 12:12 (lights on at 7:00) in a temperature- and humidity-controlled environment. Ten days prior to decapitation, individually housed rats were subjected to a scheduled feeding regimen: they received food only once per day for one hour (1100-1200). Water was available ad libitum. The animals were randomly divided into four groups and treated to different food paradigms. One group received only chow and the other ones chow in combination with a 10% Sucrose solution, a 5 % Intralipid solution or a 0.1% Saccharin solution. The rats were decapitated 2-4 hours after the end of the meal, their brains were excised, dissected and placed in RNAlater solution at room temperature for 2 h and then at -20°C until further processing.

The extraction of RNA from arcuate nucleus (ARC) was made with the RNeasy® Plus Micro Kit,²⁴ in accordance with manual, following which the treatment of the material was identical to that of the brainstem treatment with TRIZOL® from day 4 and forward.

RT-PCR analysis of gene expression in brainstem from rat

Rats were housed in LD 12:12 (lights on at 7:00) in a temperature- and humidity-controlled environment. Ten days prior to decapitation, individually housed rats were subjected to a scheduled feeding regimen: they received food only once per day for one hour (11:00-12:00). Water was available ad libitum. The animals were randomly divided into two groups, one receiving regular chow, whereas the other chow and a 10% Sucrose solution. The rats were decapitated 2-4 hours after the end of the meal, their brains were excised and the hypothalamus and brainstem were placed in RNAlater solution at room temperature for 2 h and then at -20°C until further processing.

Extraction of RNA from tissue

Extraction of RNA from brainstem was made by using TRIzol® and from ARC by using a RNeasy® Plus Micro Kit in accordance with manual.

The extraction of RNA by the TRIzol® method was preformed as described earlier but with twice the amount (1000 µl) of TRIzol® in proportion to the size of these samples.

Oxytocin activity in the Paraventricular nucleus

Mice were housed in LD 12:12 (lights on at 7:00) in a temperature- and humidity-controlled environment. They had constant access to water. Following a 16 h period of food deprivation the control group was sacrificed (initiation). The rest of the animals were fed standard laboratory chow and after a 60-90 min waiting period they were sacrificed as well (termination).

Double staining by immunochemistry

The brains excised from the mice were placed in a RNAlater-solution and stored in the refrigerator. Using the Vibratome they were then cut into thin (60 µm) slices and portioned out into the wells of a 12-well plate with up to three slices per well.

TBS (1L)

Trizma HCl 6,06 g
Trizma Base 1,39 g
NaCl 8,76 g
Dissolve in DEPC water to 1 L.

Super mix

200 ml TBS
0,5 g gelatine
1 ml Triton X-100
Mix all the ingredients and heat to 50-60°C until the gelatine dissolves.

Day 1

All steps, unless stated otherwise, were performed in room temperature on a rocking table with approximately one ml of fluid per well. In the first step the sections were rinsed in Tris Buffered Saline (TBS) (4 x 10 min) after which they were incubated for 10 min in a mixture of 10% Methanol, 3-4% Hydrogen peroxide and TBS. After the incubation the sections were again rinsed in TBS (4 x 10 min) following which the primary antibody diluted in 1:400 Super mix was added. With the antibody added the sections were transferred to a 4°C incubator and kept over night.

Day 2

The sections were rinsed in TBS (4 x 10 min) after which they were incubated for 1h in the secondary antibody diluted 1:400 in Super mix. Following this the sections were again rinsed in TBS (4 x 10 min), incubated in the ABC solution (1:1:800 Super mix) for 1 h and rinsed again in TBS (4 x 10 min). After this the sections were incubated in DAB with ~20 µl H₂O₂ for 10 min and rinsed in TBS (4 x 10 min).

- Repeat day 2 but with additional antibody for double staining

The sections were mounted by placing them on glass slides, adding a drop of Glycerol and cover glass. The edges of the cover glass were sealed with translucent varnish and left to dry horizontally. After drying the sections were studied in microscope in which the active cells of affected areas were counted and photographed.

The effect of an Oxytocin-receptor antagonist

Mice were housed in LD 12:12 (lights on at 7:00) in a temperature- and humidity-controlled environment. They had constant access to water. The antagonist was given intraperitoneal (IP) at a dose known to reduce food intake.

Immunochemistry

Sections were treated as described for double staining (above).

RESULTS AND DISCUSSION

There are several parts to the project, such as studies of behaviour, gene expression and oxytocin activity, all of them reflecting different aspects of the area of research.

Behavioural studies

All the feeding studies have in common that they are initial trials in a larger project that will be continued further.

Determination of food preference

The mice typed for food preference demonstrates the complexity of the feeding regulatory system by pointing out the difference between individuals even in a relatively small group of animals (Figure 5). Mice in general likes food high in fat which means that even the mice with a more pronounced fondness for sugar still consumed about half of their daily calories as fat (Figure 7). The total caloric intakes were more or less the same in all three groups and show that even in the presence of palatable choices there is no sign of over eating (Figure 6). However the animals that show a preference for sugar also shows a significant increase in their intake of chow (Figure 8). Since chow consists mainly of carbohydrates the results might indicate a preference not for sucrose in particular but for carbohydrates in general. The effect this may have on other studies is still unclear as these animals will be further studied to determine whether the difference is due to an innate genetic difference.

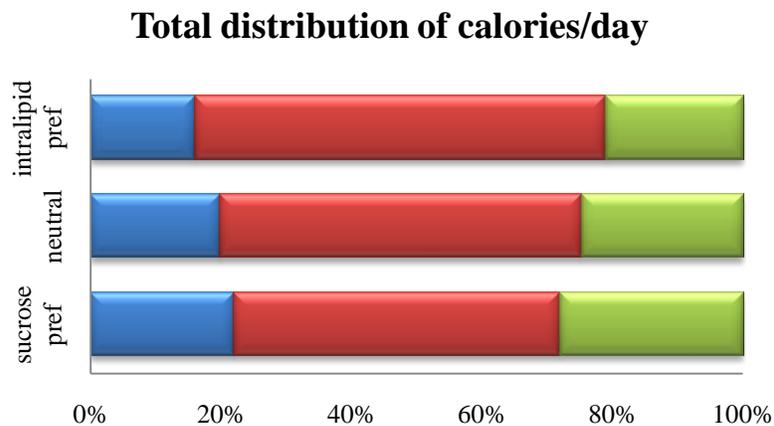


Figure 5. The total distribution of calories per day as Chow (blue), Intralipid (red) and sucrose (green) shows their preference for fat in general but also the clear shift toward sucrose for the sucrose preferring group.

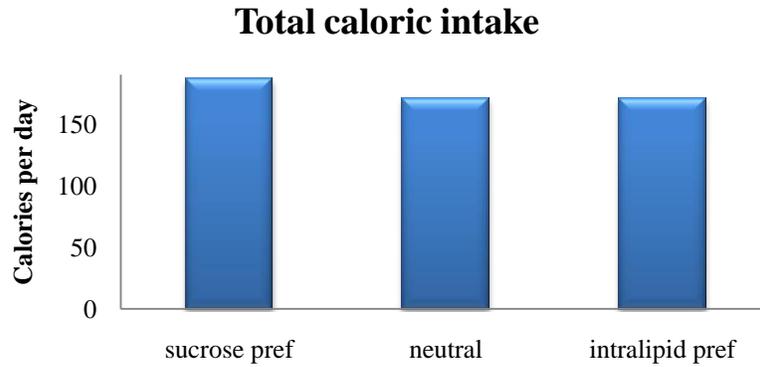


Figure 6. The total caloric intake in the groups remains the same which rules out caloric intake as a factor and confirms that the main factor behind the choice is taste.

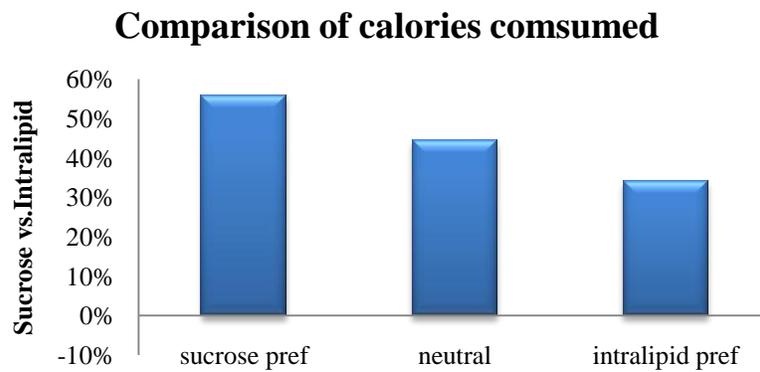


Figure 7. A comparison of the calories of Sucrose versus Intralipid consumed within the different groups.

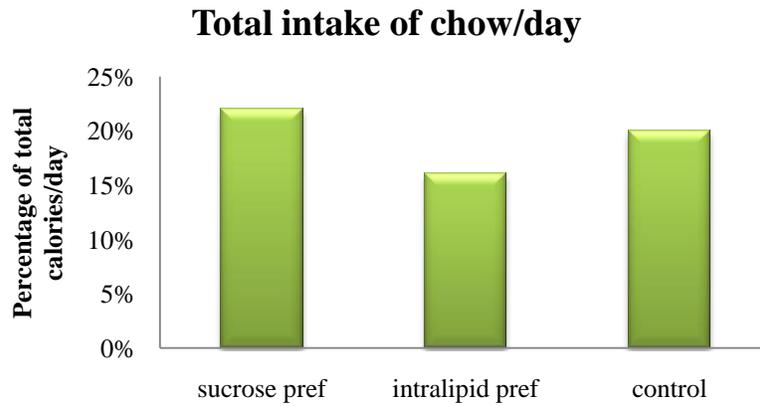


Figure 8. The clear increase in calories from chow for the group preferring sucrose suggests a preference not for sucrose in particular but for carbohydrate in general.

The effect of Leucine on the intake of palatable food

Leucine has been shown to reduce the total caloric intake. However prior studies have been performed with chow as the only choice of food and the food paradigm presented here was part of a larger set designed to evaluate the effect of Leucine on the caloric intake when presented with both chow and a more palatable choice, such as sugar. In this case the addition of Leucine did not have an effect on the intake of sugar as this was still the same in both groups (Figure 9). However the total caloric intake was significantly lower in the group fed Leucine as a supplement (Figure 10). This was accomplished by reducing the intake of chow (Figure 11). From a dietary perspective this would translate to a food supplement that would let you continue eating sweets and fast food while suppressing your taste for food with a more nutritious content. Hence you would lose the weight but most likely get quite malnourished in the process. This study will continue with the same basic layout but to evaluate the effect when the choice of palatable food is instead high in fat or when there is a larger choice of foods available.

Total intake of sucrose/day

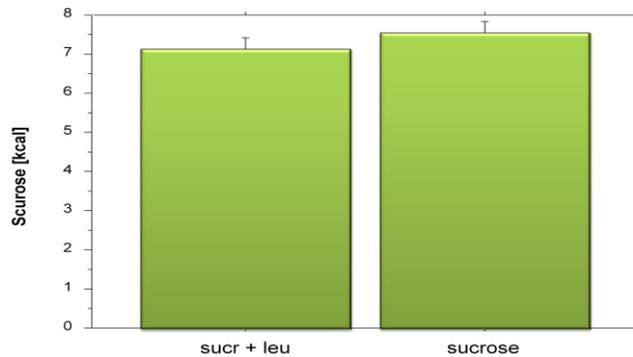


Figure 9. The total intake of sucrose was the same in both the group receiving sucrose with Leucine as for the group receiving only sucrose.

Total caloric intake/day

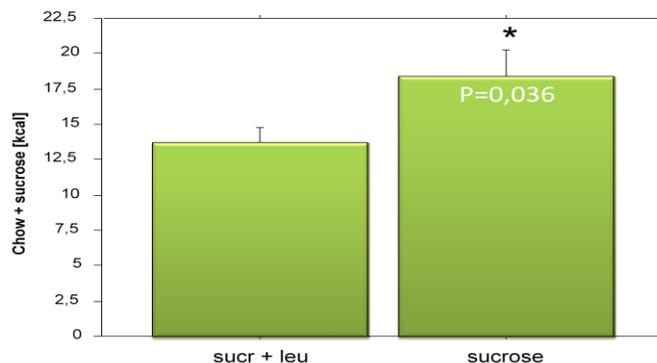


Figure 10. As expected the total caloric intake between the two groups differ significantly.

Total intake chow/day

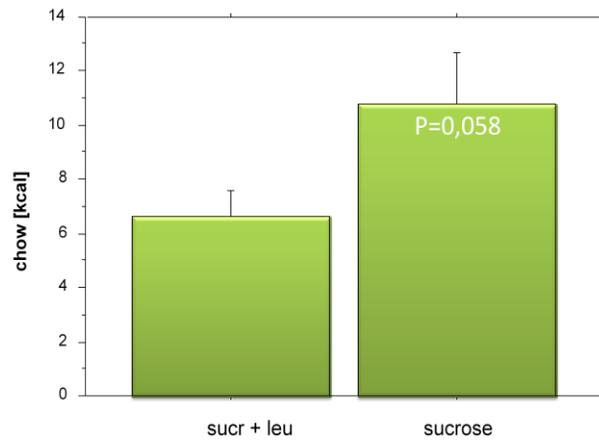


Figure 11. The difference in the intake of chow is not significant but shows a definite decrease for the group treated with Leucine.

Gene expression studies

The RT-PCR analysis's gives clues to how the energy regulatory circuitry responds to the intake of selected macronutrients. When it comes to gene expression there is a visible effect of macronutrients on the gene expression levels. The common theme for many of the genes tested seems to be down regulation of satiety signals as if to support over feeding on palatable diets and in many cases sucrose in particular.

RT-PCR analysis of gene expression in HT from Mice

The two peptides CART and POMC are expressed at the same neurons but POMC is also the precursor to two antagonistic peptides. One of the peptides (α - and β -MSH) has an inhibiting effect while the other (β -endorphin) stimulate food intake. The expression levels when fed ad libitum for this short duration of time should thereby show no significant difference as there is no negative energy state nor weight gain to compensate for. This is demonstrated by the lack of significant differences between the Intralipid-, Sucrose- and control group for both CART and POMC (Figure 12).

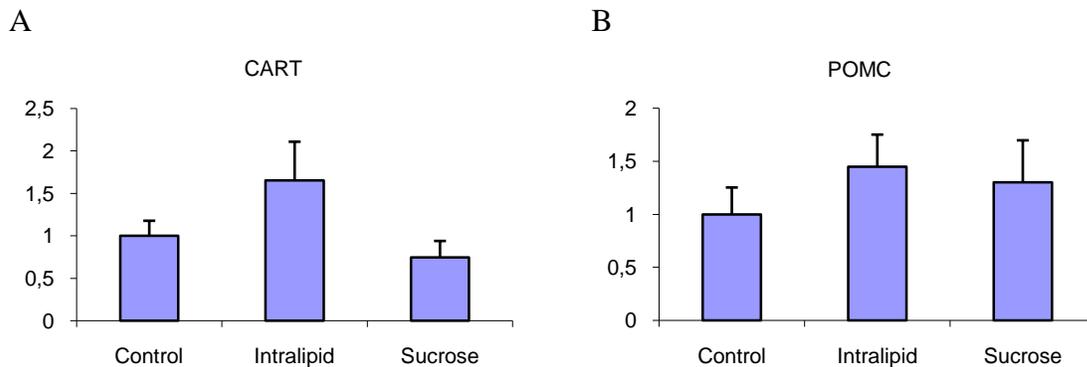


Figure 12. There is no significant difference in either CART (A) or POMC (B) which is to be expected since there is neither negative energy state nor weight gain to compensate for.

CCK is responsible for stimulating digestion of fat and as a neuropeptide mediates satiety. With its role in the digestion of fat the slight up regulation of the expression levels is not surprising neither significant compared to the group fed sucrose or to the control group (Figure 13).

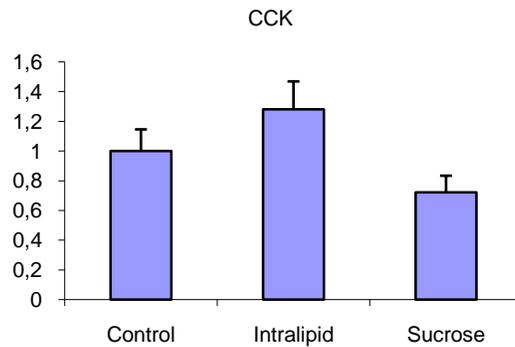


Figure 13. With its role in the digestion of fat the slight up regulation of the expression levels for Cholecystinin (CCK) is neither surprising nor significant compared to the group fed sucrose or to the control group.

With CRH the Intralipid solution actually seems to increase gene expression levels, as expected. As these animals are fed ad libitum the expression of CRH, which is a fast acting satiety peptide, should be up regulated since they are allowed to eat as much as they can. For the group fed a high sucrose diet this is not the case but here the expression of CRH is significantly down regulated (Figure 14).

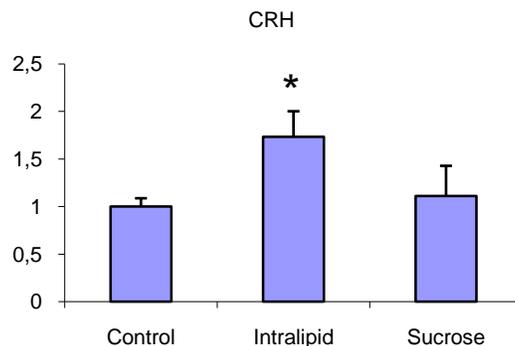


Figure 14. Corticotropin releasing hormone (CRH) is a fast regulation satiety signal and up regulation in response to feeding ad libitum is normal. The down regulation of the expression in the group fed sucrose could be an indication that sucrose suppresses satiety response.

For Oxytocin the result of a high sucrose diet seem to be suppression of satiety signals, which shows as a significant down regulation of the gene expression vs. the control group (Figure 15). The trend continues with MC4R where the up regulation for both the treated groups indicate low levels of the peptide which in turn could be a sign of a suppression of the satiety signals when fed a palatable diet, again as if to support over eating (Figure 16).

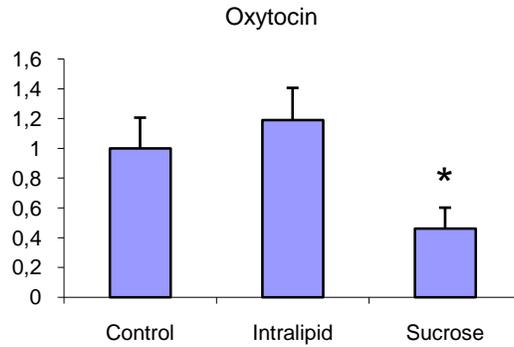


Figure 15. In the case of Oxytocin a significant down regulation of the gene expression vs. the control group can be observed, which could be an indication that a high sucrose diet suppress the satiety signals (as if to support over feeding on sucrose).

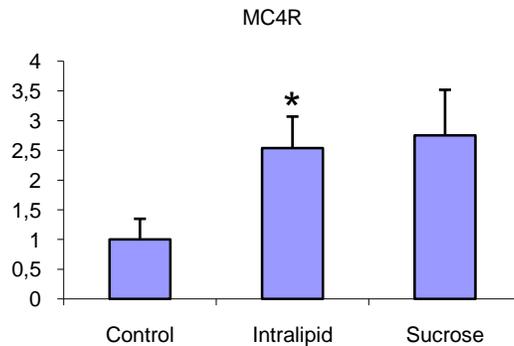


Figure 16. The up regulation of the Melanocortin receptor (MC4R) could indicate a lower concentration of the peptide as if to suppress satiety and support over feeding on a palatable diet.

AVP is a peptide hormone that regulates the body's retention of water. When the body is dehydrated AVP cause the kidneys to concentrate the urine ridding the body of salt but not water. Since all the animals had constant access to water dehydration would not be a factor and the expression level of AVP would be the same for all the groups, which is confirmed by the RT-PCR results (Figure 17).

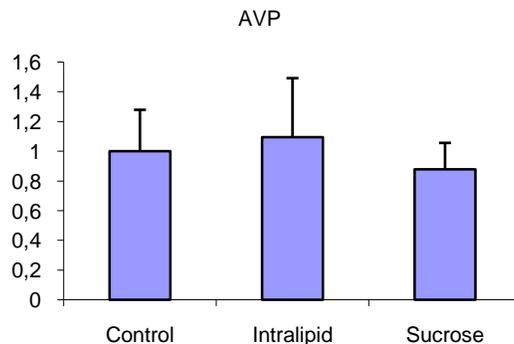


Figure 17. Since all the animals had constant access to water dehydration would not be a factor and the expression level of AVP would be the same for all the groups, which is confirmed by the RT-PCR results.

RT-PCR analysis of gene expression in ARC from rat

As earlier the antagonistic effect of the two peptides derived from POMC is demonstrated by the lack of significant differences between intralipid-, sucrose-, saccharin- and control group (Figure 18).

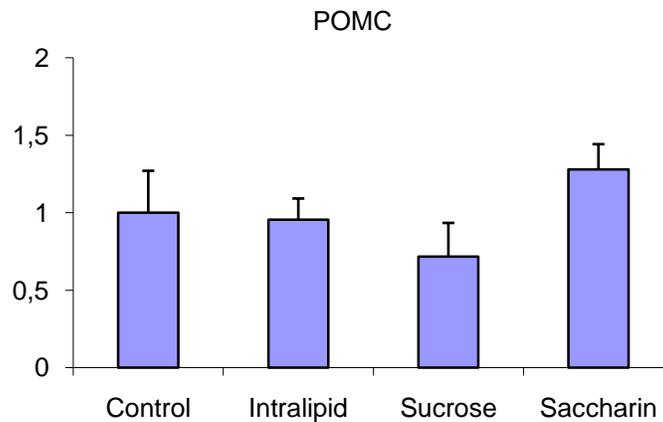


Figure 18. The antagonistic effect of the two peptides derived from POMC is demonstrated by the lack of significant differences between intralipid-, sucrose-, saccharin- and control group.

For the genes tested whose functions are more or less unknown, one that stands out is FTO. The fact that the expression of FTO changes when the subjects are treated to different food paradigms suggests a connection to the feeding regulatory circuitry. In this particular study though there is a significant up regulation in the group fed Saccharin (Figure 19). As Saccharin has no caloric or nutritional value this is interesting. Whether the observed up regulation is due to an antagonistic effect of Saccharin on satiety signals, or related to a difference in the taste of Saccharin compared to sucrose, requires further studies to determine.

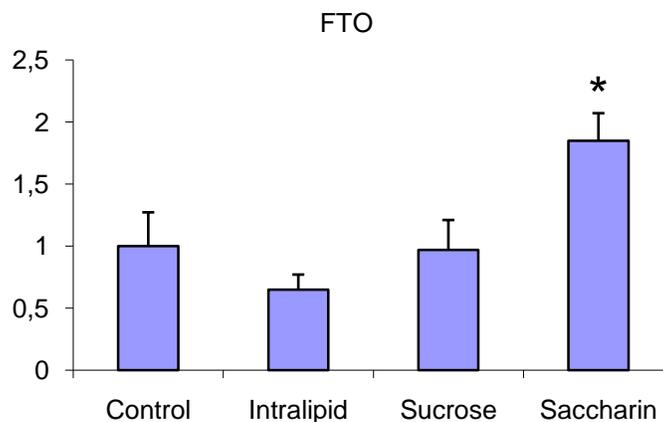


Figure 19. A significant up regulation of the FTO can be seen for the group fed Saccharin. As Saccharin

RT-PCR analysis of gene expression in brainstem from rats.

In line with previous results the expression levels of CART tend to be stable between the groups, regardless of food paradigm (Figure 20) and just as before there is no visible difference in the expression levels of CRH (Figure 21). For CRH there should be a difference as CRH is a satiety mediating peptide and the rats are fed ad libitum, hence should feel full. But as seen earlier it seems a diet high in sucrose seem to suppress the expression of CRH as if to support over feeding on sucrose.

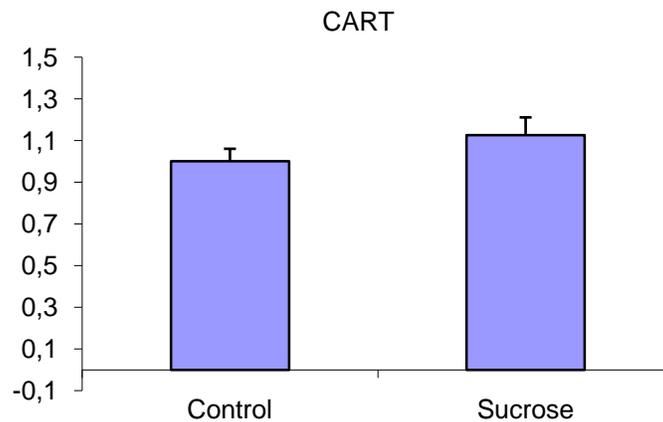


Figure 20. As earlier there is no significant difference between the two groups of the study.

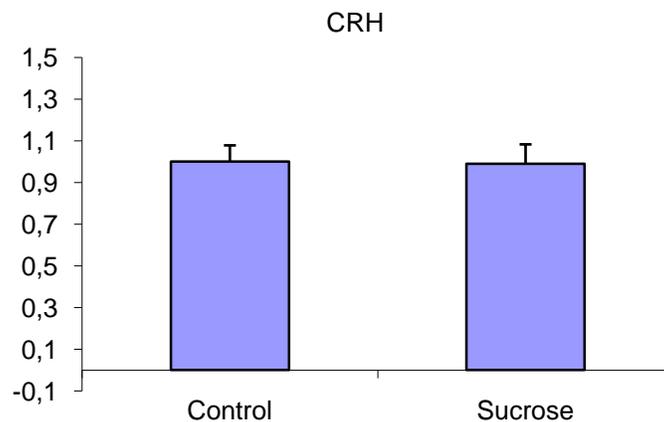


Figure 21. Corticotropin releasing hormone (CRH) is a fast regulation satiety signal and up regulation in response to feeding ad libitum is normal. The down regulation of the expression in the group fed sucrose could be an indication that sucrose suppresses satiety response.

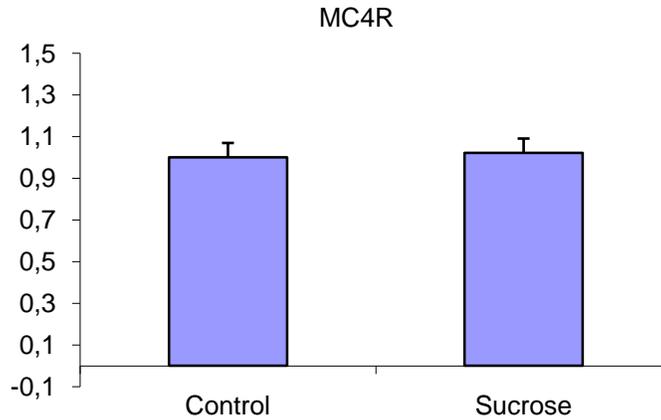


Figure 22. The indifference between the receptor levels in the two groups might indicate sucrose to have a dampening effect on the satiety signals (as if to support over feeding on an energy dense, palatable diet).

Again a significant change can be seen in the group fed a more palatable diet. On the contrary to the satiety related peptides there is a significant down regulation in the group fed Sucrose (Figure 23). Considering the negative correlation between the expression levels of FTO and orexigenic peptides observed by Fredriksson et al.¹⁶ the significant down regulation of FTO in the group fed sucrose vs. the control could indicate a corresponding up regulation of orexigenic peptides (as if to support over feeding on sucrose). However the actual function and regulation of FTO remains uncharted.

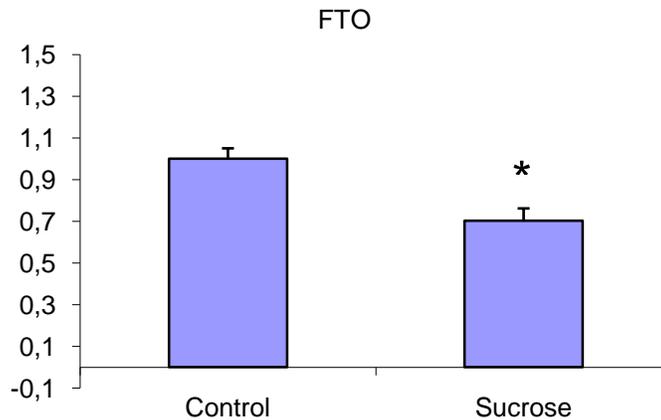


Figure 23. A significant down regulation of FTO in the group fed sucrose vs. the control can be observed and could indicate a corresponding up regulation of orexigenic peptides (as if to support over feeding on sucrose).

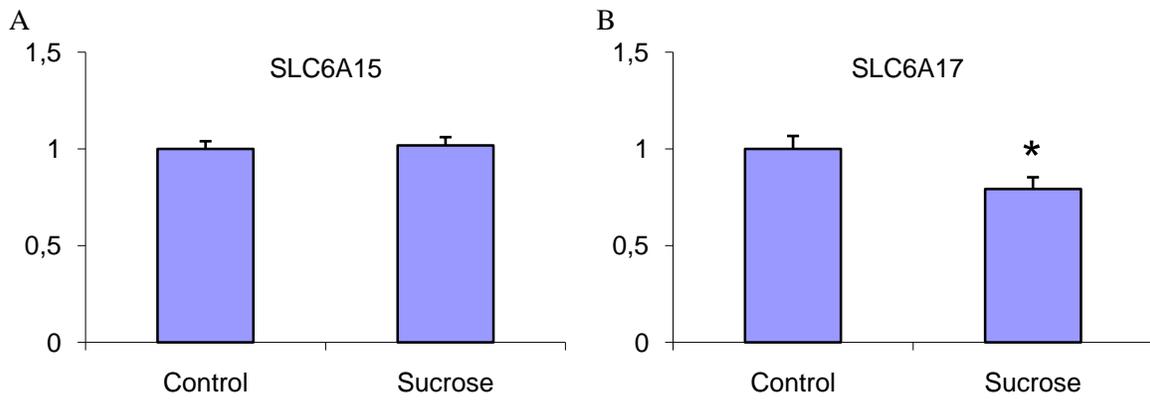


Figure 24. *SLC6A15 and SLC6A17 are amino acid transporters and the significant down regulation of SLC6A17 of animals subjected to the high sucrose diet suggest that it might be entwined to the control of energy homeostasis.*

Activity of Oxytocin

Oxytocin activity in the PVN

To show the activity of Oxytocin as a satiety mediating peptide double staining (c-FOS and Oxytocin) was performed. By staining sections taken from animals at the initiation and termination of the meal the location and difference in activity was made visible (Figure 25). The level of activity was 4,7 % (+/- 2.9) at the initiation compared to 12,3 % (+/- 4.5) at the end of the meal.

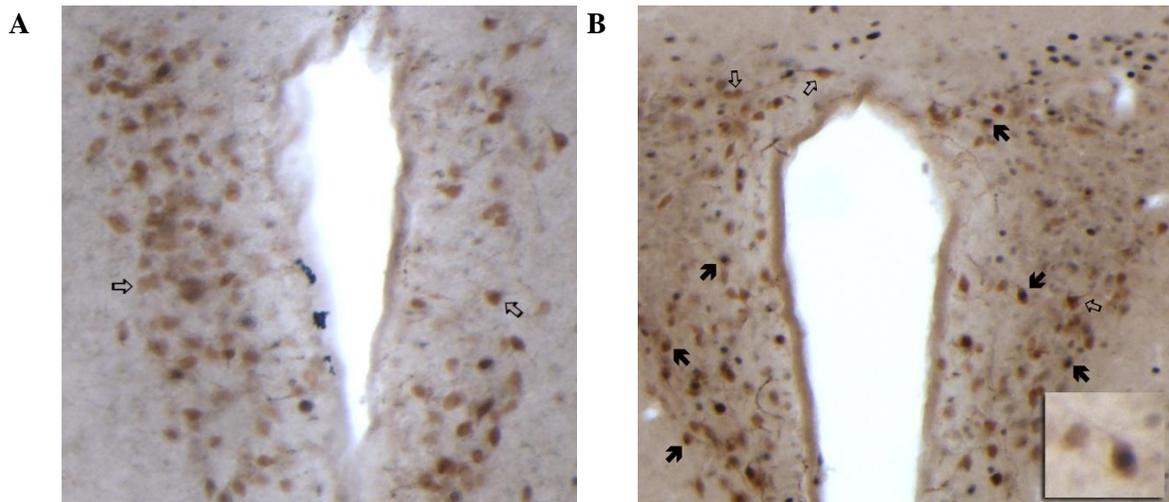


Figure 25. Double staining of active cells shows the increase in Oxytocin levels from initiation of meal (A) to termination of meal (B) as a response to feeding. Active cells are stained as brown (light arrows) and nuclei containing Oxytocin are stained black (dark arrows). Staining of the nuclei can be seen in the enlarged cut in (B).

The effect of an Oxytocin-receptor antagonist

The Oxytocin-receptor antagonist used was a novel antagonist and the staining confirms that the antagonist, who is injected IP, actually passes over the blood-brain barrier and has an affect on the regions of interest (Figure 26). The areas affected by the novel antagonist can be visualized and identified through the staining of active sites (Figure 27).

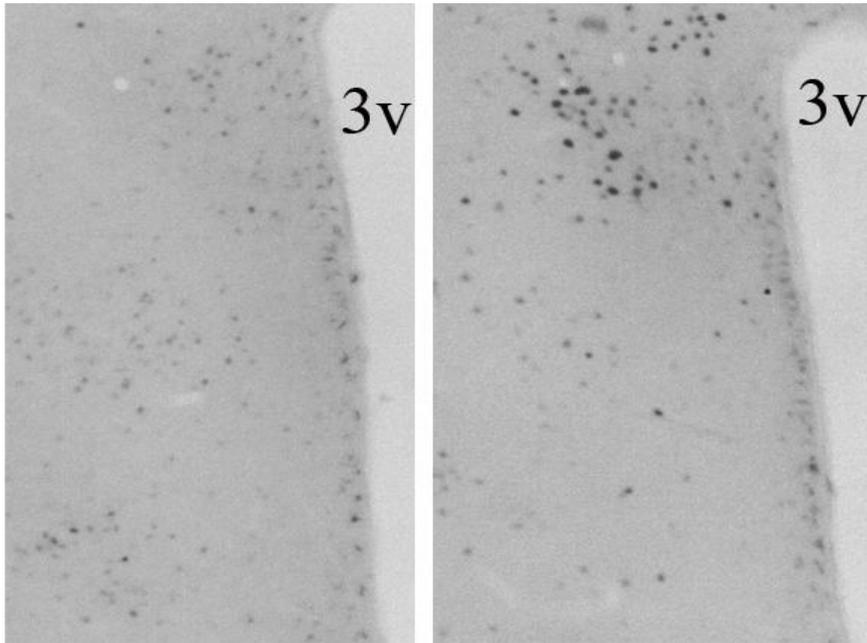


Figure 26. Activity is induced by injection of Oxytocin receptor antagonist and as the active cells are stained black the difference between the untreated animal (A) vs. treated (B) is visible and distinct.

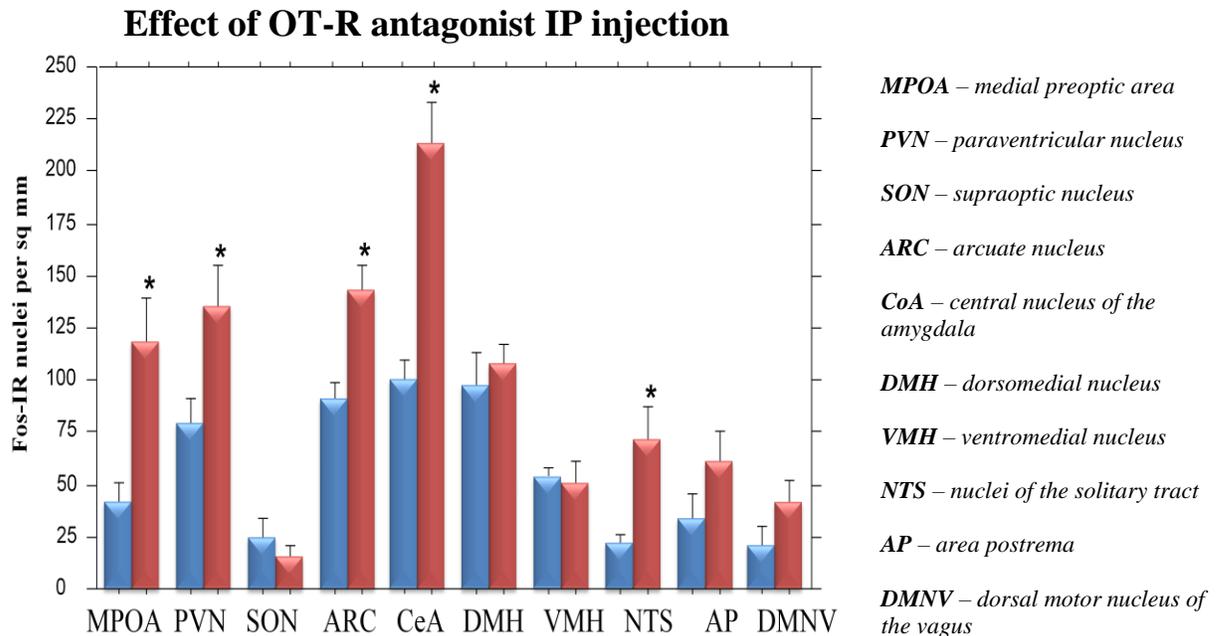


Figure 27. The effect of the Oxytocin receptor antagonist at different sites is determined by how many active nuclei can be detected. The effect on the treated animals (red) versus the untreated (blue) is apparent and in many cases significant.

To sum up the results it has been shown the intake of macronutrients such as sugar or fat has a significant effect on the feeding regulatory circuitry. This is evident from the change in gene expression levels detected by RT-PCR. To which extent however still needs further studies to determine.

One can also state that the response to said macronutrients is site specific. This is clearly shown both by the RT-PCR analysis of samples from different parts of the brain, and by the staining of sections from different areas.

It was confirmed that the novel Oxytocin-antagonist passes the blood-brain barrier and that it acts through the central circuitry. The areas affected were visualized and charted by staining sections from different parts of the brain.

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Appendix 1 – Table of primers with forward and reverse sequences.

Primer	Forward	Reverse
AVP	gctcaacactacgctctc	cttgggcagttctggaag
AVP	tgctcaacactacgctctctg	cctcctcttgggcagttctg
bAct HKG (mouse)	ccttcttgggtatggaatcctgtg	cagcactgtgtggcatagagg
bAct HKG (rat)	cactgccgcacacctctct	aaccgctcattgccgatagtg
bTUB HKG (rat)	cggaaggaggcggagagc	agggtgcccatgccagagc
CART	acgcattccgatctacgag	ttacaagcacttcaagagg
CART	tgcttgtgaaggggtgacagc	ttaaagcggctccagggacaa
CCK	cttgaggagggtggaatgag	cgtgacagatttaccattggg
CRH	gttgagaaactgaagagaaagg	actgttgttctgcgaggtac
CRH	taccaagggaggagaagagag	ggacgacagagccaccag
Cyclo HKG (mouse)	tttgggaaggtgaaagaagg	acagaaggaatggtttgatgg
Cyclo HKG (rat)	gagcgttttgggtccaggaat	aatgcccgcaagtcaagaaa
FTO	aacaccaggctcttcacc	cacttcacatcgcaggac
FTO	ggctgcttgtttcgggacg	tacttgccacggctgacc
FTO	gatgtcagagcgtcagagag	aaggatcaggagtgtgagtc
GAPDH HKG (mouse)	gccttccgtgttctacc	gcctgcttaccaccttc
GAPDH HKG (rat)	acatgccgctggagaaacct	gcccaggatgcccttagtgg
H3b HKG (mouse)	ccttgggtctgtttga	cagttggatgtccttggg
H3b HKG (rat)	atcgcaagctcccccttcag	tggaaagcgcaggtctgtttg
MC4R	tggcgaggcttcacattaaga	caaggtaatgcacctca
MC4R	cgctccagtaccataacatc	gaagaggacgcctgacac
OXY	acgctgcttcggaccaag	gcaagggcaggtagtctcc
OXY	cccattctatccttgcctg	gaatcgtcttattgccattacc
OXY	cggtggatctcgactgaac	tagcaggcggaggtcagag
POMC	tgggtcacttccgctggg	tctccgcacgcctctg
POMC	gaacgccatcatcaagaac	ctaagaggctagaggatc
RPL19 HKG (mouse)	aatcgcaatgccaactc	ggaatggacagtcacagg
RPL19 HKG (rat)	tcgcaatgccaactctctg	agccccgggaatggacagtcac
SDCA HKG (rat)	gggagtgccgtggtgcattg	ttcgccatagccccagtag
SLC6A15	gcatcggagaatttctgagc	agcgacgaatgatgaacacc
SLC6A15	tgcatggatcaaggagaaggc	gcgacgaatgaaaacgactgg
SLC6A17	ccttcatcaacttctcacctc	cgaccacacacttctcattc
SLC6A17	tggagcagcagaagcc	gcagcctctgaagatttattg
SUCB HKG (mouse)	tggtggaacggagacaag	cagcggtagacagagaag