UPPSALA UNIVERSITY

Department of Neuroscience, Unit of Functional Pharmacology

Bachelor thesis, 30 credits

Functional and neuroanatomical expression characterization of newly identified genes associated with increased body mass

Λειτουργικός και νευροανατομικός χαρακτηρισμός της έκφρασης προσφάτως εντοπισθέντων γονιδίων που σχετίζονται με αυξημένη μάζα σώματος

by Georgia Petrou

Research Project Submitted in Partial Fulfilment

of the Requirements for the Degree of

Bachelor of Biochemistry and Biotechnology, University of Thessaly

Uppsala , July 2013

Helgi Schiöth. *Professor at Department of Neuroscience, Functional Pharmacology, Uppsala University*

Nikolaos Balatsos. *Assistant professor at Department of Biochemistry and Biotechnology, Biochemistry, University of Thessaly*

Theologia Sarafidou. *Lecturer at department of Biochemistry and Biotechnology, Animal Molecular Genetics, University of Thessaly*

There is no sincerer love than the love of food.

George Bernard Shaw

Table of contents

Abbreviations ... 6

Abstract (English/Greek) ... 6

Introduction ... 7

Molecular circuits regulating food intake behavior and energy homeostasis ... 8

Short-term regulation of food intake ... 8

Long-term regulation of food intake ... 9

Food reward and Palatability ... 10

Obesity-associated genes from GWAS ... 12

ETV5 ... 13

NUDT3 ... 13

MTCH2 ... 14

SH2B1 ... 15

Epigenetics of obesity ... 15

What do we aim for? ... 16

Why mice? ... 16

Materials and Methods ... 18

Tissue collection and sectioning ... 18

Design and synthesis of RNA probes ... 18

In situ hybridization ... 18

Results ... 19

CNS expression of mouse *Nudt3*, *Etv5* and *Mtch2* in adult male C57Bl6/J mice ... 19

Etv5 ... 19

Nudt3 ... 22

Mtch2 ... 24

Sh2b1 ... 27

DISCUSSION ... 29

The areas and nuclei where the obesity related genes were expressed ... 29

~Hypothalamus ... 29

~Ventral Tegmental Area-Nucleus Accumbens-Substantia Nigra ... 30

~Amygdala ... 30

~Thalamus ... 32

~Piriform Cortex ... 32

~Hippocampus ... 32

~Cerebral cortex-cingulate cortex 32
~Caudate Putamen 32
How the obesity-associated SNPs act on the expression of the nearby genes? Possible mechanisms 32
Ideas for future research 34
Conclusion 35
Aknowledgements 35
ADDITIONAL FOLDER 35
Etv5 expression 35
Diencephalon 36
Telencephalon 36
Other 37
Nudt3 expression 37
Diencephalon 38
Telencephalon 39
Other 39
Mtch2 expression 40
Diencephalon 40
Telencephalon 41
Other 41
Sh2b1 expression 42
Diencephalon 43
Telencephalon 43
Other 44
REFERENCES 44

Abbreviations

BAX~ BCL2-associated X protein,BBB ~blood–brain barrier, BMI~ Body Mass Index, CCK ~ cholecystokinin, CNS ~ Central Nervous System, DAG ~ diacylglycerol, DAPI~ 4',6-diamidino-2-phenylindole, DEPC~ Diethylpyrocarbonate, DGKG ~ diacylglycerol kinase gamma, DIG~ digoxigenin, ERK ~ extracellular-signal-regulated kinase, ETV5~ Ets variant gene 5, FTO ~ fat mass and obesity associated, GLP-1~ glucagon-like-peptide-1, GWAS~ Genome-Wide Association Studies, LEPR~ Leptin Receptor, *MTCH2*~ mitochondrial carrier homolog 2, Nudix~ Nucleoside Diphosphate Linked Moiety X, NUDT3~ nucleoside diphosphate linked moiety X-type motif 3 gene, PA ~ phosphatidic acid , PBS~ Phosphate-buffered saline, PBT~ PBS with 0,5% Triton X-100, PFA~ Paraformaldeyde, POMC~ proopiomelanocortin, PYY_{3-36}~ peptide YY_{3-36}, RT~ Room Temperature, SFA ~ subcutaneous fat area , SNP~ Single Nucleotide Polymorphism, SSC~ Saline-Sodium Citrate, tBID ~ truncated BID, TBS~ Tris-Buffered Saline Nudix, TBST~ TBS with 0,1% Tween-20, ZDF~ Zucker diabetic fatty, ZL ~ Zucker lean

Abstract (English/Greek)

During the recent years the heritability of obesity has been given a lot of focus. Large scale genome wide association studies (GWAS) have identified a number of single nucleotide polymorphisms (SNPs) found to be associated with variations of Body Mass Index (BMI) among the population. Four of the genes close to such polymorphisms are Etv5, Nudt3, Mtch2 and Sh2b1. As the central nervous system is a major component in food intake behavior, *in situ* hybridization experiments were conducted in order to map the expression of the genes in the brain. The results revealed high expression of the genes in food reward related regions such as the ventral tegmental area (VTA), the nucleus accumbens (Acb), and the amygdala (Amy), as well as in energy homeostasis related regions like the arcuate, paraventricular and ventromedial hypothalamic nuclei (Arc, Pa, VMH), suggesting a possible role in food intake behavior through food reward system and energy balance.

Τα τελευταία χρόνια έχει δοθεί μεγάλη έμφαση στην κληρονομικότητα της παχυσαρκίας. Μελέτες συσχέτισης του γονιδιώματος (GWAS) μεγάλης κλίμακας ταυτοποίησαν έναν αριθμό μονονουκλεοτιδικών πολυμορφισμών (SNPs) οι οποίοι βρέθηκε ότι σχετίζονται με παρεκκλίσεις του δείκτη μάζας σώματος (BMI) μεταξύ του πληθυσμού. Τα γονίδια Etv5, Nudt3, Mtch2 και Sh2b1 βρίσκονται κοντά σε τέτοιου είδους πολυμορφισμούς. Καθώς το κεντρικό νευρικό σύστημα αποτελεί σημαντική συνιστώσα για τη συμπεριφορά πρόσληψης της τροφής, διεξήχθησαν πειράματα *in situ* υβριδοποίησης με στόχο τη χαρτογράφηση της έκφρασης των γονιδίων στον εγκέφαλο. Τα αποτελέσματα έδειξαν υψηλή έκφραση των γονιδίων σε περιοχές που σχετίζονται με την επιβραβευτική τροφή, όπως το κοιλιακό καλυπτρικό πεδίο (VTA), ο επικλινής πυρήνας (Acb), και τα αμύγδαλα (Amy), καθώς και σε περιοχές σχετιζόμενες με την ενεργειακή ομοιόσταση, όπως ο τοξοειδής, ο παρακοιλιακός, και ο έσω κοιλιακός πυρήνας του υποθαλάμου (Arc, Pa, VMH), προτείνοντας ένα πιθανό ρόλο στη συμπεριφορά πρόσληψης τροφής μέσω του συστήματος επιβράβευσης και της ενεργειακής ισορροπίας.

Introduction

Due to the increasing wealth in western societies, palatable food availability has increased tremendously and a more sedentary lifestyle has been adopted. Coinciding with these changes, the prevalence of obesity has increased rapidly. In 2009-2010 more than one third of U.S. adults and almost 17% of U.S. youth were obese (Ogden, Carroll et al. 2012).

Obesity is an important cause of morbidity and mortality, as well as reduced life expectancy in developed world. Every year at least 2,8 million people die as a result of being overweight or obese (World Health Organization 2013). There is evidence that it is a risk factor for diabetes type II, cardiovascular diseases, some cancer types (colon, breast endometrial and gallbladder cancer), osteoporosis, osteoarthritis and other diseases(Goran, Ball et al. 2003, Lahmann, Hoffmann et al. 2004, Friedenreich, Cust et al. 2007, Larsson and Wolk 2007, Larsson and Wolk 2007, Grazio and Balen 2009, Migliaccio, Greco et al. 2011).

The crucial question is what causes obesity. That is not an easy question to answer, as the underlying mechanisms are difficult to be completely clarified. In overall, obesity is caused by perturbations of the balance between food intake and energy expenditure (Obesity: Prevention and Treatment, James M. Rippe, Theodore J. Angelopoulos, CRC Press). This balance is regulated by a complex physiological system that demands the integration of sundry peripheral signals and central coordination in the brain (Obesity: Prevention and Treatment, James M. Rippe, Theodore J. Angelopoulos, CRC Press).

Obesity is a polygenic disease, even if there are exceptions where it is caused by single mutations in genes encoding leptin, leptin receptor (LEPR), prohormone convertase 1 and proopiomelanocortin (POMC), or syndromic forms of it, such as Prader-Willi syndrome (Jackson, Creemers et al. 1997, Montague, Farooqi et al. 1997, Clement, Vaisse et al. 1998, Krude, Biebermann et al. 1998, Delrue and Michaud 2004). Through large-scale Genome-Wide Association Studies (GWAS), a number of single nucleotide polymorphisms (SNPs) in loci that are correlated with increased BMI and obesity have been identified. Several of these likely causal variants in the associated regions are highly expressed or act in the central nervous system (CNS), but how they regulate obesity is still obscure (Herrera, Keildson et al. 2011). Our target was to map the expression of some of these genes through *in situ* hybridization in mice brain, trying to elucidate their functional role.

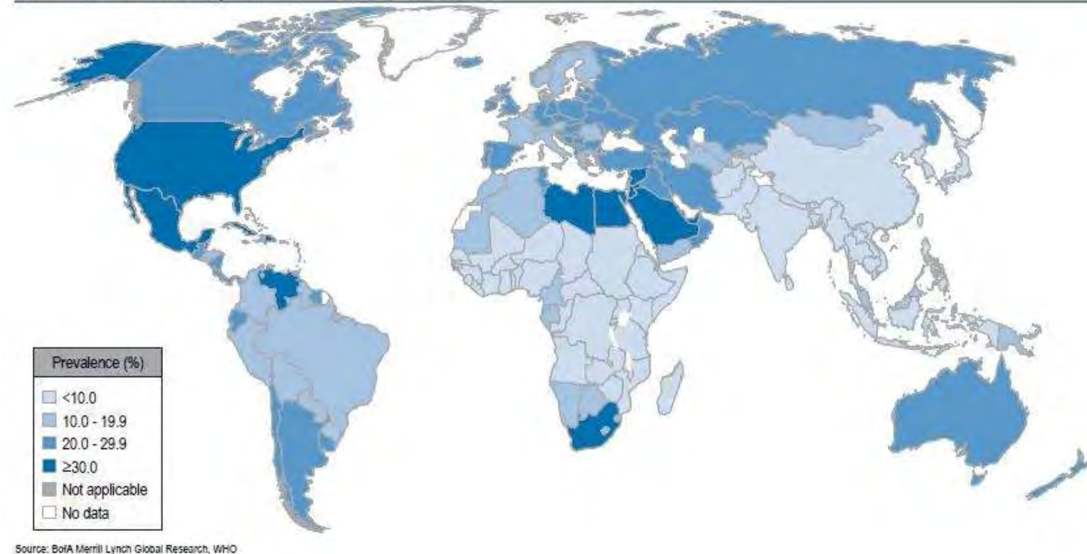


Figure 1. World-wide obesity prevalence according to WHO

Molecular circuits regulating food intake behavior and energy homeostasis

Knowledge of the regulation of food intake and energy homeostasis is a crucial point to the understanding of body weight and obesity. Multiple adiposity signals that regulate food intake and energy homeostasis exist, while many Central Nervous System (CNS) pathways participate in the response to those.

Short-term regulation of food intake

Although several peptides such as ghrelin (Cummings, Purnell et al. 2001) are identified to stimulate feeding, there is no unifying explanation for how eating is initiated and the perception of hunger.

Satiety refers to the reduced interest in food after a meal, while satiation is the feeling of fullness that causes meal termination. These two responses make sure that eating stops before gastric capacity is reached, and that an appropriate length of time passes, so the disposition of the ingested nutrients can be allowed before the next meal starts (Davis and Smith 1990). Contrary to hunger perception, the molecular circuits of satiety perception, involving gastric distention and peptide signals from gastrointestinal tract cells, are quite well understood (Cummings and Overduin 2007).

Gastric distension is sensed by mechanoreceptor neurons in the stomach and the signal is transferred to the hindbrain via vagal afferent and spinal sensory nerves (Ritter 2004). Some of the satiation/satiety –inducing peptides released from intestinal enteroendocrine cells are cholecystokinin (CCK), glucagon-like-peptide-1 (GLP-1), oxyntomodulin, peptide YY_{3-36} (PYY_{3-36}), apolipoprotein A-IV, and enterostatin (Cummings and Overduin 2007). Other satiety –inducing peptides, such as pancreatic polypeptide (Katsuura, Asakawa et al. 2002),

glucagon(Geary and Smith 1982), and amylin (Lutz, Del Prete et al. 1994), are released by the endocrine pancreas.

The main anabolic hormones excreted from the periphery are ghrelin and CCK. Ghrelin is an acylated peptide secreted from the gastric mucosa that stimulates feeding and is involved in feeding initiation (Cummings, Purnell et al. 2001). So, the levels of circulating ghrelin peak just before meal onset and decline very quickly after the meal (Cummings and Overduin 2007). Nevertheless, mice lacking ghrelin do not display altered meal patterns (Wortley, Anderson et al. 2004), and that is the reason that the contribution of ghrelin to feeding initiation remains vague. CKK is a satiation peptide secreted by the duodenal and jejunal mucosa in response to fat and protein consumption, and decreases food intake rapidly but briefly via the activation of vagal afferents (Kissileff, Pi-Sunyer et al. 1981). Meal size is increased by interruptions that agitate CCK signaling, showing the physiological role of CCK in satiation (Moran, Ameglio et al. 1992).

Also, peptide YY_{3-36} (PYY_{3-36}), which is released from the ileal L cells of the gastrointestinal tract after the meal, in proportion to the ingested calories, has been shown that reduces appetite, food intake, and weight gain (Batterham, Cowley et al. 2002). This is achieved by the modulation of the activity of Neuropeptide Y (NPY) and POMC neurons in the arcuate nucleus of the hypothalamus (Batterham and Bloom 2003).

Long-term regulation of food intake

Very important regulators of food intake and energy balance are the catabolic hormones leptin and insulin.

Leptin, which is primarily synthesized in the adipocytes of white adipose tissue and its circulation is proportional to the body fat mass, plays a crucial role in energy homeostasis by informing the brain about energy balance and the status of stored fat(Morton, Cummings et al. 2006). Leptin acts as a negative feedback regulator of adiposity in the brain, as it limits fat mass by limiting energy intake and reinforcing energy expenditure (Morton, Cummings et al. 2006). So, reduced leptin signaling leads to increased food intake, positive energy balance and fat accumulation (Morton, Cummings et al. 2006). Leptin receptors (LEPR) reside in mesolimbic dopaminergic neurons in hypothalamic areas such as the arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamic area (LHA), dorsomedial and ventromedial hypothalamic nuclei (DMH, VMH), and ventral premammillary nuclei (PMv), as well as in many extrahypothalamic regions such as the solitary nucleus (NTS), the ventral tegmental area, and the periaqueductal gray matter (Figlewicz and Sipols 2010).

Despite the fact that leptin administration results in weight loss, the enthousiasm for it as a therapeutic approach for obesity decreased rapidly, because it was discovered that leptin resistance is a very common phenomenon in obese people (Heymsfield, Greenberg et al. 1999). Leptin resistance is defined by the failure of elevated leptin levels to suppress feeding and mediate weight loss. So, fat mass and leptin levels in the circulation are both increased. Although the cellular mechanisms triggering leptin insensitivity are not elucidated yet, possible causes are the failure of circulating leptin to reach its targets in the brain across the blood–brain barrier (BBB) and the inhibition of the intracellular LEPR signaling cascade (Munzberg, Bjornholm et al. 2005).

Insulin is produced within the β-cells of the islets of Langerhans in pancreas as a response to increased blood sugar. The hormone is crucial for the transport of glucose into the cells and causes the liver to convert glucose into glycogen for storage. When stores of glycogen reach the maximum level, the raised insulin levels stimulate the conversion of glucose into lipids for long-term storage in adipocytes. Apart from this aspect, insulin also acts in the brain by binding on the insulin receptors in the hypothalamic region. Through this interaction the brain gets informed about the energy status of the body, and a decrease in food intake is stimulated (Begg and Woods 2012). However, when more insulin is required to maintain a normal level of blood glucose, we have the condition of insulin resistance. Insulin resistance can probably be explained by the decreased insulin receptors in the brain, when the circulating insulin is not enough to decrease the appetite as it is supposed to do (Obici, Feng et al. 2002).

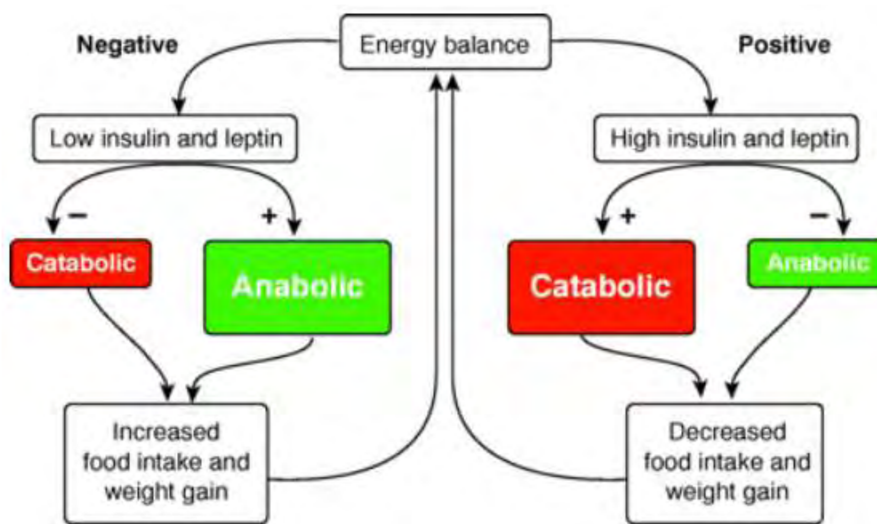


Figure 2. Scheme depicting the long term regulation of energy balance by the catabolic hormones insulin and leptin.

Food reward and Palatability

Over millions of years, a lot of instinct-based behaviors essential for survival have evolved, and one of them is the food reward system. In times when the environmental conditions are scarce and dangerous, the food reward gives the essential motivation to overcome those difficulties, and eat (Berthoud, Zheng et al. 2012). So, food reward is the process by which the consummation of a specific food reinforces behaviors that endorse the acquisition and consumption of the food. Furthermore, palatability is defined as the hedonic value associated with food. The palatability of food has an effect on satiation but not on subsequent satiety (De Graaf, De Jong et al. 1999). Behavioral experiments on rats have shown that rats prefer to stay in places where they have previously gotten rewarding food such as human "snacks" high in fat and sugar, than eat normal chow (Jarosz, Kessler et al. 2007). Also, it has been reported that rats-models for binge eating endured even foot shock in order to get palatable food (Oswald, Murdaugh et al. 2011). So, it is not surprising that processed food manufactures struggle to make their products as much palatable and rewarding as can be.

The central nervous system circuits that are involved in food reward are incorporated with the circuits regulating energy homeostasis, so as the food-searching behavior is adapted to the energy needs of that time (Figlewicz and Sipols 2010). A region that is a good example of that, as it participates in both processes is the lateral hypothalamic area (LHA) ((Hoebel and Teitelbaum 1962)/(Powley and Keesey 1970)). Other brain regions that participate in the reward system are the ventral tegmental area, substantia nigra, amygdala, nucleus accumbens, orbitofrontal cortex, striatum, and insula(Kenny 2011). Food reward is regulated by dopamine signaling, while palatability by opioid peptides (Davis, Levitan et al. 2009)). Obese humans and rats have showed less striatal D2 receptor availability than those that were lean (Volkow, Wang et al. 2008)/(Fetissov, Meguid et al. 2002)), so their striatum responded less to palatable food. As a result, they overate in order to balance the lack of reward, leading to weight gain (Wang, Volkow et al. 2002). Also, regular intake of high-fat and high-sugar foods leads to downregulation of postsynaptic D2 receptors, decreased D2 sensitivity, and reduced reward sensitivity in rodents (Johnson and Kenny 2010) showing that overeating can lead to a further damage of the striatal responsivity to food. D_2 receptor mRNA was also downregulated in both the VMH and LHA in obese rats, compared with lean rats (Fetissov, Meguid et al. 2002). Finally, when palatable food such as sucrose are consumed, an increase in dopamine levels and turnover occurs in the nucleus accumbens (Acb) (Hajnal, Smith et al. 2004). The pathway that mediates this increase is the mesolimbic dopamine system that originates in the ventral tegmental area (VTA) and projects to the nucleus accumbens (Acb).

Regarding the regulation of palatability by opioids, it has been shown that opioid receptor agonists increased the intake of palatable food compared to standard chow in rodents, while opioid antagonists decreased it (Olszewski and Levine 2007).

By measurement of positive and negative orofacial expressions when tasting pleasurable or aversive stimuli, it has been shown that the nucleus accumbens shell (Acbsh) and ventral pallidum are μ-opioid receptor mediated “hotspots” for liking (Pecina and Berridge 2005). Indeed, it was demonstrated that nucleus accumbens injection of an opioid receptor antagonist transiently suppressed such sucrose-evoked positive hedonic orofacial reactions (Shin, Pistell et al. 2010).

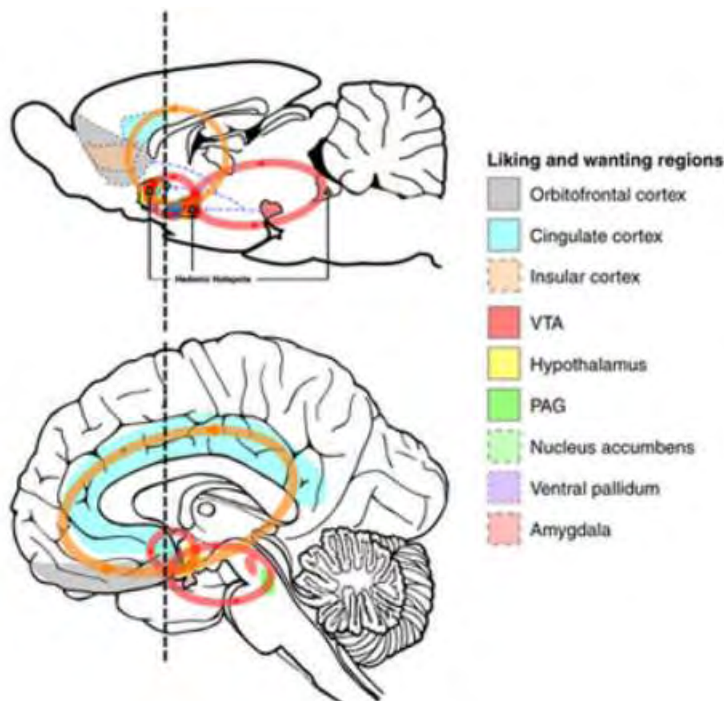


Figure 3. Comparison of liking and wanting-related regions between mouse and human brain.

Obesity-associated genes from GWAS

Until just a few years ago, the genetic factors of obesity were widely unknown, except from a few forms of monogenic extreme obesity. During the last years, extensive knowledge of the human genome contributed to the development of new tools that make the simultaneous analysis of thousands of genes easy. Several high-throughput forward approaches are used in the effort to unveil the gene networks associated with many diseases, including common obesity. Such approach is GWAS, that allows the scanning of the entire human genome in an unbiased manner, using statistical methods to establish associations between chromosomal loci and a specific phenotype. Thus, GWAS is really useful in order to reveal associations between SNPs and different measures of obesity, such as increased BMI (Internet source: http://www.nature.com/scitable/topicpage/genome-wide-association-studies-gwas-and-obesity-752, 4/8/2013).

The first locus associated with increased BMI that showed strong statistical significance, was identified through GWAS in 2007, and mapped to a gene which at the time had unknown function. This gene is now known as fat mass and obesity associated (FTO)(Frayling, Timpson et al. 2007). The FTO encodes an mRNA demethylase. High expression of it has been observed in distinct nuclei of the hypothalamus, brainstem, and extended amygdala (Fredriksson, Hagglund et al. 2008). It has also been suggested that children homozygotes for the risk variant rs9939609 of the FTO are prone to impulsivity towards food (Velders, De Wit et al. 2012).

Since the first GWAS report for obesity, an increasing number of loci have been shown to be associated with BMI and other measures of obesity or fat distribution. The loci chosen for our research are near the genes *ETV5*, *NUDT3*, *MTCH2*, and *SH2B1*.

ETV5

The SNPs rs7647305 (Thorleifsson, Walters et al. 2009) and rs9816226 (Speliotes, Willer et al. 2010), located in introns of the 3rd chromosome, around 7,4 kb upstream of the Ets variant gene 5 (ETV5), have been significantly associated with increased BMI. ETV5 is a transcription factor that plays a role in development and cancer (Thorleifsson, Walters et al. 2009). It is widely expressed, mostly in brain and placenta, and plays an important role in the pancreatic development through expression in cells proximal to pancreatic mesenchyma(Kobberup, Nyeng et al. 2007)). Also, ETV5 knockout mice have been shown to be infertile, with complete stem/progenitor spermatogonia loss, probably due to changes in the chemokine production (Simon, Ekman et al. 2010).

rs7647305 is located 30,7 kb downstream of the gene that expresses diacylglycerol kinase gamma (DGKG), which phosphorylates diacylglycerol (DAG) to phosphatidic acid (PA) playing a critical role in lipid metabolism. DGKG is highly expressed in the retina (Kai, Sakane et al. 1994) and the cerebellum (Goto, Funayama et al. 1994). The other body tissues express a truncated DGKG, lacking 25 aminoacids. Also, rs7647305 is located 178,5 kb upstream of the arginine/serine/rich 10 splicing factor (SFRS10) which is implicated in pre-mRNA splicing (Tacke, Tohyama et al. 1998). SFRS is predominantly expressed in brain, liver and testis (Nayler, Cap et al. 1998).

During radioactive in situ hybridization experiments on rats, the animals with restricted access to food showed 34% significant decrease of ETV5 expression in the ventral tegmental area (VTA) and Substantia nigra (SN). Also , the animals treated with high fat high sugar (HFHS) diet showed a significant decrease of ETV5 expression in the arcuate hypothalamic nucleus (Arc) and ventromedial hypothalamic nucleus (VMH) (Boender, van Rozen et al. 2012).

Also, ETV5 knockout mice have been shown to have reduced body weight compared to wild type mice, suggesting that ETV5 provides protection against gain loss (Schlesser, Simon et al. 2008). Finally, a study conducted on Zucker lean (ZL) and Zucker diabetic fatty (ZDF) rats showed that the highest ETV5 expression was detected in the hypothalamus, suggesting a potential role of it in the energy balance regulation. In ZL rats the expression difference between hypothalamus and the adipose tissue was significant and in mesenterial fat the expression was higher than in kidney fat ($p = 0.018$). Also, in ZDF rats the hypothalamic amount of ETV5 mRNA was significantly bigger than in the kidney fat and subcutaneous fat. In mesenterial fat it was significantly higher expressed than in kidney and subcutaneous fat (Schmid, Heid et al. 2012).

NUDT3

The SNP rs206936, located in an intron of the 6th chromosome, near nucleoside diphosphate linked moiety X-type motif 3 gene (NUDT3) has been significantly associated with increased BMI (Speliotes, Willer et al. 2010). NUDT3 is an enzyme (hydrolase) that belongs to the Nudix (Nucleoside Diphosphate Linked Moiety X) protein family. Nudix proteins function as homeostatic checkpoints at crucial stages in metabolic pathways of nucleoside phosphates, providing protection from elevated levels of potentially deleterious intermediates and metabolites, that can promote mutations, by catalyzing their hydrolysis (Safrany, Caffrey et

al. 1998). So, NUDT3 can be described as a cell cleaning gene. It has also been demonstrated for NUDT3 to act as a negative regulator of the extracellular-signal-regulated kinase 1/2 (ERK1/2) pathway (Chu, Alapat et al. 2004).

A current study demonstrated that NUDT3, together with some other Nudix family proteins, has *in vitro* decapping activity in monomethylated and unmethylated capped RNA(Song, Bail et al. 2013). Therefore, NUDT3 may be a critical determinant of mRNA decapping and stability.

In a very recent study, it was shown that rs206936 was significantly associated with BMI and subcutaneous fat area (SFA) in Japanese women, but not with any metabolic disorders (Kitamoto, Kitamoto et al. 2013).

rs206936 has also been recently associated with ADHD (Albayrak, Putter et al. 2013) and melanoma risk (Li, Liang et al. 2013).

MTCH2

The SNPs rs10838738 (Willer, Speliotes et al. 2009), rs4752856 - in the linkage disequilibrium region of rs10838738-(Renstrom, Payne et al. 2009), and rs3817334 (Speliotes, Willer et al. 2010), located in introns of the 11th chromosome, near the mitochondrial carrier homolog 2 (*MTCH2)* gene have been significantly associated with increased BMI. *MTCH2* is a mitochondrial membrane protein that appears to lead to mitochondrial depolarization. The substrate transported is not known yet (Uniprot). Unlike most family members of the mitochondrial carrier family, MTCH2 is located on the outer membrane of the mitochondria. It also appears to control the regulation of cell proliferation (Leibowitz-Amit, Tsarfaty et al. 2006) and apoptosis. In particular, it co-localizes with a protein complex that includes apoptosis regulating truncated BID (tBID) and BCL2-associated X protein (BAX), while it is essential for tBID recruitment to the mitochondria (Zaltsman, Shachnai et al. 2010).

Studies showed that carriers of the risk allele rs10838738 consumed less polysaccharides (Bauer, Elbers et al. 2009). Moreover, a high expression of MTCH2 was observed in adipose tissue. MTCH2 mRNA and protein expression was also raised in isolated fat cells of obese women and during adipocyte differentiation (Kulyte, Ryden et al. 2011).

Also, MTCH2 was more highly expressed in subcutaneous and mesenterial fat of ZDF, than in ZL rats, while high expression in the hypothalamus of both animal groups was observed (Schmid, Heid et al. 2012).

Finally, besides rs10838738 SNP, all other SNPs at *MTCH2* showed an association with endometrial cancer that was consistent with the directions of association with BMI reported in previous GWAS (Delahanty, Beeghly-Fadiel et al. 2011).

SH2B1

The SNPs rs7498665 (Thorleifsson, Walters et al. 2009) and rs7359397 (Speliotes, Willer et al. 2010), located in introns of the 16th chromosome, near the sarcoma homology 2B1 gene (SH2B1), have been significantly associated with increased BMI. SH2B adapter protein 1 is a cytoplasmic adaptor protein, that binds via its Src homology 2 (SH2) domain to multiple proteins, incuding Insulin receptor (Nelms, O'Neill et al. 1999), insulin receptor substrate 1 (Morris, Cho et al. 2009) and Janus kinase 2 (Rui, Mathews et al. 1997).

Taking into account the interaction of SH2B1 with the insulin receptor, its substrate, and JAK2, the role of SH2B1 in glucose and energy homeostasis is quite reasonable. SH2B1 acts as an endogenous insulin sensitizer. By directly binding to insulin receptors and their substrates, it promotes insulin receptor catalytic activity and inhibits tyrosine dephosphorylation of the substrates (Morris, Cho et al. 2009). Another study has shown that SH2B1-null mice were obese, leptin resistant, insulin resistant and glucose intolerant, and had hyperlipidemia, hyperphagia and hyperglycemia. Thus, it is really interesting that their condition could be reversed by targeted SH2B1 expression in neurons, and also the JAK2-mediated leptin signaling showed improvement in the hypothalamus (Li, Zhou et al. 2007). This fact suggests that the beneficial effects of SH2B1 on obesity, are mediated through the CNS, probably by boosting the hypothalamic leptin sensitivity. It should also be noted, that SH2B1 expression is high in the brain, especially the hypothalamus. Moreover, supporting the previous evidence, neuron-specific overexpression of SH2B1 dose-dependently protected against high-fat diet–induced leptin resistance and obesity (Ren, Zhou et al. 2007).

In humans, large deletions in SH2B1 have been associated with severe early-onset obesity (Bochukova, Huang et al. 2010). Also, rs7498665 is not only associated with increased BMI in humans, but also with increased total fat, saturated fat, and monounsaturated fat intake (Bauer, Elbers et al. 2009).

Finally, in a recent study, SH2B1 SNPs and four other SNPs close to it, were analysed for association with triglyceride levels using sequential oligogenic linkage analysis routines (SOLAR). A stronger signal was observed for rs8045689, a SNP near SH2B1. rs8045689 is located in an intron of spinster homolog 1 (SPNS1) which encodes a protein structurally similar to a sphingolipid transporter. Sphingolipids are synthesized from fatty acids, and it is possible that sphingolipid metabolism disorders may influence triglyceride levels. Moreover, an association with triglyceride levels was also discovered for rs4788102, which is also located near SH2B1 (Vastermark, Jacobsson et al. 2012).

Epigenetics of obesity

Last but not least, even though so many genes are linked to increased BMI, obesity cannot always be explained through the genome. So, epigenetic mechanisms also seem to contribute in the complex problem of obesity. The most important epigenetic mechanism is DNA methylation. During DNA methylation, DNA methyltransferases add a methyl group on the cytosines in CpG dinucleotides, inducing a more compact and rigid nucleosome structure, not reachable from the transcription factors (Choy, Wei et al. 2010). As a result, transcription is prevented, silencing the gene. Through the lack of methylation, or demethylation, the gene is “turned on”, normally expressed. So, changes in the methylation

pattern can promote the expression of a gene that has typically been silent or silence a gene that is usually active (Rhee, Phelan et al. 2012). Generally, the epigenome is highly dynamic, changing in response to nutrition, exercise, and aging (Franks and Ling 2010).

Several studies demonstrate that an obesogenic *in utero* environment is able to increase the risk for obesity. Exposure to excess saturated fat during early development disturbed the hypothalamic expression of genes mediating orexigenic and anorexigenic signaling of the offspring (Page, Malik et al. 2009) and affected the offspring's weight/adiposity phenotypes (Carmody, Wan et al. 2011). Moreover, the chemical bisphenol A, used in plastic bottles, had a hypomethylating effect in a region upstream to *Agouti* gene of mice, increasing their susceptibility in obesity. This effect could be negated by providing the mother with methyl donor supplements, like folic acid (Dolinoy, Huang et al. 2007). Also, according to a recent cohort, paternal obesity has been associated with epigenetic abnormalities in newborns. The Insulin-like growth factor 2 (IGF2) gene was likely to be less methylated in children whose fathers were obese, than in children with non-obese fathers (Soubry, Schildkraut et al. 2013).

Of course, epigenetic changes can also happen postnatally, due to environmental factors. After six months of aerobic exercise, scientists observed differential methylation pattern of 473,753 CpG sites in adipose tissue from healthy men. The genes whith altered methylation pattern include obesity, diabetes and fat storage-related genes (Ronn, Volkov et al. 2013).

What do we aim for?

The exact function of most of the genes associated with obesity through GWAS is not known. The aim of our study is to try to elucidate the function of the chosen genes (*Etv5, Nudt3, Mtch2, Sh2b1*), by mapping their expression in the central nervous system of the mouse, via *in situ* hybridization on mouse brain.

It is crucial to understand how the genes are expressed in different brain regions and neurotransmitter systems, as our ultimate goal is to investigate the molecular circuits regulating food intake and food intake preferences.

Why mice?

The mouse is the most important mammalian model organism in biomedical research nowadays. The main advantage of the mouse as a model organism is its impressive similarity to human when it comes to anatomy, physiology, and genetics. Mouse genetic research is applicable to human disease, as over 95% of the mouse genome is similar to human (Internet source: http://research.jax.org/mousegenetics/advantages/advantages-of-mouse.html, 1/8/2013).

Moreover, mice have some unique technological advantages that make them ideal for human disease research. Transgenic mice carrying any gene of interest can be created through gene transfer technology, while selected genes can be deliberately mutated (Internet source: http://genome.wellcome.ac.uk/doc_WTD020804.html, 1/8/2013).

Mice are a really convenient tool for the investigation of CNS regulation of food intake, as the arrangement of their brain is nearly identical with the human brain (Figure 4), and they both use the same neurotransmitters and receptors, the same proteins for synaptic vesicle release and recycling, and similar signaling mechanisms. Moreover, humans and mice share the same conserved food reward pathway (Internet source: http://learn.genetics.utah.edu/content/addiction/genetics/neurobiol.html, 1/8/2013).

All the leading mouse models for obesity research are *Cpefat*, *Lepob*, *Leprdb* and *tub* (developed at The Jackson Laboratory). We used the C57BL/6J wild type strain (Figure 5), which is prone to diet-induced obesity, and so suitable for investigation of the epigenetic mechanisms within adipose tissue that underlie diet-induced obesity (Koza, Nikonova et al. 2006).

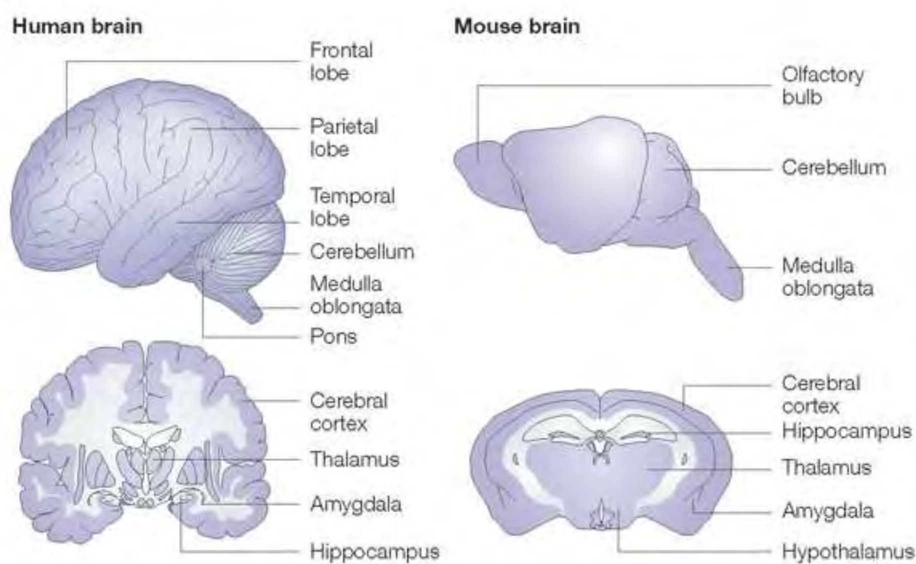


Figure 4. Comparative architecture of human and mouse brain. Sideviews of the whole brains (up) and coronal sections (down).

Figure 5. Male C57BL/6J mouse

Materials and Methods

The experiments conducted included extensive *in situ* hybridization of RNA probes for the genes of interest on mouse brain tissue.

Tissue collection and sectioning

All animal procedures were approved by the local ethical committee in Uppsala and followed the guidelines of European Communities Council Directive (C419/12).

Adult male C57Bl6/J mice (Taconic M&B) were housed in appropriate controlled environment (21°C, 12:12 hours Light/Dark Cycle), with *ad libitum* access to chow and water. The mice were anesthetized by intraperitoneally injection with 100 mg/kg pentobarbital sodium. Transcardial perfusion was then performed through the left ventricle with phosphate-buffered saline (PBS) followed by 4% formaldehyde (HistoLab). The brain was excised and kept in 4% formaldehyde overnight. Afterwards, the brain was washed in 0.1 M PBS, embedded in 4% agarose and 50 µm sections were cut on a Leica VT1000S vibratome (Leica Microsystems, Germany). The sections were preserved in 0.1 M PBS in the refrigerator.

Design and synthesis of RNA probes

Antisense probes were generated from commercial mouse cDNA clones (BioScience). The gene was cloned into a plasmid using standard procedures. The plasmids were purified with the Qiagen Plasmid midi kit and linearized with restriction enzyme (Fermentas). The cleaved vector was purified with the GeneJet PCR Purification Kit (#K0701, Thermoscientific) and the probes were synthesized using 1 µg cleaved vector and 40 U RNA polymerase (Roche) in the presence of digoxigenin (DIG) RNA Labeling mix (Roche), transcription buffer (Roche), and RNase Inhibitor (RiboLock, Thermoscientific). To remove template DNA, DNase I was used (2 units, Thermoscientific). Labeled probes were quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and stored at -32°C.

In situ hybridization

The bench and all the equipment used were meticulously sprayed surface decontamination solution that destroys RNases (Molecular Bio Products), and all the solutions were made with RNase free diethylpyrocarbonate (DEPC)-treated water. Sections were washed 3×5 minutes with PBS with 0.5% Triton X-100 (PBT) at room temperature (RT). The sections were bleached with 6% Hydrogen Peroxide (H_2O_2) (VWR International) diluted in PBT for 15 minutes, and washed 3×5 minutes with 0.1 M PBT. Then they were permeabilized in 0.5% Triton-X-100 in PBT, and washed again 3×5 minutes with 0.1 M PBT. Tissue was digested with 20 µg/ml proteinase K (Invitrogen) solution in PBT for 6 minutes, washed 3×5 minutes with PBT, post-fixed with 4% paraformaldehyde (Histolab) for 20-25 minutes, and washed again 3×5 minutes with PBT. Afterwards, sections were pre-hybridized in hybridization buffer (*50% formamide (Sigma), 5xSSC, 1% SDS, 50 mg/ml yeast transfer RNA (Sigma), 50 mg/ml heparin, all diluted in PBT*) for 2 hours at 58°C. Hybridization buffer was replaced with probe diluted in hybridization buffer (1µg/ml, heat-denatured for 5 minutes at 80°C), the plate was sealed thoroughly, and sections were left hybridizing over night at 58 ^{o}C.

Sections were washed 3×30 minutes with wash buffer II (*50% formamide (Sigma), 2xSSC, 1% SDS, all diluted in PBT*), and then 3×30 minutes with wash buffer III (*50% formamide (Sigma), 0.2xSSC, 0.1% Tween-20, all diluted in PBT*) at 65 ^{o}C. Afterwards, tissue was washed 2×5 minutes with Tris-Buffered Saline with 0.1% Tween-20 (TBST), and incubated in blocking solution (1% blocking reagent, Roche, in TBST) for 2 hours in RT. The blocking solution was replaced by the anti-DIG antibody conjucated to alkaline phosphatase (1:5000, Roche) diluted in blocking solution, and the slides were left incubating at 4 ^{o}C overnight.

Sections were washed with 2mM levamisole diluted in TBST 5×10 minutes at RT. Then, ready-to-use BM Purple AP substrate precipitating (Roche) was added on the sections. The plate was covered with tin foil and the sections were left developing for about 2.5 hours at 37 ^{o}C and washed 3×5 minutes with PBS. Three five-minute washes with PBS followed. Sections were carefully placed on Superfrost Plus slides (Menzel-Gläser), mounted with Mowiol antifade media (33.3% glycerol, 16.7% Mowiol 4–88, 0.02% Thimerosal, and 2% n-propyl gallate in PBS, all from Sigma Aldrich) and covered with glass cover slips.

Pictures of the slides were taken with the MIRAX MIDI slide scanner (Zeiss). The software used for taking the pictures was Panoramic Scanner (3DHISTECH), and for resizing and adjusting them Panoramic Viewer (3DHISTECH) and Adobe Photoshop CS3 were used.

Results

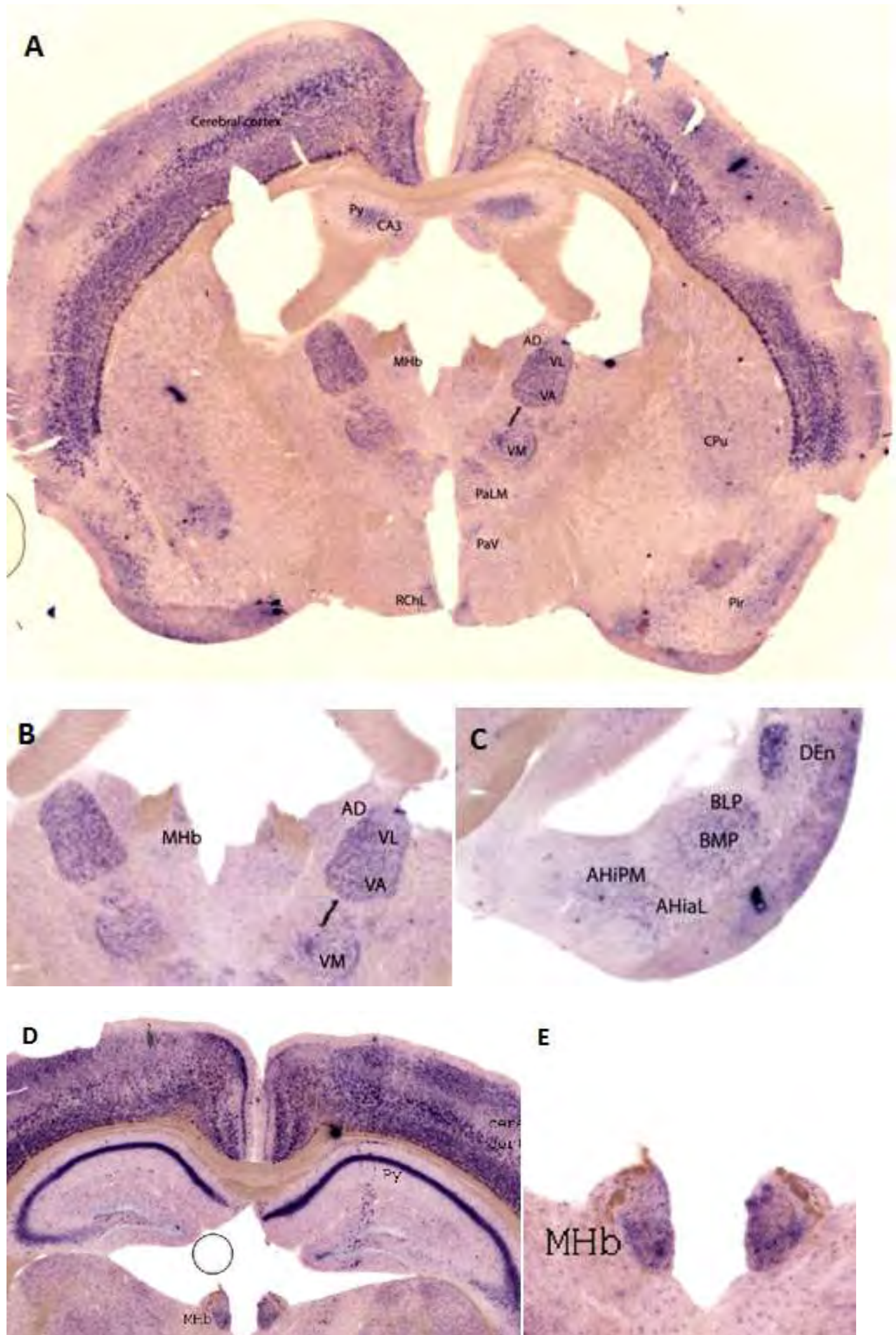
CNS expression of mouse *Nudt3*, *Etv5* and *Mtch2* in adult male C57Bl6/J mice

Coronal sections were used to screen male adult mouse brains for areas expressing *Etv5*, *Nudt3*, *Mtch2* and *Sh2b1* with *in situ* hybridization. The expression of these genes is ubiquitous, but with certain differences between tissues. Generally, expression was predominantly found in the cortex, thalamus, hypothalamus, hippocampus and amygdala. Although the mRNA expression of the genes was mapped in the whole brain (See Additional Folder), we focused on the brain regions that are known to be implicated in feeding and feeding behavior.

Etv5

In the diencephalon, *Etv5* was highly expressed in the anteroventral thalamic nucleus (AV), while in the ventral and lateral part of paraventricular hypothalamic nucleus (PaV, PaLM), and in the arcuate hypothalamic nucleus (Arc) the *Etv5* expression level was medium. In the telencephalon, *Etv5* was highly expressed in the medial (CeM), lateral (CeL), and capsular (CeC) divisions of the central amygdaloid nucleus, as well as in the ventromedial part of the lateral amygdaloid nucleus (LaVM). In the anterior and posterior part of the basolateral amygdaloid nucleus (BLA, BLP), as well as in the anterior and posterior part of the basomedial amygdaloid nucleus (BMA, BMP), medium *Etv5* expression level was detected. Also, high expression of *Etv5* was found in the regions of the intraamygdaloid division of stria terminalis (STIA) and the parabrachial pigmented nucleus of the ventral tegmental area (VTA). In the core and shell of the accumbens nucleus (Acbc, Acbsh), and in the anterolateral

and posteromedial amygdalo-hippocampal area (AHiAL, AHiPM) the *Etv5* expression level was medium.



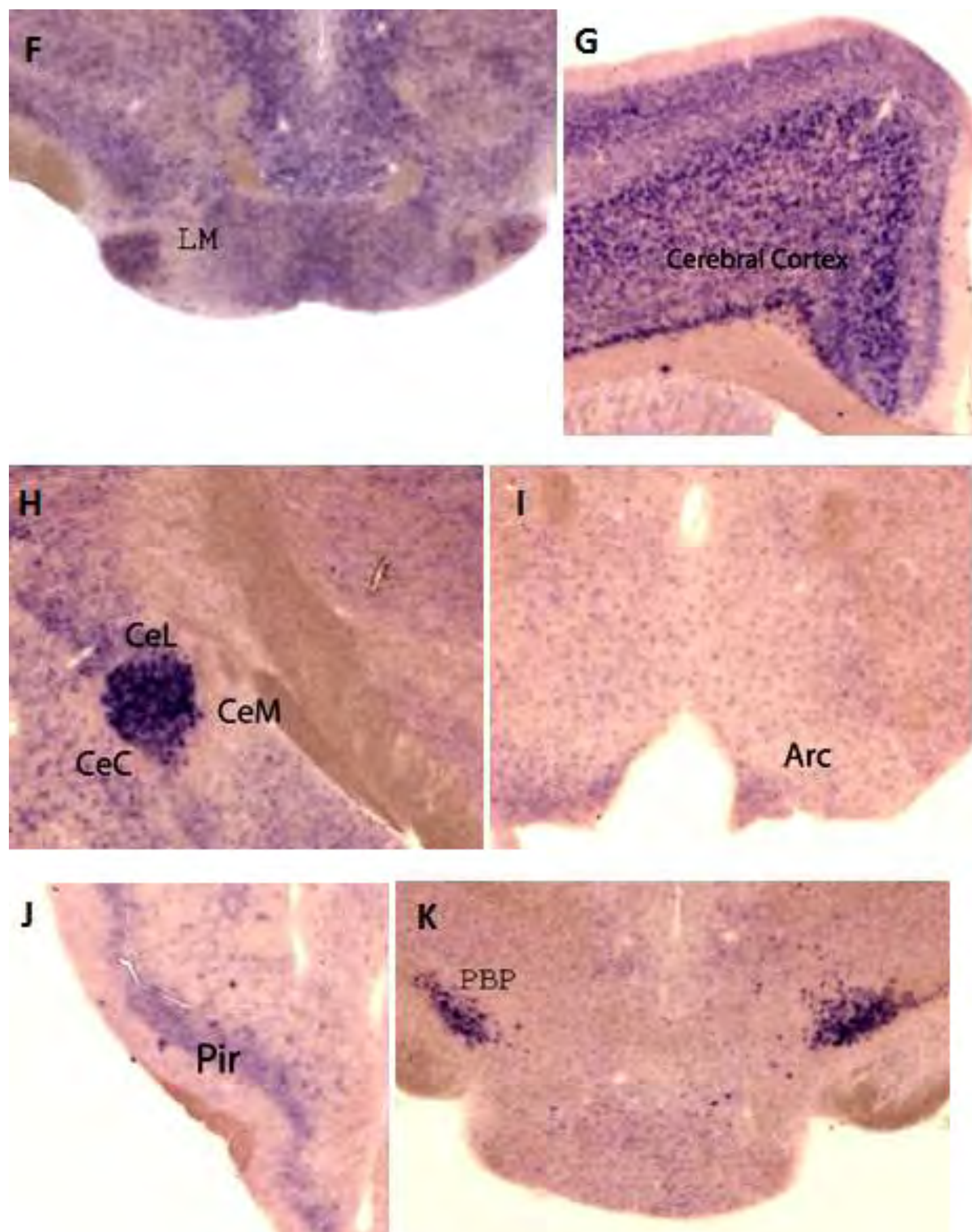
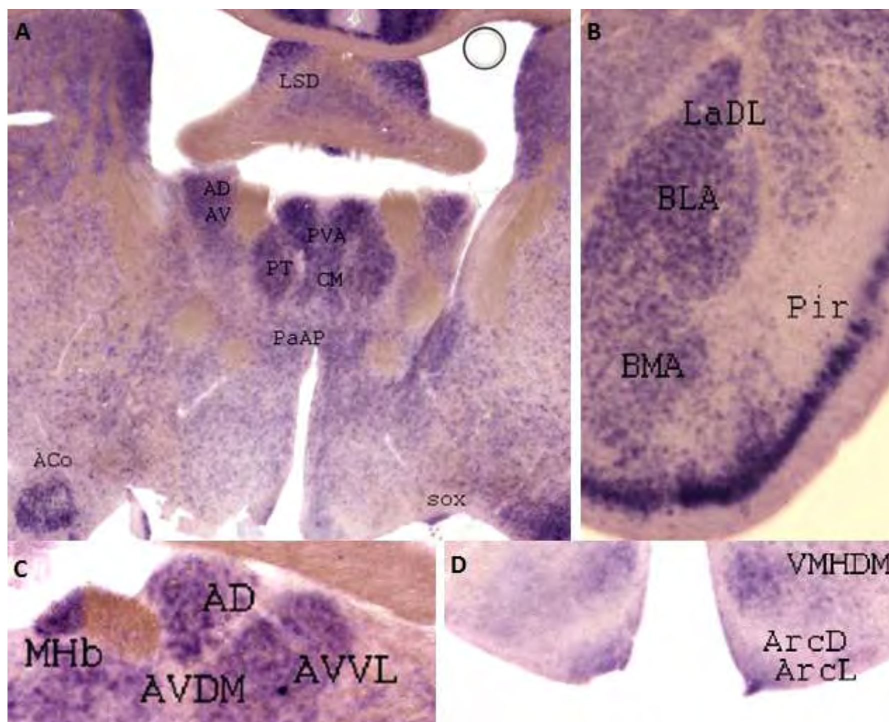


Figure 6. Etv5 expression in mouse brain. *In situ hybridization on free floating sections using 1 mg probe/ml of DIG-labeled mouse Etv5 probe (purple staining). 50 µm coronal mouse brain sections, visualized as overview, or close up pictures. Abbreviations;* ***A****. Caudate Putamen (CPu), Pyramidal Tract (Py), Field Ca3 Of Hippocampus (CA3), Medial Habenular Nucleus (MHb), Anterodorsal Thalamic Nucleus (AD), Ventrolateral Thalamic Nucleus (VL), Ventral Anterior Thalamic Nucleus (VA), Ventromedial Thalamic Nucleus (VM), Paraventricular Hypothalamic Nucleus Lateral Magnocellular Part (PaLM), Paraventricular*

*Hypothalamic Nucleus Ventral Part (PaV), Caudate Putamen (CPu), Piriform Cortex (Pir), Retrochiasmatic Area Lateral Part (RChL), **B**. Medial Habenular Nucleus (MHb), Anterodorsal Thalamic Nucleus (AD), Ventrolateral Thalamic Nucleus (VL), Ventral Anterior Thalamic Nucleus (VA), Ventromedial Thalamic Nucleus (VM), **C**. Dorsal Endopiriform Nucleus (DEn), Basolateral Amygdaloid Nucleus Posterior Part (BLP), Basomedial Amygdaloid Nucleus Posterior Part (BMP), Amygdalohippocampal Area Posteromedial Part (AHiPM), Amygdalohippocampal Area Anterolateral Part (AHiAL), **D**. Pyramidal Tract (Py), Medial Habenular Nucleus (MHb), **E.** Medial Habenular Nucleus (MHb), **F**. Lateral Mammillary Nucleus (LM), **G**. Cerebral Cortex, **H**. Central Amygdaloid Nucleus Medial Division (CeM), Central Amygdaloid Nucleus Lateral Division (CeL), Central Amygdaloid Nucleus Capsular Division (CeC), **I**. Arcuate Hypothalamic Nucleus (Arc), **J**. Piriform Cortex (Pir), **K**. Parabrachial Pigmented Area (PBP)*

Nudt3

In the diencephalon, the *Nudt3* expression was medium in the posterior, lateral magnocellular, and medial parvicellular part of the paraventricular hypothalamic nucleus (PaPo, PaLM, PaMP) and in the ventromedial hypothalamic nucleus (VMH). Also, low *Nudt3* expression was observed in the lateral and dorsomedial part of the arcuate hypothalamic nucleus (ArcL, ArcD). Moreover, in the telencephalon, medium *Nudt3* expression level was observed in the anterior part of the cortical amygdaloid nucleus (ACo), in the anterior and posterior part of the basolateral amygdaloid nucleus (BLA, BLP), as well as in the anterior and posterior part of the basomedial amygdaloid nucleus (BMA, BMP), and in the dorsolateral, ventrolateral and ventromedial part of the lateral amygdaloid nucleus (LaDL, LaVL, LaDM). Also, high expression of *Nudt3* was observed in the regions of the posteromedial part of the amygdalo-hippocampal area (AHiPM), the dorsal tier of the compact part of substantia nigra, and the parabrachial pigmented area (PBP)of the Ventral Tegmental Area (VTA).



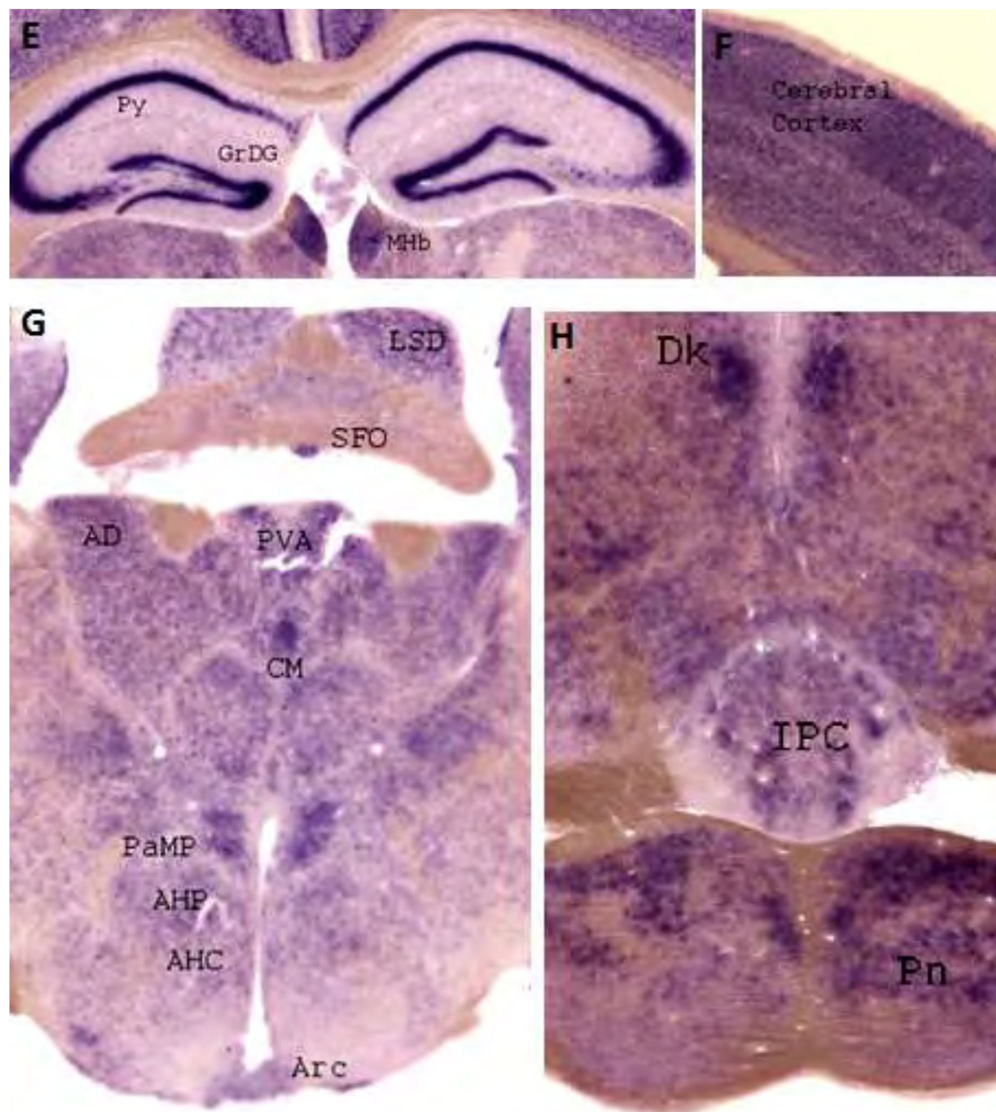
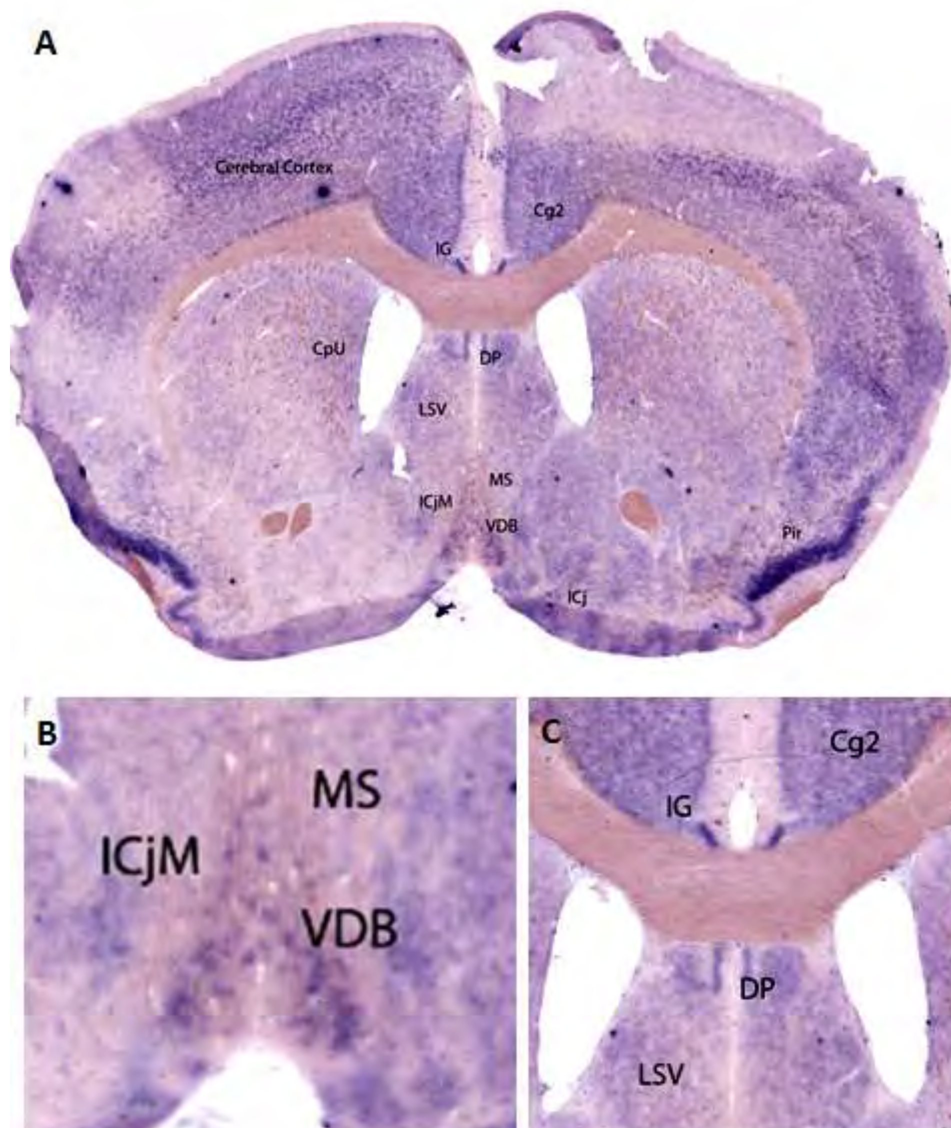


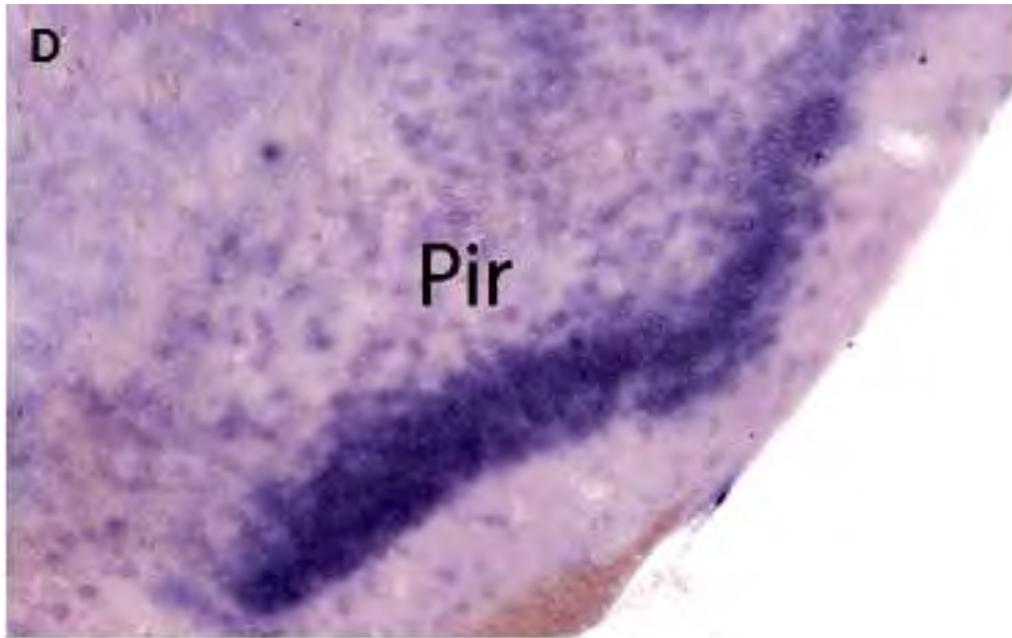
Figure 7. Nudt3 expression in mouse brain. *In situ hybridization on free floating section using 1 mg probe/ml of DIG-labeled mouse Nudt3 probe (purple staining). 50 µm coronal mouse brain sections, visualized as close up pictures. Abbreviations;* ***A****. Lateral Septal Nucleus Dorsal Part (LSD), Anterodorsal Thalamic Nucleus (AD), Anteroventral Thalamic Nucleus (AV), Paraventricular Thalamic Nucleus Anterior Part (PVA), Paratenial Thalamic Nucleus (PT), Central Medial Thalamic Nucleus (CM), Paraventricular Hypothalamic Nucleus Anterior Parvicellular Part (PaAP), Anterior Cortical Amygdaloid Nucleus (ACo), Supraoptic Decussation (sox),* ***B****. Lateral Amygdaloid Nucleus Dorsolateral Part (LaDL), Basolateral, Basolateral Amygdaloid Nucleus Anterior Part (BLA), Basomedial Amygdaloid Nucleus Anterior Part (BMA), piriform cortex (Pir),* ***C****. Medial Habenular Nucleus (MHb), Anteroventral Thalamic Nucleus Dorsomedial Part (AVDM), Anteroventral Thalamic Nucleus Ventrolateral Part (AVVL),* ***D****.Ventromedial Hypothalamic Nucleus Dorsomedial Part (VMHDM), Arcuate Hypothalamic Nucleus Dorsal Part (ArcD), Arcuate Hypothalamic Nucleus Lateral Part (ArcL),* ***E****. Pyramidal Tract (Py), Granular Layer Of The Dentate Gyrus (GrDG), Medial Habenular Nucleus (MHb,)* ***F****. Cerebral Cortex,* ***G****. Subfornical Organ (SFO), Paraventricular Hypothalamic Nucleus Medial Parvicellular Part (PaMP), Anterior Hypothalamic Area Posterior Part (AHP), Anterior Hypothalamic Area Central Part (AHC), Arcuate Hypothalamic Nucleus (Arc),* ***H****.*

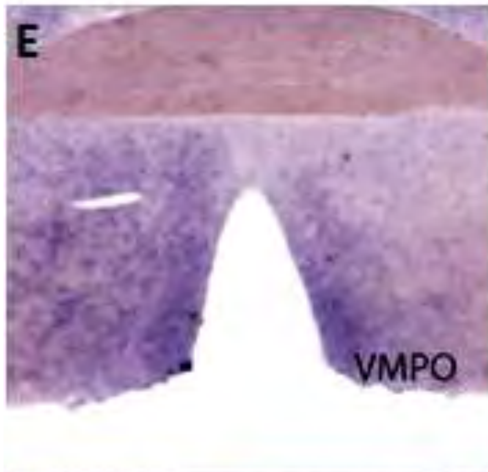
Nucleus Of Darkschewitsch (Dk), Interpeduncular Nucleus Caudal Subnucleus (IPC), Paranigral Nucleus (PN)

Mtch2

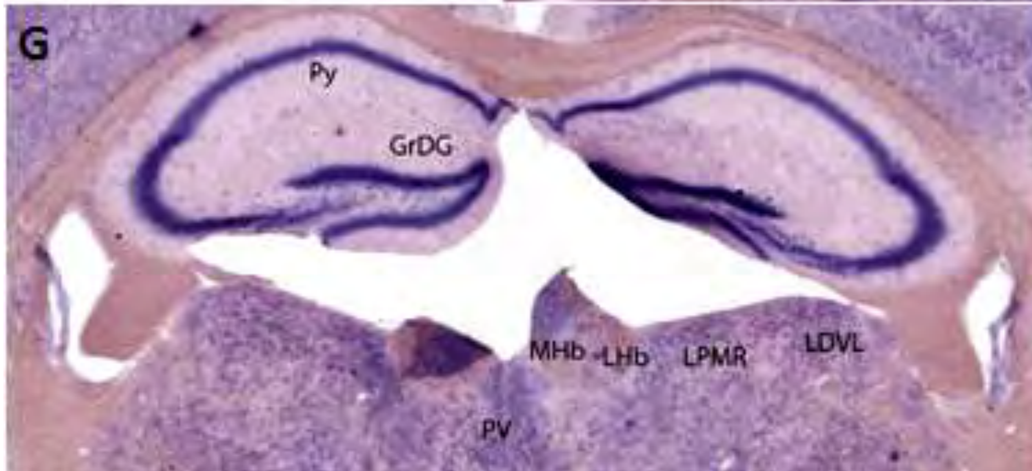
In the diencephalon, the *Mtch2* expression level was medium in the dorsomedial part of the ventromedial hypothalamic nucleus (VMHDM). In the telencephalon, medium *Nudt3* expression was detected in the anterior part of the basomedial amygdaloid nucleus (BMA). Also, medium expression level of *Mtch2* was observed in the regions of caudate putamen (CPu) and the reticular part of substantia nigra (SNR).




D
Pir


E
VMPO

F
Cerebral Cortex


G
Py
GrDG
MHb
LHb
LPMR
LDVL
PV

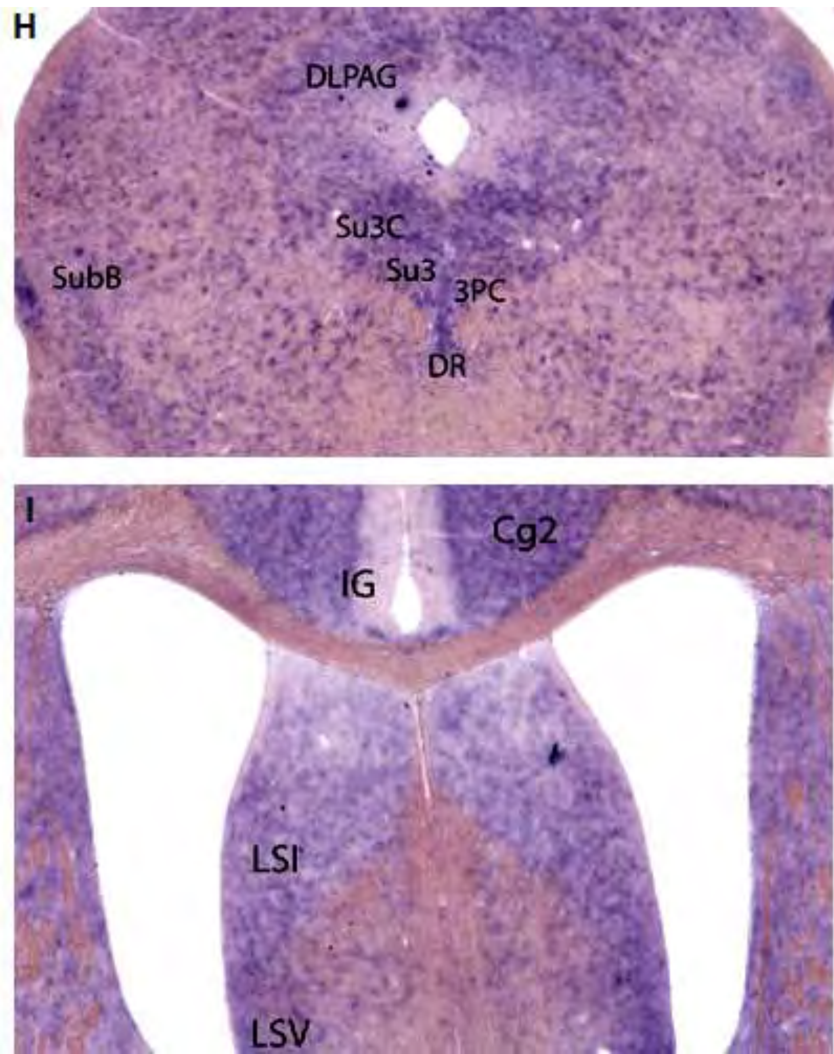
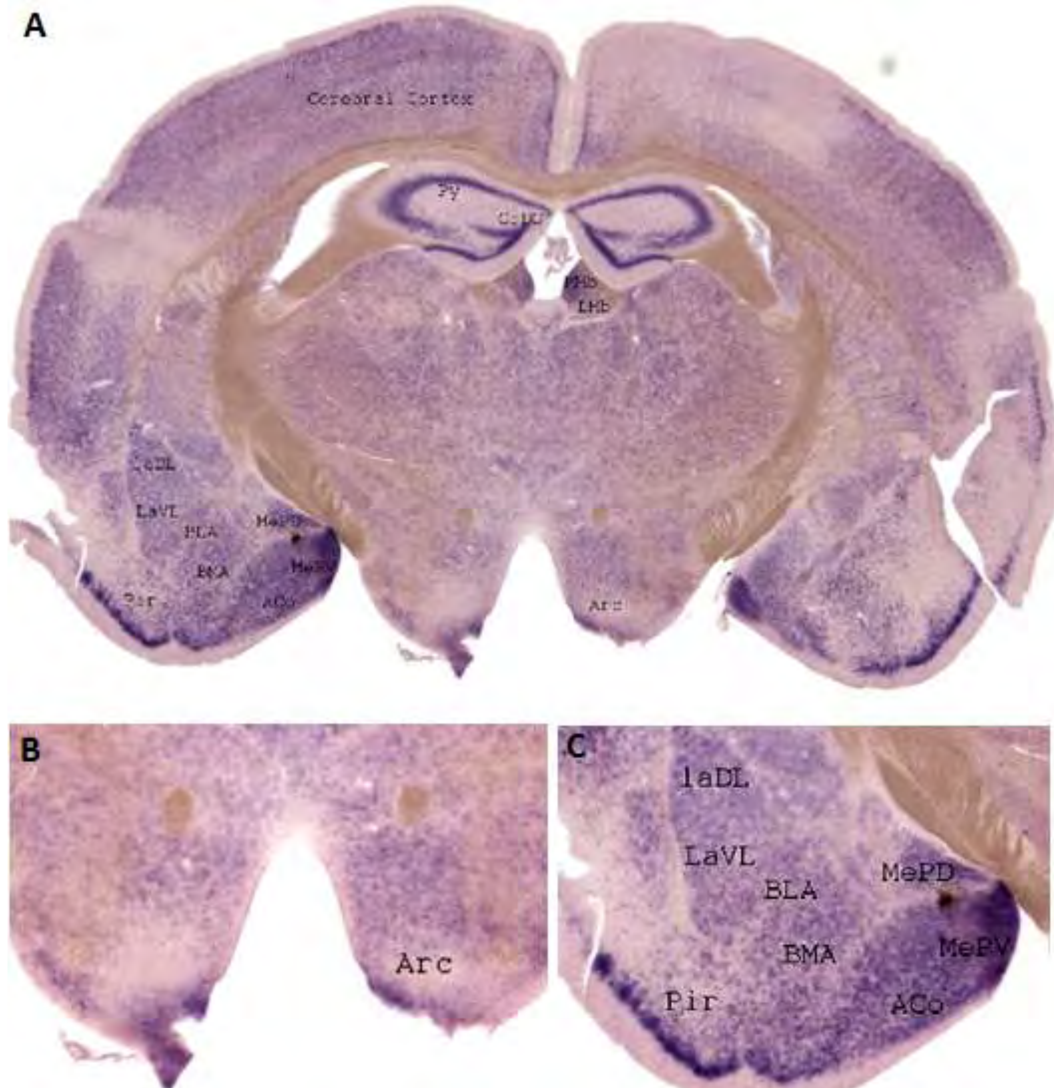


Figure 8. Mtch2 expression in mouse brain. *In situ hybridization on free floating section using 1 mg probe/ml of DIG-labeled mouse Nudt3 probe (purple staining). 50 µm coronal mouse brain sections, visualized as overview, or close up pictures. Abbreviations;* ***A****. Cingulate Cortex Area 2 (Cg2), Indusium Griseum (IG), Dorsal Peduncular Cortex (DP), Lateral Septal Nucleus Ventral Part (LSV), Caudate Putamen (CPu), Medial Septal Nucleus (MS), Nucleus Of The Vertical Limb Of The Diagonal Band (VDB), Islands Of Calleja Major Island (ICjM), Island Of Calleja (ICj), Piriform Cortex (Pir), Ventromedial Posterior Nucleus (VMPO),* ***B****. Medial Septal Nucleus (MS), Nucleus Of The Vertical Limb Of The Diagonal Band (VDB), Islands Of Calleja Major Island (ICjM),* ***C****. Cingulate Cortex Area 2 (Cg2), Indusium Griseum (IG), Dorsal Peduncular Cortex (DP), Lateral Septal Nucleus Ventral Part (LSV),* ***D****. Piriform Cortex (Pir),* ***E****. Ventromedial Posterior Nucleus (VMPO),* ***F****. Cerebral Cortex,* ***G****. Pyramidal Tract (py), Granular Layer Of The Dentate Gyrus (GrDG), Paraventricular Thalamic Nucleus (PV), Medial Habenular Nucleus (MHb), Lateral Habenular Nucleus (LHb), Lateral Posterior Thalamic Nucleus Mediorostral Part (LPMR), Laterodorsal Thalamic Nucleus Ventrolateral Part (LDVL),* ***H****. Dorsolateral Periaqueductal Gray (DLPAG), subbrachial nucleus (SubB), Supraoculomotor Cap (Su3C), Supraoculomotor Periaqueductal Gray (Su3), oculomotor nucleus, parvicellular part (3PC), Dorsal Raphe Nucleus (DR),* ***I****. Lateral septal nucleus, intermediate part (LSI), Lateral Septal Nucleus Ventral Part (LSV), Cingulate Cortex Area 2 (Cg2), Indusium Griseum (IG)*

Sh2b1

In the diencephalon, *Sh2b1* expression level was medium in the arcuate nucleus (Arc). In the telencephalon, medium *Sh2b1* expression was detected in the anterior cortical amygdaloid nucleus (ACo), the anterior and lateral parts of the basomedial amygdaloid nucleus (BMA, BLA), the dorsolateral and ventrolateral parts of the lateral amygdaloid nucleus (LaDL, LaVL), as well as in the , posterodorsal and posteroventral parts of the medial amygdaloid nucleus (MePD, MePV).



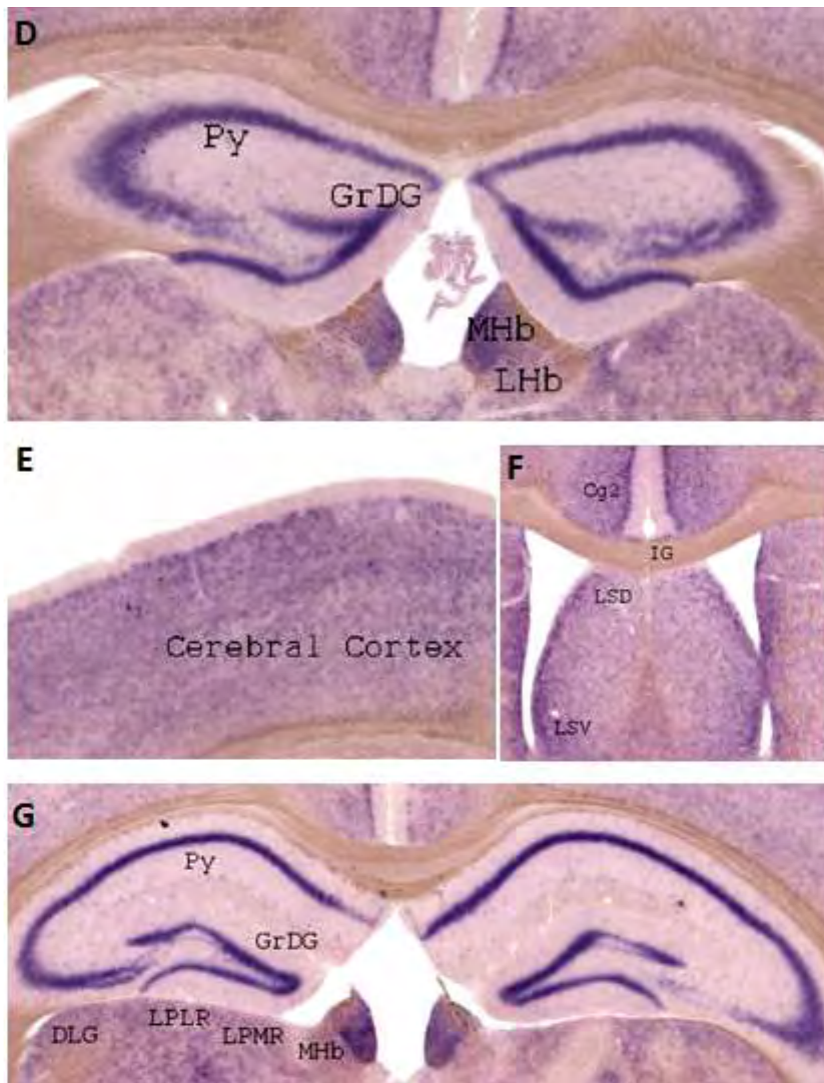


Figure 9. Sh2b1 expression in mouse brain. *In situ hybridization on free floating section using 1 mg probe/ml of DIG-labeled mouse Nudt3 probe (purple staining). 50 µm coronal mouse brain sections, visualized as overview, or close up pictures. Abbreviations;* ***A****. Granular dentate gyrus (GrDG), Pyramidal cell hippocampus (Py)****,*** *Lateral habenular nucleus (LHb), Medial habenular nucleus (MHb), Arcuate Nucleus (Arc), Anterior Cortical Amygdaloid Nucleus (ACo), Basomedial amygdaloid nucleus, anterior part (BMA), Basomedial amygdaloid nucleus lateral part (BLA), Lateral Amygdaloid Nucleus Dorsolateral Part (LaDL), Lateral Amygdaloid Nucleus Ventrolateral Part (LaVL), Medial Amygdaloid Nucleus Posterodorsal Part (MePD), Medial Amygdaloid Nucleus Posteroventral Part (MePV),* ***B****. Arcuate Nucleus (Arc),* ***C****. Anterior Cortical Amygdaloid Nucleus (ACo), Basomedial amygdaloid nucleus, anterior part (BMA), Basomedial amygdaloid nucleus lateral part (BLA), Lateral Amygdaloid Nucleus Dorsolateral Part (LaDL), Lateral Amygdaloid Nucleus Ventrolateral Part (LaVL), Medial Amygdaloid Nucleus Posterodorsal Part (MePD), Medial Amygdaloid Nucleus Posteroventral Part (MePV) ,* ***D****. Granular dentate gyrus (GrDG), Pyramidal cell hippocampus (Py)****,*** *Lateral habenular nucleus (LHb), Medial habenular nucleus (MHb),* ***E****. Cerebral Cortex,* ***F****. Cingulated cortex area 2 (Cg2), Indusium griseum (IG), Lateral septal nucleus, dorsal part (LSD), Lateral septal nucleus, ventral part (LSV),* ***G****. Dorsal Lateral Geniculate Nucleus (DLG), Lateral posterior thalamic nucleus, mediorostral part (LPMR),*

Lateral Posterior Thalamic Nucleus, Laterorostral Part (LPLR), Granular dentate gyrus (GrDG), Pyramidal cell hippocampus (Py), Medial habenular nucleus (MHb)

DISCUSSION

The areas and nuclei where the obesity related genes were expressed

The obesity related genes were expressed in a lot of brain areas and nuclei (see additional folder and results), a lot of which have been previously related with obesity.

- ✓ Etv5 expression in the amygdala, the ventral tegmental area and the accumbens nucleus strongly suggest the gene's role in food reward, as those regions are reward network components. Also, expression in the paraventricular and arcuate hypothalamic nuclei, suggests a probable role of Etv5 in energy balance.
- ✓ Nudt3 expression in food-reward network components, like the amygdala and the ventral tegmental area, suggest a role of it in food reward. Expression in hypothalamic regions that participate in energy-related feeding behavior, like the paraventricular and anteroventral thalamic nuclei, suggest a role of the gene in energy balance.
- ✓ Mtch2 expression only in the ventromedial hypothalamic nucleus, and in the basomedial amygdaloid nucleus, not so strongly suggest a possible role in energy intake and food reward, respectively.
- ✓ Finally, Sh2b1 expression in the hypothalamic arcuate nucleus, the most important hypothalamic component in energy-associated feeding behavior, and in many amygdaloid nuclei, suggests a role of it in energy balance and food reward, respectively.

Moreover, all the genes showed very high expression in the hippocampal formation that has been recently associated with food intake, in piriform cortex that participates in the olfactory system, in the cerebral and cingulated cortex, and in other brain regions that are potentially associated with food intake behavior.

Previous bibliography about the regions where expression was high, and their relation with obesity and food intake behavior will be listed, aiming in the elucidation of their function in food intake behavior.

~Hypothalamus

The arcuate nucleus (Arc) and the ventromedial hypothalamus (VMH) are hypothalamic regions that integrate peripheral metabolic signals and receive input from multiple other metabolic centers in the brain. The Arc includes two neuronal populations with different functional roles. One population is orexigenic and expresses neuropeptide Y and AGRP (agouti-related protein), while the other is anorexigenic and expresses POMC and CART (cocaine- and amphetamine-regulated transcript) (Garfield, Lam et al. 2009). So, energy

sufficiency leads to low NPY/AgRP expression and high POMC/CART expression in the Arc, which promote satiety (Ziotopoulou, Mantzoros et al. 2000) while energy lack results in high NPY/AgRP expression and low POMC/CART expression that promote hunger (Pinto, Roseberry et al. 2004). VMH is described as the satiety center. Lesions in the VMH led rats to overeating and obesity, while electrical stimulation of it led to increased satiety as the animals stopped eating (Treatment of the Obese Patient (Contemporary Endocrinology), Robert F. Kushner, Daniel H. Bessesen, page 4).

Paraventricular hypothalamic nucleus is known to be involved in hunger regulation. Lesions of the nucleus led to increased meal size in rat experiments(Shor-Posner, Azar et al. 1985). Also, paraventricular hypothalamic nucleus, as well as arcuate nucleus, ventromedial hypothalamic nucleus, and lateral hypothalamic area are regions where a lot of leptin receptors are, explaining their effect on energy balance (Guyenet and Schwartz 2012).

~Ventral Tegmental Area-Nucleus Accumbens-Substantia Nigra

Contrary to the hypothalamic centers that control homeostatic feeding, the substantia nigra (SN) and the ventral tegmental area (VTA) are regions related with motivation for food, as they are the sole sources of striatal and limbic forebrain dopamine (Zhou and Palmiter 1995). In the VTA and SN extensive co-expression of insulin and leptin receptors with a dopamine neuron marker, suggesting that midbrain dopamine neurons are direct targets of insulin and leptin, participating in the mediation of the effects of these hormones on reward-seeking behavior (Figlewicz, Evans et al. 2003).

As previously mentioned (See Introduction-Food reward and palatability), the nucleus accumbens plays a crucial role in food reward and palatability mediated by dopamine and opioids, respectively. Also, it has been discovered that GLP-1 release in accumbens nucleus of rats reduces the hedonic value of food, as antagonists of the GLP-1 receptors have been shown to increase meals size and palatability in rats (Dossat, Diaz et al. 2013).

~Amygdala

Generally, the amygdala are known for their role in memory processing and emotions, especially fear and stress. With regard to food, the amygdala are implicated in food reward, detecting the flavor intensity and palatability of a certain food and inducing the desire to eat more. As a result, amydgala are strongly associated with obesity and food intake behavior (Yale Scientific 2012).

During electrophysiology experiments in rats, it was shown that the central amygdaloid nucleus influences gut-associated neurons in the dorsal vagal complex. After electrical stimulation of the nucleus, altered basal firing rates in the gut-associated neurons of the solitary tract and dorsal motor nucleus of the vagus were observed, as well as modulated neuronal response to gastrointestinal stimuli in the dorsal vagal complex. This neuronal circuitry seems to explain how gastrointestinal activity is regulated by the amygdala (Zhang, Cui et al. 2003)

Moreover, *in situ* hybridization experiments revealed the presence of ghrelin receptor mRNA in several amygdaloid nuclei, with the highest levels of expression in the ventrolateral (LaVL)

and ventromedial (LaVM) parts of the lateral amygdaloid nucleus (Alvarez-Crespo, Skibicka et al. 2012).

Melanin-concentrating hormone (MCH) is an orexigenic peptide involved in food intake behavior, controlling both short and long term food intake in rats (Della-Zuana, Presse et al. 2002). Neurons containing MCH are mostly located in the lateral hypothalamic area (LHA). In a recent study, it was shown that neurons of the anterior part of basomedial amygdaloid nucleus (BMA) and the anterior cortical amygdaloid nucleus (CoA) innervate MCH neurons in the LHA, suggesting that this pathway may have a role in food intake (Niu, Yokota et al. 2012). LHA is characterized as hunger center. Lesions in the LHA of rats have resulted in loss of interest in food, and decreased body weight. When the LHA was electrically stimulated, it caused eating in satiated animals (Treatment of the Obese Patient (Contemporary Endocrinology), Robert F. Kushner (Editor), Daniel H. Bessesen, page 4).

Corticotropin-releasing factor type 1 (CRF_1) receptor antagonists, having the ability to reduce the motivational effects of withdrawal, have been proposed as novel therapeutic targets for addictive disorders. As palatable food intake is closely related to addiction, scientists investigated whether CRF_1 receptors in the basolateral (BLA) and central (CeA) amygdaloid nucleus can mediate excessive eating of palatable food in diet cycled rats. One rat group had *ad libitum* access to a chow diet 7 days a week (*Chow/Chow,* control group), and the other had free access to chow for 5 days a week, followed by 2 days of *ad libitum* acces to a highly palatable, chocolate-flavored, high-sucrose diet (*Chow/Palatable* group). Microinfusion of the CRF_1 receptor antagonist R121919 into the BLA reduced the hypophagia of regular chow diet in *Chow/Palatable* rats, without affecting it in *Chow/Chow* rats. On the other hand, intra-CeA R121919 completely blocked the excessive eating of palatable food in *Chow/Palatable* rats, without affecting regular chow intake in control *Chow/Chow* rats (left panel). These results indicate that the CRF–CRF_1 receptor system in the CeA is a key mediator of excessive eating of palatable food and the withdrawal-dependent negative affect, whereas in the BLA it mediates the subjects' aversive responses induced by reward reduction (Iemolo, Blasio et al. 2013).

As serotonin and the medial amygdaloid nucleus (MeA) has been known as two components critical in food intake regulation, scientists examined the effects of the serotonin reuptake inhibitor, zimelidine, on rat feeding behaviour in order to investigate the serotonergic system in the MeA. Zimelidine microinjection into the MeA provoked dose dependent hypophagic effects in fasted rats, while microinjection of a 5-HT(2C) receptor antagonist blocked this hypophagic effect, suggesting the role of MeA 5-HT(2C) receptors in the modulation of the hypophagic effect caused by zimelidine (Scopinho, Fortaleza et al. 2012).

Finally, when naloxone was infused into the ventral pallidum or nucleus accumbens shell, and food deprivation was induced, changes in sucrose palatability were blocked, and incentive learning was not affected. On the other hand, naloxone infusion into the basolateral amygdala blocked incentive learning and did not affect sucrose palatability. These changes in palatability and incentive learning regulated by opioids suggest the complex role of endogenous opioids in reward system, and show the role of these regions to neural processes that rule rewarding events and desire (Wassum, Ostlund et al. 2009).

~Thalamus

The ventrolateral thalamic nucleus has been shown to play a crucial role in the learning of performing tasks that require coordination and planning of movement (Subcortical Functions in Language and Memory, Bruce A. Crosson). This could be a component in food reward behavior, as many times the acquisition of the desired food requires the performance of a specific motor task, such us the pressing of a lever (Sharma, Hryhorczuk et al. 2012).

The anterodorsal and anteroventral thalamic nuclei might not be directly associated with food intake, but they have a crucial role in spatial memory (van Groen, Kadish et al. 2002). Since spatial memory is important in food reward behaviour (a good example are the rats remembering the place where they got palatable food (Jarosz, Kessler et al. 2007)), the expression of the genes in those nuclei might show their involvement in food reward.

~Piriform Cortex

Apart from taste, touch, temperature and texture, the smell and the appearance of food contribute to the whole picture of palatability. The piriform cortex (Pir) is a basic component of the olfactory system, the sensory system used for smell (Kenny 2011). It has been suggested that the anterior piriform cortex plays a role in neuroperception of deficiencies or imbalances in physiologically essential amino acids. Diets deficient in aminoacids has been shown to induce the expression of c-fos in the anterior piriform cortex (Cummings 1997).

~Hippocampus

The hippocampus, a brain structure involved in learning, memory, and spatial navigation, has recently been associated with food intake control. Leptin administration to the ventral hippocampus suppressed food intake, body weight and memory consolidation for the spatial location of food. So, ventral hippocampal leptin signaling seems to contribute to the inhibition of food-related memories provoked by coherent stimuli (Kanoski, Hayes et al. 2011).

~Cerebral cortex-cingulate cortex

Scientists investigated that there was an increase in the binding of CCK to its receptors in the cerebral cortex of ob/ob mice, due to an increase of the receptor sites, suggesting that CCK binding and action in specific regions may be associated with obesity (Saito, Williams et al. 1981). Also, a neuronal representation of taste is found in the cingulate cortex, which is activated by many pleasant stimuli, suggesting the role of it in motivation and emotion (Rolls 2008).

~Caudate Putamen

Restoration of dopamine production within the caudate putamen of hypophagic dopamine-deficient mice restored their feeding on regular chow, suggesting a role in food intake behavior for the region (Szczypka, Kwok et al. 2001).

How the obesity-associated SNPs act on the expression of the nearby genes? Possible mechanisms

The obesity-associated SNPs of interest are located in introns near the genes on which we did research. Even if introns are removed through splicing, and their sequence does not

participate in the transcript, it is sure that they can affect gene expression. This is obvious from the fact that intronless and intron-containg versions of the same gene have shown different expression profile. Moreover, introns have been shown to be extremely crucial in transgene expression. By the addition of just one intron in transgenic mice the efficiency of transcription could dramatically increase up to 100 times (Brinster, Allen et al. 1988). The possible mechanisms of intron function are several. First of all, introns are important for efficient splicing. Also, elements that regulate the transcription, such as repressors and enhancers, can be binded on binding sites that are located in introns. So, a difference in the sequence of a transcriptional regulatory element could dramatically effect the extent of gene expression. Moreover, it has been shown that splicing signals can enhance the action of RNA polymerase, enhancing transcription level (Furger, O'Sullivan et al. 2002). When it comes to pre-mRNA processing events, on the one hand, the nuclear cap-binding complex promotes the excision of the 5′-most intron (Le Hir, Nott et al. 2003). On the other hand, 3′-end formation is reciprocally linked to splicing, involving direct contacts between splicing and polyadenylation machineries (Proudfoot, Furger et al. 2002). Furthermore, sequences in introns are required for the chemical alteration of exonic nucleotides through RNA editing (Reenan 2001). Finally, it has been shown that some introns are mobile genetic elements (Lambowitz and Belfort 1993). All these possible roles that introns have in the regulation of gene expression, can explain why an SNP in a crucial sequence of the intron could lead to big expression changes.

As the obesity-associated SNPs rs7647305 and rs9816226 are located in an intron near ETV5, they could probably increase the expression of the ETV5 transcription factor by the disturbance of the sequence of a transcriptional regulatory element binding site in the intron.

Except from their proximity to ETV5, the obesity associated SNPs located in the intron near ETV5 are also near genes that encode other proteins. rs7647305, is located in an intron near the diacylglycerol kinase gamma, which reverses the normal flow of glycerolipid biosynthesis by phosphorylating diacylglycerol back to phosphatidic acid. As diacylglycerol is a precursor of triacylglycerol, a decreased expression and action level of diacylglycerol kinase gamma will lead to increased triacylglycerol levels. So, one possible mechanism of how rs7647305 acts is by disturbing the sequence of a DGKG gene suppressor binding site in the intron, and decreasing the expression level of DGKG. On the other hand, rs9816226, is located near a splicing factor. So, the SNP could probably affect the expression of the splicing factor, disturbing splicing.

The obesity-related SNP rs206936 is located in an intron near NUDT3. As NUDT3 has shown in vitro decapping activity in methylated and unmethylated RNA, a possible action mechanism of it in obesity is by affecting mRNA decapping and stability. This SNP could probably change the sequence of a suppressor or enhancer binding site, changing the extent of decapping activity. Increased decapping leads in increased mRNA degradation by exonucleases, while decreased decapping results mRNAs with 5′cap, stable and resistant to exonucleases, available for splicing and translation. Interesting is the fact that when the body is under nutrient starvation condition, the decapping is stimulated in order to save

energy from protein synthesis, and decapping is also promoted when premature stop codons are recognized.

The obesity associated SNPs located in an intron near the MTCH2 gene, could affect the expression of the protein through a sequence change in a binding site of a transcriptional regulatory element. Since MTCH2 regulates cell proliferation and apoptosis, and obesity is associated with increased fat cell renewal (Arner and Spalding 2010), the distorted control of fat cell renewal is a possible mechanism by which MTCH2 contributes to obesity (Kulyte, Ryden et al. 2011).

Concerning the SNPs located in an intron near the SH2B1 gene, they could decrease the expression of the protein through a sequence change in a binding site of a transcriptional regulatory element. A disturbance in the expression of the SH2B1 protein can have important effects in energy and glucose homeostasis, as SH2B1 binds insulin receptors and their substrates, promoting insulin receptor catalytic activity.

Finally, all the obesity-associated intron SNPs could cause a splicing site mutation, resulting in introns remaining in mature mRNA. Also, the SNPs could affect RNA editing, resulting in altered RNA structure, or even degradation.

Ideas for future research

The results gained from the *in situ* hybridization experiments are very interesting, as the expression of the genes of interest in food intake-related brain areas shows their function in food intake behavior and energy balance through CNS. However, in order to gain more information about the function of the genes, more different experiments should be conducted. Double *in situ* could allow the co-localization of the mRNA of the genes. Also, immnohistochemistry experiments for the cellular localization of the protein, as well as c-Fos immunohistochemistry experiments combined with in situ hybridization for the gene compared between feeding termination and feeding initiation, could shed more light to the function of the genes. For higher specificity and sensitivity, *in situ* proximity ligation assay (PLA) could be used for the detection of the protein. Moreover, the expression level of the genes could be charted through real time PCR. The expression could be charted after different conditions, such as consumption of palatable food or food deprivation.

Also, since the genes seem to have an expression predominant in the amygdala and other food intake behavior-related brain regions, behavioral studies on wild type and knock out mice for the genes would be definitely interesting. Behavioral experiments during which the animal has to perform a task in order to obtain a highly palatable food reward (pressing a lever to receive the reward, learning a specific route finding its way through a maze) are good examples. Also it will be interesting to study the stress-induced eating of palatable food, as well as the battle between fear or pain, and palatable food.

Finally, clinical studies should be conducted on genotyped subjects. Functional magnetic resonance imaging (fMRI) of the subjects' brain at different times could shed more light on the expression and function of the polymorphisms. Psychological tests like the Stroop can

also contribute to create a more complete picture of how the polymorphisms affect the different brain regions.

The eventual characterization of the obesity-associated gene products will reveal their etiological pathways, suggesting them as molecular targets for pharmacological interventions that will probably lead to effective treatments. Still, many pieces of the puzzle are missing.

Conclusion

In conclusion, our data allow as to link those four obesity-associated genes (Etv5, Nudt3, Mtch2, Sh2b1) with the regulation of food intake and food intake preferences by the CNS. The association of the genes with food reward seems to be stronger than the association with homeostatic feeding. Although our results seem to bring us one step closer to finding out the function of those genes, further investigation should be conducted in order to completely elucidate their functional role.

Aknowledgements

First of all, I would like to express my very great appreciation to professor Helgi Schiöth for accepting me in his group and giving me the chance to take part in such a promising project. Then, I would like to thank my tutor Anders Eriksson for his great help with my project. I would also like to thank Ida Hansson for her help.

Then, I would like to express my very great appreciation to assistant professor Nikolaos Balatsos for his great help and for accepting to be my supervisor, and to lecturer Theologia Sarafidou for accepting to participate in my jury.

Finally, I would like to thank my friend and colleague Nataly Perez Martinez for her assistance through these 6 months, and my friends George Flamourakis and Elena Gkanatsiou for joining me in this unforgettable journey.

ADDITIONAL FOLDER

-, no apparent expression

+, low expression

++, medium expression

+++, high expression

The regions are organized in alphabetical order under each subsection.

Etv5 expression

Diencephalon
Thalamus

Anterodorsal thalamic nucleus (AD) ++

Anteromedial thalamic nucleus (AM) ++

Anteroventral thalamic nucleus (AV) +++

Dorsal lateral geniculate nucleus (DLG) ++

Lateral posterior thalamic nucleus , mediorostral part (LPMR) +

Laterodorsal thalamic nucleus, dorsomedial part (LDDM) +

Medial habenular nucleus (MHb) ++

Ventrolateral thalamic nucleus, anterior part (VA) +++

Ventrolateral thalamic nucleus, lateral part (VL) +++

Ventromedial thalamic nucleus, medial part (VM) ++

Hypothalamus

Arcuate hypothalamic nucleus (Arc)++

Paraventricular hypothalamic nucleus, ventral part (PaV) ++

Paraventricular Hypothalamic Nucleus, Lateral Magnocellular Part (Palm) ++

Telencephalon
Cerebral cortex

Layer 1-

Layer 2++

Layer 3+

Layer 4 +++

Layer 5++

Layer 6+++

Amygdala

Basolateral amygdaloid nucleus, anterior part (BLA)++

Basolateral amygdaloid nucleus, posterior part (BLP) ++

Basomedial amygdaloid nucleus, anterior part (BMA) ++

Basomedial amygdaloid nucleus, posterior part (BMP) ++

Central amygdaloid nucleus, capsular division (CeC) +++

Central amygdaloid nucleus, lateral division (CeL) +++

Central amygdaloid nucleus, medial division (CeM) +++

Lateral amygdaloid nucleus, ventromedial part (LaVM) +++

Hippocampal formation

Pyramidal cell hippocampus (Py) +++

Granular dentate gyrus (GrDG) +

Other

Accumbens nucleus core (Acbc) ++

Accumbens nucleus shell (Acbsh) ++

Amygdalo-hippocampal area, anterolateral (AHiAL) ++

Amygdalo-hippocampal area, posteromedial (AHiPM) ++

Caudate putamen (CPu) +

Dorsal endopyriform nucleus (DEn) +++

External capsule (EC) +++

Field CA3 hippocampus (CA3) ++

Lateral septal nucleus, dorsal part (LSD) ++

Lateral septal nucleus, intermediate part (LSI) ++

Molecular dentate gyrus (MoDG) +

Parabrachial pigmented nucleus (PBP) +++

Piriform cortex (Pir) ++

Retrochiasmatic area (RChL) ++

Stria terminalis intraamygdaloid division (STIA) +++

Ventral endopyrifrom nucleus (Ven) +++

Nudt3 expression

Diencephalon

Thalamus

Anterodorsal thalamic nucleus (AD) ++

Anteroventral Thalamic Nucleus (AV) ++

Anteroventral Thalamic Nucleus Dorsomedial Part (AVDM) ++

Anteroventral Thalamic Nucleus Ventrolateral Part (AVVL) ++

Central medial thalamic nucleus (CM) ++

Dorsal lateral geniculate nucleus (DLG) ++

Lateral posterior thalamic nucleus, mediocaudal part (LPMC) ++

Laterodorsal thalamic nucleus, dorsomedial part (LDDM) +

Medial habenular nucleus (MHb) ++

Parafascicular thalamic nucleus (PF) ++

Paraventricular Thalamic Nucleus Anterior Part (PVA) ++

Ventral posterior thalamic nucleus, parvicellular part (VPPC) ++

Ventral posteromedial thalamic nucleus (VPM) ++

Hypothalamus

Anterior Hypothalamic Area, Central Part (AHC) +

Anterior Hypothalamic Area, Posterior Part (AHP) +

Arcuate hypothalamic nucleus (Arc) +

Arcuate hypothalamic nucleus, dorsomedial part (ArcD) +

Arcuate hypothalamic nucleus, lateral part (ArcL) +

Lateral mammillary nucleus (LM) ++

Paraventricular hypothalamic nucleus, lateral magnocellular part (PaLM) ++

Paraventricular Hypothalamic Nucleus, Medial Parvicellular Part (PaMP) ++

Paraventricular hypothalamic nucleus, posterior part (PaPo) ++

Supraoptic nucleus (SO) ++

Ventromedial hypothalamic nucleus (VMH) ++

Telencephalon

Cerebral cortex

Layer 1-

Layer 2++

Layer 3++

Layer 4 ++

Layer 5++

Layer 6+

Amygdala

Anterior cortical amygdaloid nucleus (ACo) +++

Basolateral amygdaloid nucleus, anterior part (BLA) ++

Basolateral amygdaloid nucleus, posterior part (BLP) ++

Basomedial amygdaloid nucleus, anterior part (BMA) ++

Basomedial amygdaloid nucleus, posterior part (BMP) ++

Lateral amygdaloid nucleus, dorsolateral part (LaDL) ++

Lateral amygdaloid nucleus, ventrolateral part (LaVL) ++

Lateral amygdaloid nucleus, ventromedial part (LaDM) ++

Medial Amygdaloid Nucleus Posterodorsal Part (MePD) ++

Medial Amygdaloid Nucleus Posteroventral Part (MePV) ++

Hippocampal formation

Granular Dentate Gyrus (GrDG) +++

Pyramidal cell hippocampus (Py) +++

Other

Amygdalo-hippocampal area, posteromedial part (AHiPM) ++

Caudate putamen (CPu) +

Cingulated cortex, area 2 (Cg2) +++

Dorsal peduacular cortex (DP)++

Dorsal subiculum (DS) +++

Indusium griseum (IG) ++

Interfascicular nucleus (IF)+

Interpeduncular fossa (IPF) +

Interstitial nucleus of Cajal (InC) +

Lambdoid septal zone (Ld) ++

Lateral septal nucleus, dorsal part (LSD) ++

Medial septal nucleus (MS) ++

Nucleus of Darkschewitsch (Dk) +

Nucleus of posterior commissure (PCom) ++

Nucleus of the optic tract (OT) ++

Nucleus of the vertical limb of the diagonal band (VBD) ++

Piriform Cortex (Pir)+++

Paranigral Nucleus (PN) +++

Pontine Nuclei (Pn) ++

Retromammilary nucleus, lateral part (RML) ++

Retromammilary nucleus, medial part (RMM) ++

Retrosplenial granular cortex b (RSGb) +++

Retrosplenial granular cortex c (RSGc) +++

Supraoptic Decussation (sox) ++

Substantia nigra, compact part , dorsal tier (SNCD) ++

Ventral tuberomammillary nucleus (VTM) +++

Zonal layer of the superior colliculus (Zo) +++

Mtch2 expression

Diencephalon

Thalamus

Lateral habenular nucleus (LHb) +

Lateral posterior thalamic nucleus, mediorostral part (LPMR) ++

Laterodorsal thalamic nucleus, ventrolateral (LDVL) ++

Medial geniculate nucleus, dorsal part (MGD) ++

Medial geniculate nucleus, medial part (MGM) ++

Medial geniculate nucleus, ventral part (MGV) ++

Medial habenular nucleus (MHb) ++

Paraventricular thalamic nucleus (PV) ++

Hypothalamus

Ventromedial hypothalamic nucleus, dorsomedial part (VMHDM) ++

Telencephalon

Cerebral cortex

Layer 1-

Layer 2++

Layer 3++

Layer 4 +++

Layer 5++

Layer 6+++

Amygdala

Basomedial amygdaloid nucleus, anterior part (BMA) ++

Hippocampal formation

Granular dentate gyrus (GrDG) +++

Pyramidal cell hippocampus (Py) +++

Other

Caudate putamen (CpU) +

Cingulated cortex area 2 (Cg2) ++

Dentate gyrus (DG) +++

Dorsal peduncular cortex (DP) ++

Dorsal raphe nucleus (DR) ++

Dorsolateral periaqueductal gray (DLPAG) ++

Dorsomedial periaqueductal gray (DMPAG) ++

Habenular commissure (hbc) +++

Indusium griseum (IG) ++

Interpeduncular nucleus, caudal subnucleus (IPC) +

Interpeduncular nucleus, lateral subnucleus (IPL) +

Interpeduncular nucleus, rostral subnucleus (IPR) +

Interpeduncular nucleus, intermediate subnucleus (IPI) +

Island of Calleja, major island (ICjM) ++

Lateral septal nucleus, intermediate part (LSI) ++

Lateral septal nucleus, ventral part (LSV) ++

Medial septal nucleus (MS) +

Nucleus of Darkschewitsch (Dk) ++

Nucleus of the vertical limb of the diagonal band (VDB) +++

Oculomotor nucleus, parvicellular part (3PC) ++

Piriform cortex (Pir) +++

Pontine nuclei (Pn) ++

Red nucleus +

Subbrachial nucleus (SubB) +++

Substantia nigra, reticular part (SNR) +

Subthalamic nucleus (STh) ++

Superior cerebellar penducle (scp) +

Supraoculomotor periaqueductal gray (Su3C) ++

Ventromedial preoptic nucleus (VMPO) +++

Sh2b1 expression

Diencephalon

Thalamus

Dorsal Lateral Geniculate Nucleus (DLG) ++

Lateral habenular nucleus (LHb) +

Lateral posterior thalamic nucleus, mediorostral part (LPMR) ++

Lateral Posterior Thalamic Nucleus, Laterorostral Part (LPLR) ++

Medial habenular nucleus (MHb) ++

Hypothalamus

Arcuate Nucleus (Arc) ++

Telencephalon

Cerebral cortex

Layer 1-

Layer 2++

Layer 3++

Layer 4 ++

Layer 5+

Layer 6+

Amygdala

Anterior Cortical Amygdaloid Nucleus (ACo)+++

Basomedial amygdaloid nucleus, anterior part (BMA) ++

Basomedial amygdaloid nucleus, lateral part (BLA) ++

Lateral Amygdaloid Nucleus, Dorsolateral Part (LaDL) ++

Lateral Amygdaloid Nucleus, Ventrolateral Part (LaVL) ++

Medial Amygdaloid Nucleus Posterodorsal Part (MePD) ++

Medial Amygdaloid Nucleus Posteroventral Part (MePV) ++

Hippocampal formation

Granular dentate gyrus (GrDG) +++

Pyramidal cell hippocampus (Py) +++

Other

Cingulated cortex area 2 (Cg2) +++

Indusium griseum (IG) ++

Lateral septal nucleus, dorsal part (LSD) ++

Lateral septal nucleus, ventral part (LSV) ++

REFERENCES

Albayrak, O., et al. (2013). "Common obesity risk alleles in childhood attention-deficit/hyperactivity disorder." Am J Med Genet B Neuropsychiatr Genet **162B**(4): 295-305.

Alvarez-Crespo, M., et al. (2012). "The amygdala as a neurobiological target for ghrelin in rats: neuroanatomical, electrophysiological and behavioral evidence." PLoS One **7**(10): e46321.

Arner, P. and K. L. Spalding (2010). "Fat cell turnover in humans." Biochem Biophys Res Commun **396**(1): 101-104.

Batterham, R. L. and S. R. Bloom (2003). "The gut hormone peptide YY regulates appetite." Ann N Y Acad Sci **994**: 162-168.

Batterham, R. L., et al. (2002). "Gut hormone PYY(3-36) physiologically inhibits food intake." Nature **418**(6898): 650-654.

Bauer, F., et al. (2009). "Obesity genes identified in genome-wide association studies are associated with adiposity measures and potentially with nutrient-specific food preference." Am J Clin Nutr **90**(4): 951-959.

Begg, D. P. and S. C. Woods (2012). "The central insulin system and energy balance." Handb Exp Pharmacol(209): 111-129.

Berthoud, H. R., et al. (2012). "Food reward in the obese and after weight loss induced by calorie restriction and bariatric surgery." Ann N Y Acad Sci **1264**(1): 36-48.

Bochukova, E. G., et al. (2010). "Large, rare chromosomal deletions associated with severe early-onset obesity." Nature **463**(7281): 666-670.

Boender, A. J., et al. (2012). "Nutritional state affects the expression of the obesity-associated genes Etv5, Faim2, Fto, and Negr1." Obesity (Silver Spring) **20**(12): 2420-2425.

Brinster, R. L., et al. (1988). "Introns increase transcriptional efficiency in transgenic mice." Proc Natl Acad Sci U S A **85**(3): 836-840.

Carmody, J. S., et al. (2011). "Respective contributions of maternal insulin resistance and diet to metabolic and hypothalamic phenotypes of progeny." Obesity (Silver Spring) **19**(3): 492-499.

Choy, J. S., et al. (2010). "DNA methylation increases nucleosome compaction and rigidity." J Am Chem Soc **132**(6): 1782-1783.

Chu, C., et al. (2004). "Ectopic expression of murine diphosphoinositol polyphosphate phosphohydrolase 1 attenuates signaling through the ERK1/2 pathway." Cell Signal **16**(9): 1045-1059.

Clement, K., et al. (1998). "A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction." Nature **392**(6674): 398-401.

Cummings, D. E. and J. Overduin (2007). "Gastrointestinal regulation of food intake." J Clin Invest **117**(1): 13-23.

Cummings, D. E., et al. (2001). "A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans." Diabetes **50**(8): 1714-1719.

Cummings, S. L. (1997). "Neuropeptide Y, somatostatin, and cholecystokinin of the anterior piriform cortex." Cell Tissue Res **289**(1): 39-51.

Davis, C. A., et al. (2009). "Dopamine for "wanting" and opioids for "liking": a comparison of obese adults with and without binge eating." Obesity (Silver Spring) **17**(6): 1220-1225.

Davis, J. D. and G. P. Smith (1990). "Learning to sham feed: behavioral adjustments to loss of physiological postingestional stimuli." Am J Physiol **259**(6 Pt 2): R1228-1235.

De Graaf, C., et al. (1999). "Palatability affects satiation but not satiety." Physiol Behav **66**(4): 681-688.

Delahanty, R. J., et al. (2011). "Association of obesity-related genetic variants with endometrial cancer risk: a report from the Shanghai Endometrial Cancer Genetics Study." Am J Epidemiol **174**(10): 1115-1126.

Della-Zuana, O., et al. (2002). "Acute and chronic administration of melanin-concentrating hormone enhances food intake and body weight in Wistar and Sprague-Dawley rats." Int J Obes Relat Metab Disord **26**(10): 1289-1295.

Delrue, M. A. and J. L. Michaud (2004). "Fat chance: genetic syndromes with obesity." Clin Genet **66**(2): 83-93.

Dolinoy, D. C., et al. (2007). "Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development." Proc Natl Acad Sci U S A **104**(32): 13056-13061.

Dossat, A. M., et al. (2013). "Nucleus accumbens GLP-1 receptors influence meal size and palatability." Am J Physiol Endocrinol Metab.

Fetissov, S. O., et al. (2002). "Expression of dopaminergic receptors in the hypothalamus of lean and obese Zucker rats and food intake." Am J Physiol Regul Integr Comp Physiol **283**(4): R905-910.

Figlewicz, D. P., et al. (2003). "Expression of receptors for insulin and leptin in the ventral tegmental area/substantia nigra (VTA/SN) of the rat." Brain Res **964**(1): 107-115.

Figlewicz, D. P. and A. J. Sipols (2010). "Energy regulatory signals and food reward." Pharmacol Biochem Behav **97**(1): 15-24.

Franks, P. W. and C. Ling (2010). "Epigenetics and obesity: the devil is in the details." BMC Med **8**: 88.

Frayling, T. M., et al. (2007). "A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity." Science **316**(5826): 889-894.

Fredriksson, R., et al. (2008). "The obesity gene, FTO, is of ancient origin, up-regulated during food deprivation and expressed in neurons of feeding-related nuclei of the brain." Endocrinology **149**(5): 2062-2071.

Friedenreich, C., et al. (2007). "Anthropometric factors and risk of endometrial cancer: the European prospective investigation into cancer and nutrition." Cancer Causes Control **18**(4): 399-413.

Furger, A., et al. (2002). "Promoter proximal splice sites enhance transcription." Genes Dev **16**(21): 2792-2799.

Garfield, A. S., et al. (2009). "Role of central melanocortin pathways in energy homeostasis." Trends Endocrinol Metab **20**(5): 203-215.

Geary, N. and G. P. Smith (1982). "Pancreatic glucagon and postprandial satiety in the rat." Physiol Behav **28**(2): 313-322.

Goran, M. I., et al. (2003). "Obesity and risk of type 2 diabetes and cardiovascular disease in children and adolescents." J Clin Endocrinol Metab **88**(4): 1417-1427.

Goto, K., et al. (1994). "Cloning and expression of a cytoskeleton-associated diacylglycerol kinase that is dominantly expressed in cerebellum." Proc Natl Acad Sci U S A **91**(26): 13042-13046.

Grazio, S. and D. Balen (2009). "[Obesity: risk factor and predictor of osteoarthritis]." Lijec Vjesn **131**(1-2): 22-26.

Guyenet, S. J. and M. W. Schwartz (2012). "Clinical review: Regulation of food intake, energy balance, and body fat mass: implications for the pathogenesis and treatment of obesity." J Clin Endocrinol Metab **97**(3): 745-755.

Hajnal, A., et al. (2004). "Oral sucrose stimulation increases accumbens dopamine in the rat." Am J Physiol Regul Integr Comp Physiol **286**(1): R31-37.

Herrera, B. M., et al. (2011). "Genetics and epigenetics of obesity." Maturitas **69**(1): 41-49.

Heymsfield, S. B., et al. (1999). "Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial." JAMA **282**(16): 1568-1575.

Hoebel, B. G. and P. Teitelbaum (1962). "Hypothalamic control of feeding and self-stimulation." Science **135**(3501): 375-377.

Iemolo, A., et al. (2013). "CRF-CRF Receptor System in the Central and Basolateral Nuclei of the Amygdala Differentially Mediates Excessive Eating of Palatable Food." Neuropsychopharmacology.

Jackson, R. S., et al. (1997). "Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene." Nat Genet **16**(3): 303-306.

Jarosz, P. A., et al. (2007). "Conditioned place preferences (CPPs) to high-caloric "snack foods" in rat strains genetically prone vs. resistant to diet-induced obesity: resistance to naltrexone blockade." Pharmacol Biochem Behav **86**(4): 699-704.

Johnson, P. M. and P. J. Kenny (2010). "Dopamine D2 receptors in addiction-like reward dysfunction and compulsive eating in obese rats." Nat Neurosci **13**(5): 635-641.

Kai, M., et al. (1994). "Molecular cloning of a diacylglycerol kinase isozyme predominantly expressed in human retina with a truncated and inactive enzyme expression in most other human cells." J Biol Chem **269**(28): 18492-18498.

Kanoski, S. E., et al. (2011). "Hippocampal leptin signaling reduces food intake and modulates food-related memory processing." Neuropsychopharmacology **36**(9): 1859-1870.

Katsuura, G., et al. (2002). "Roles of pancreatic polypeptide in regulation of food intake." Peptides **23**(2): 323-329.

Kenny, P. J. (2011). "Common cellular and molecular mechanisms in obesity and drug addiction." Nat Rev Neurosci **12**(11): 638-651.

Kenny, P. J. (2011). "Reward mechanisms in obesity: new insights and future directions." Neuron **69**(4): 664-679.

Kissileff, H. R., et al. (1981). "C-terminal octapeptide of cholecystokinin decreases food intake in man." Am J Clin Nutr **34**(2): 154-160.

Kitamoto, A., et al. (2013). "NUDT3 rs206936 is associated with body mass index in obese Japanese women." Endocr J.

Kobberup, S., et al. (2007). "ETS-family genes in pancreatic development." Dev Dyn **236**(11): 3100-3110.

Koza, R. A., et al. (2006). "Changes in gene expression foreshadow diet-induced obesity in genetically identical mice." PLoS Genet **2**(5): e81.

Krude, H., et al. (1998). "Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans." Nat Genet **19**(2): 155-157.

Kulyte, A., et al. (2011). "MTCH2 in human white adipose tissue and obesity." J Clin Endocrinol Metab **96**(10): E1661-1665.

Lahmann, P. H., et al. (2004). "Body size and breast cancer risk: findings from the European Prospective Investigation into Cancer And Nutrition (EPIC)." Int J Cancer **111**(5): 762-771.

Lambowitz, A. M. and M. Belfort (1993). "Introns as mobile genetic elements." Annu Rev Biochem **62**: 587-622.

Larsson, S. C. and A. Wolk (2007). "Obesity and colon and rectal cancer risk: a meta-analysis of prospective studies." Am J Clin Nutr **86**(3): 556-565.

Larsson, S. C. and A. Wolk (2007). "Obesity and the risk of gallbladder cancer: a meta-analysis." Br J Cancer **96**(9): 1457-1461.

Le Hir, H., et al. (2003). "How introns influence and enhance eukaryotic gene expression." Trends Biochem Sci **28**(4): 215-220.

Leibowitz-Amit, R., et al. (2006). "Mimp, a mitochondrial carrier homologue, inhibits Met-HGF/SF-induced scattering and tumorigenicity by altering Met-HGF/SF signaling pathways." Cancer Res **66**(17): 8687-8697.

Li, X., et al. (2013). "Obesity-related genetic variants, human pigmentation, and risk of melanoma." Hum Genet **132**(7): 793-801.

Li, Z., et al. (2007). "SH2B1 enhances leptin signaling by both Janus kinase 2 Tyr813 phosphorylation-dependent and -independent mechanisms." Mol Endocrinol **21**(9): 2270-2281.

Lutz, T. A., et al. (1994). "Reduction of food intake in rats by intraperitoneal injection of low doses of amylin." Physiol Behav **55**(5): 891-895.

Migliaccio, S., et al. (2011). "Is obesity in women protective against osteoporosis?" Diabetes Metab Syndr Obes **4**: 273-282.

Montague, C. T., et al. (1997). "Congenital leptin deficiency is associated with severe early-onset obesity in humans." Nature **387**(6636): 903-908.

Moran, T. H., et al. (1992). "Blockade of type A, not type B, CCK receptors attenuates satiety actions of exogenous and endogenous CCK." Am J Physiol **262**(1 Pt 2): R46-50.

Morris, D. L., et al. (2009). "SH2B1 enhances insulin sensitivity by both stimulating the insulin receptor and inhibiting tyrosine dephosphorylation of insulin receptor substrate proteins." Diabetes **58**(9): 2039-2047.

Morton, G. J., et al. (2006). "Central nervous system control of food intake and body weight." Nature **443**(7109): 289-295.

Munzberg, H., et al. (2005). "Leptin receptor action and mechanisms of leptin resistance." Cell Mol Life Sci **62**(6): 642-652.

Nayler, O., et al. (1998). "Human transformer-2-beta gene (SFRS10): complete nucleotide sequence, chromosomal localization, and generation of a tissue-specific isoform." Genomics **53**(2): 191-202.

Nelms, K., et al. (1999). "Alternative splicing, gene localization, and binding of SH2-B to the insulin receptor kinase domain." Mamm Genome **10**(12): 1160-1167.

Niu, J. G., et al. (2012). "Projections from the anterior basomedial and anterior cortical amygdaloid nuclei to melanin-concentrating hormone-containing neurons in the lateral hypothalamus of the rat." Brain Res **1479**: 31-43.

Obici, S., et al. (2002). "Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats." Nat Neurosci **5**(6): 566-572.

Ogden, C. L., et al. (2012). "Prevalence of obesity in the United States, 2009-2010." NCHS Data Brief(82): 1-8.

Olszewski, P. K. and A. S. Levine (2007). "Central opioids and consumption of sweet tastants: when reward outweighs homeostasis." Physiol Behav **91**(5): 506-512.

Oswald, K. D., et al. (2011). "Motivation for palatable food despite consequences in an animal model of binge eating." Int J Eat Disord **44**(3): 203-211.

Page, K. C., et al. (2009). "Maternal and postweaning diet interaction alters hypothalamic gene expression and modulates response to a high-fat diet in male offspring." Am J Physiol Regul Integr Comp Physiol **297**(4): R1049-1057.

Pecina, S. and K. C. Berridge (2005). "Hedonic hot spot in nucleus accumbens shell: where do mu-opioids cause increased hedonic impact of sweetness?" J Neurosci **25**(50): 11777-11786.

Pinto, S., et al. (2004). "Rapid rewiring of arcuate nucleus feeding circuits by leptin." Science **304**(5667): 110-115.

Powley, T. L. and R. E. Keesey (1970). "Relationship of body weight to the lateral hypothalamic feeding syndrome." J Comp Physiol Psychol **70**(1): 25-36.

Proudfoot, N. J., et al. (2002). "Integrating mRNA processing with transcription." Cell **108**(4): 501-512.

Reenan, R. A. (2001). "The RNA world meets behavior: A-->I pre-mRNA editing in animals." Trends Genet **17**(2): 53-56.

Ren, D., et al. (2007). "Neuronal SH2B1 is essential for controlling energy and glucose homeostasis." J Clin Invest **117**(2): 397-406.

Renstrom, F., et al. (2009). "Replication and extension of genome-wide association study results for obesity in 4923 adults from northern Sweden." Hum Mol Genet **18**(8): 1489-1496.

Rhee, K. E., et al. (2012). "Early determinants of obesity: genetic, epigenetic, and in utero influences." Int J Pediatr **2012**: 463850.

Ritter, R. C. (2004). "Gastrointestinal mechanisms of satiation for food." Physiol Behav **81**(2): 249-273.

Rolls, E. T. (2008). "Functions of the orbitofrontal and pregenual cingulate cortex in taste, olfaction, appetite and emotion." Acta Physiol Hung **95**(2): 131-164.

Ronn, T., et al. (2013). "A six months exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue." PLoS Genet **9**(6): e1003572.

Rui, L., et al. (1997). "Identification of SH2-Bbeta as a substrate of the tyrosine kinase JAK2 involved in growth hormone signaling." Mol Cell Biol **17**(11): 6633-6644.

Safrany, S. T., et al. (1998). "A novel context for the 'MutT' module, a guardian of cell integrity, in a diphosphoinositol polyphosphate phosphohydrolase." EMBO J **17**(22): 6599-6607.

Saito, A., et al. (1981). "Alterations of brain cerebral cortex CCK receptors in the ob/ob mouse." Endocrinology **109**(3): 984-986.

Schlesser, H. N., et al. (2008). "Effects of ETV5 (ets variant gene 5) on testis and body growth, time course of spermatogonial stem cell loss, and fertility in mice." Biol Reprod **78**(3): 483-489.

Schmid, P. M., et al. (2012). "Expression of fourteen novel obesity-related genes in Zucker diabetic fatty rats." Cardiovasc Diabetol **11**: 48.

Scopinho, A. A., et al. (2012). "Medial amygdaloid nucleus 5-HT(2)c receptors are involved in the hypophagic effect caused by zimelidine in rats." Neuropharmacology **63**(2): 301-309.

Sharma, S., et al. (2012). "Progressive-ratio responding for palatable high-fat and high-sugar food in mice." J Vis Exp(63): e3754.

Shin, A. C., et al. (2010). "Reversible suppression of food reward behavior by chronic mu-opioid receptor antagonism in the nucleus accumbens." Neuroscience **170**(2): 580-588.

Shor-Posner, G., et al. (1985). "Deficits in the control of food intake after hypothalamic paraventricular nucleus lesions." Physiol Behav **35**(6): 883-890.

Simon, L., et al. (2010). "ETV5 regulates sertoli cell chemokines involved in mouse stem/progenitor spermatogonia maintenance." Stem Cells **28**(10): 1882-1892.

Song, M. G., et al. (2013). "Multiple Nudix family proteins possess mRNA decapping activity." RNA **19**(3): 390-399.

Soubry, A., et al. (2013). "Paternal obesity is associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study (NEST) cohort." BMC Med **11**: 29.

Speliotes, E. K., et al. (2010). "Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index." Nat Genet **42**(11): 937-948.

Szczypka, M. S., et al. (2001). "Dopamine production in the caudate putamen restores feeding in dopamine-deficient mice." Neuron **30**(3): 819-828.

Tacke, R., et al. (1998). "Human Tra2 proteins are sequence-specific activators of pre-mRNA splicing." Cell **93**(1): 139-148.

Thorleifsson, G., et al. (2009). "Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity." Nat Genet **41**(1): 18-24.

van Groen, T., et al. (2002). "Role of the anterodorsal and anteroventral nuclei of the thalamus in spatial memory in the rat." Behav Brain Res **132**(1): 19-28.

Vastermark, A., et al. (2012). "Polymorphisms in sh2b1 and spns1 loci are associated with triglyceride levels in a healthy population in northern Sweden." J Genet **91**(2): 237-240.

Velders, F. P., et al. (2012). "FTO at rs9939609, food responsiveness, emotional control and symptoms of ADHD in preschool children." PLoS One **7**(11): e49131.

Volkow, N. D., et al. (2008). "Low dopamine striatal D2 receptors are associated with prefrontal metabolism in obese subjects: possible contributing factors." Neuroimage **42**(4): 1537-1543.

Wang, G. J., et al. (2002). "The role of dopamine in motivation for food in humans: implications for obesity." Expert Opin Ther Targets **6**(5): 601-609.

Wassum, K. M., et al. (2009). "Distinct opioid circuits determine the palatability and the desirability of rewarding events." Proc Natl Acad Sci U S A **106**(30): 12512-12517.

Willer, C. J., et al. (2009). "Six new loci associated with body mass index highlight a neuronal influence on body weight regulation." Nat Genet **41**(1): 25-34.

Wortley, K. E., et al. (2004). "Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference." Proc Natl Acad Sci U S A **101**(21): 8227-8232.

Yale Scientific (2012). The experience of eating. WWW document: http://www.yalescientific.org/2011/04/the-experience-of-eating. Date visited 5 August 2013.

Zaltsman, Y., et al. (2010). "MTCH2/MIMP is a major facilitator of tBID recruitment to mitochondria." Nat Cell Biol **12**(6): 553-562.

Zhang, X., et al. (2003). "The central nucleus of the amygdala modulates gut-related neurons in the dorsal vagal complex in rats." J Physiol **553**(Pt 3): 1005-1018.

Zhou, Q. Y. and R. D. Palmiter (1995). "Dopamine-deficient mice are severely hypoactive, adipsic, and aphagic." Cell **83**(7): 1197-1209.

Ziotopoulou, M., et al. (2000). "Differential expression of hypothalamic neuropeptides in the early phase of diet-induced obesity in mice." Am J Physiol Endocrinol Metab **279**(4): E838-845.