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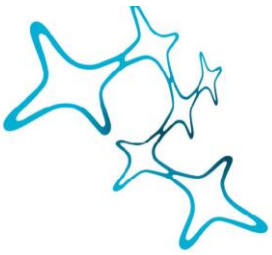

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Hedonic interruption of the physiological controls of eating: sites and mechanisms

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LIST OF ABBREVIATIONS

2-way ANOVA	Two factor analysis of variance
2-AG	2-arachidonoyl-*sn*-glycerol
3V	Third (III) ventricle
5-HT	5-hydroxy-tryptamine, serotonin
5-HT2A	5-hydroxy-tryptamine receptor 2A
α-MSH	α -melanocyte-stimulating hormone
Acetyl-CoA	Acetyl coenzyme A
ACTH	Adrenal corticotropin releasing hormone
AGEs	Advanced glycosylated end products
AgRP	Agouti-related peptide
AP	Area postarema
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BBB	Blood-brain barrier
BMI	Body mass index
BNST	Bed nucleus of the stria terminalis
BW	Body weight
CART	Cocaine- and amphetamine-regulated transcript
CB1	Cannabinoid receptor 1
CeA	Central amygdala
CCK	Cholecystokinin
CNVII	Cranial nerve VII
CNS	Central nervous system
CRH	Corticotropin releasing hormone
DA	Dopamine
DAB	Diaminobenzidine
DMH	Dorsomedial hypothalamus
DMNX	Dorsal motor nucleus of the vagus
DREADD	Designer receptor exclusively activated by designer drugs
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase-1 and -2
Exp 1	Experiment 1

FAs	Fatty acids
FI	Food intake
FITC	Fluorescein isothiocyanate
FEOs	Food-entrainable oscillators
fMRI	Functional magnetic resonance imaging
FTO	Fat mass and obesity-associated gene
GABA	Gamma-aminobutyric acid
Gal	Galanin
Gal1R	Galanin receptor 1
GCTA	Genome-wide Complex Trait Analysis
GHS-R	Growth hormone secretagogue receptor
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
GLUT4	glucose transporter 4
GPCR	G-protein coupled receptor
GWAS	Genome-wide association studies
HDL	High density lipoprotein
HF	High-fat
HFD	High-fat diet
HPA axis	Hypothalamic pituitary adrenal axis
ICV	Intra-cerebroventricular
IHC	Immunohistochemistry
i.p.	Intraperitoneal
ISHH	*In* situ hybridization histochemistry
IRS	Insulin receptor substrate proteins
JAK2	Janus kinase-2
KO	Knock-out
LC	Locus coeruleus
LDL	Low density lipoprotein
LF	Low-fat
LepRb	Long isoform of leptin receptor
LH	Lateral hypothalamus
M	Mean (average value)
MC4R	Melanocortin receptor 4
MCH	Melanin-concentrating hormone

MOR	μ-opioid receptors
mPFC	Medial prefrontal cortex
mRNA	Messenger RNA
MUFAs	Monounsaturated fatty acids
MZ	Monozygotic
NAc	Nucleus accumbens
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract (Nucleus tractus solitarius)
OECD	Organization for Economic Cooperation & Development
OEA	Oleoylethanolamide
OFC	Orbitofrontal cortex
OX	Orexin
OXM	Oxyntomodulin
OXY	Oxytocin
PBN	Parabranchial nucleus
PF	Palatable food
PI3k	Phosphatidylinositol-3 kinase
POMC	Proopiomelanocortin
PPARα	Peroxisome proliferation activating receptor alpha
PUFAs	Polyunsaturated fatty acids
PVN	Paraventricular nucleus
PYY	Peptide tyrosine-tyrosine
WHO	World Health Organisation
RNA	Ribonucleic acid
SC	Standard chow
SCN	Suprachiasmatic nucleus
SD	Standard deviation of mean
SEM	Standard error of the mean
SNPs	Single nucleotide polymorphisms
SOCS-3	Suppressor of cytokine signalling 3
STAT3	Signal transducer and activator of transcription 3
T2DM	Type 2 diabetes mellitus
TRH	Thyrotropin releasing hormone
TNFα	Tumor necrosis factor alpha
TZDs	Thiazolidinediones

VMH	Ventromedial hypothalamus
VLDL	Very low density lipoprotein
VP	Ventral pallidum
VTA	Ventral tegmental area
WAT	White adipose tissue
ZT	Zeitgeber time

‘

ABSTRACT

Food is consumed to obtain energy but also for its pleasant sensory properties. Food consumption that is driven by motivation to gain hedonic simulation poses a challenge to the homeostatic mechanisms that regulate energy balance because of increased overeating in excess of metabolic requirements. The neural circuits that serve as an interface for the processing and integration of information regarding physiological state and hedonic drive, and the generation of feeding behaviour, are poorly understood. This work focused on the neural responses to physiological signals of hunger and satiety during initial exposures of adult male mice to novel palatable foods; understanding such early events, namely the neuroadaptations underlying reward, memory and central homeostatic circuits was considered likely to help cast light on the mechanisms that initiate overeating and obesity. Our first approach was to develop a simple food choice paradigm to test how palatability might compete out the effects of the major signal of satiety, leptin. Expectedly, the palatable food was ingested almost exclusively and in excess of normal caloric need; importantly, however, exogenous leptin administration failed to curtail overeating of the palatable food, indicating hedonic overriding of a potent satiety-signalling molecule. Hedonic feeding when mice were satiated was associated with activation of various brain areas concerned with hedonic processing and reward as well as hypothalamic nuclei involved in the regulation of eating. Among the latter, prominent neuronal activation was observed in the lateral hypothalamus (LH), an area responsible for the integration of information about metabolic status and various extrinsic factors that influence feeding and motivated behaviour. Notably, in the presence of palatable food, leptin failed to suppress the activity of appetite-inducing orexin neurons in the LH, although leptin signalling per se was seen to be intact. Subsequent exploration of possible explanations for the lack of leptin-induced inhibition of orexin neurons revealed potential mediation of the actions of leptin on orexin neurons by the galanin receptor 2, a neuronal loop that seems to be interrupted by hedonic experiences. These findings support previous suggestions that the LH serves as the “battle ground” upon which hedonic inputs prevail over the homeostatic regulators of eating behaviour.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Feeding – an essential behaviour

1.1.1. Meeting the energy demands of brain and body

The primary purpose of eating is to obtain essential macronutrients: carbohydrates, fats and proteins. Carbohydrates are essential for normal physiological function and are the preferred energy source for muscles, brain and red blood cells (Berg et al., 2002). Of all organs, the brain is the biggest consumer of energy. Although it represents just 2% of the body mass, it accounts for 20% of the organism's oxygen consumption (Attwell and Laughlin, 2001) and approximately 60% of its glucose usage, some 60-70% of which is used to power transport mechanisms that maintain the Na^+-K^+ membrane potential and facilitate neurotransmission (Berg et al., 2002; Peters et al., 2004). While amino and fatty acids can also be used to generate energy (see below), the brain depends on glucose-derived energy almost exclusively (in exceptional cases, e.g. extreme starvation and during neuronal maturation, ketone bodies generated by the liver may partially replace glucose) (Morris, 2005). Recent studies have also shown that long-chain fatty acids (FA) can rapidly (and in substantial amounts) traverse the blood-brain-barrier (Schönfeld and Reiser, 2013) and it is estimated that fatty acid oxidation may contribute up to 20% of the brain's energy needs (Ebert et al., 2003).

Consumption of macronutrients triggers a set of catabolic processes, resulting in the synthesis of high energy phosphate bonds in adenosine triphosphate (ATP). The latter is, in turn, hydrolysed into adenosine diphosphate (ADP) or adenosine monophosphate (AMP) which are essential for the maintenance and growth of cells. Amino acids, monosaccharides and fatty acids can all be used for energy generation; following their entry into the citric acid cycle, conversion into acetyl coenzyme A (Acetyl-CoA) and subsequent ATP production. Amino and fatty acids additionally serve to repair and regenerate tissue.

The initial steps of food metabolism occur in the gastrointestinal tract, first in the oral cavity and then the stomach. Small intestinal enterocytes are responsible for nutrient absorption; these cells are equipped with amino acid, glucose and fatty acid transporters. Further passage of amino acids and glucose from the intestinal cells into the portal blood is mediated by other specific transporters. Long-chain fatty acids (FA) and cholesterol, packaged into chylomicrons in the enterocytes, first enter lymphatic capillaries and are carried through the subclavian vein into the general circulation from where they are taken up into tissues and cells via cognate transporters (Stahl et al., 1999; Kohlmeier, 2003). In the context of the experimental part of this dissertation, it is

important to note that both, the stomach and intestine have sensors that monitor the amount of ingested nutrients that serve to regulate food intake at an optimal level where intake matches physiological demands. This regulation is mediated by chemical signals and neural afferents that send information regarding fullness (satiety) to the brain (see 1.2.1).

As glucose is a major source of energy, it is perhaps not surprising that glucose-rich foods are highly liked; humans have easier access to such foods and can fulfil this "liking" more easily than other species, thus also satisfying their brains' greater demands for energy to meet their more demanding cognitive tasks (Aiello and Wheeler, 1995). In nature, short carbohydrates are usually sweet (e.g. fruits, honey, milk) and are frequently enriched in food that also contain minerals and vitamins that are vital for a variety of biochemical processes. It is likely that appropriate sensory systems have evolved to find such energy-dense and nutrient-rich foods.

Intense liking of sweet foods during early postnatal life serves to fuel maximal periods of growth (Ventura and Mennella, 2011). This preference is thought to be innate, as human infants, from disparate cultures, prefer sugar solutions over water from very early life (Maller and Turner, 1973). On the other hand, adult humans differ widely in their liking of sweet items, with genetic, sex, environment and age playing important modifying roles (Keskitalo et al., 2007; Ventura and Mennella, 2011).

Dietary fats also play an essential nutritional role by providing essential fatty acids, storage of fat-soluble vitamins and oxidizable energy depots. In fact, diets with relatively low fat contents can meet these crucial nutritional requirements (Mela, 1995), but nevertheless most humans and animals show a strong preference for fatty foods, especially because of their easily-sensed texture and aromatic properties. This often leads to intake rates that exceed physiological needs (Drewnowski and Greenwood, 1983; Takeda et al., 2001) and overweight or obesity. Compounding this problem is the fact that a mixture of sucrose and fat greatly enhances the hedonic properties of food. Interestingly, fasting does not influence preference for sweet or fatty foods (Drewnowski and Greenwood, 1983), indicating that these preferences are not strictly under the usual physiological controls of energy intake.

It is important and interesting to observe that, while blood glucose levels are tightly regulated and, in humans are maintained with the narrow range of 4.4-6.1 mmol/L (82-110 mg/dL) through the actions of insulin, those of fat are not known to be subject to any similar regulatory mechanisms. The critical role of glucose in brain functioning is illustrated by the fact that mental functions may be severely compromised if blood levels fall below 3 mM (39.6 mg/dL; close to the K_M value of GLUT3, the main glucose transporter in brain cells) (Berg et al., 2002). On the other hand, it should also be noted that elevated glucose levels over an extended period are toxic for the brain and other tissues, as is seen in patients suffering from diabetes (Fowler, 2008); the rapid rise in

blood glucose after eating (up to 7.8 mmol/L (140 mg/dL) (International Diabetes Federation, 2007) is usually only a transient event; under normo-physiological conditions, excess glucose is rapidly taken up by peripheral tissues (predominantly skeletal muscle) or converted into glycogen in the liver from which it can be back-converted into glucose if required. Additionally, all commonly-ingested sugars (glucose, fructose, galactose) as well as glycogen have the potential to be converted into fat (adipose tissue); from an evolutionary perspective, sugar and fat consumption appear to be an efficient way to build energy reserves for periods of food scarcity.

1.1.2. Overeating and obesity

Energy intake in excess of actual needs results in overweight and obesity (Australian National Health and Medical Research Council (NHMRC) and New Zealand Ministry of Health (MoH); Berg et al., 2002; Klurfeld et al., 2013). As discussed in the previous section, easy access to highly likeable foods can lead to their over-consumption, posing a challenge to homeostatic and glucoregulatory mechanisms (Cordain et al., 2005) that have not adapted to respond sufficiently fast to the changing food environment.

Worldwide rates of obesity have doubled in the last 30 years; presently, some 40% of people are overweight (OECD, 2014; World Health Organization, 2015) (Figure 1.1), mainly because of the availability of cheap, high-energy and tasteful foods, food cultures as well as socio-economic status, gender, work habits, physical exertion (OECD, 2014) and exposure to environmental toxins (Grun and Blumberg, 2006; Manikkam et al., 2013). Obesity imposes high costs on individual health (see section 1.1.3) and on healthcare systems (1-3% of healthcare budgets in most countries; 5-10% in the USA); these costs can be expected to rise dramatically in coming years because of the growing size of the obese population and high incidence of comorbidities suffered by obese subjects (OECD, 2014). In addition, obesity is associated with indirect costs (decreased productivity, premature death and changes in public transportation, hospital and other public spaces infrastructures (World Obesity Federation, 2012).

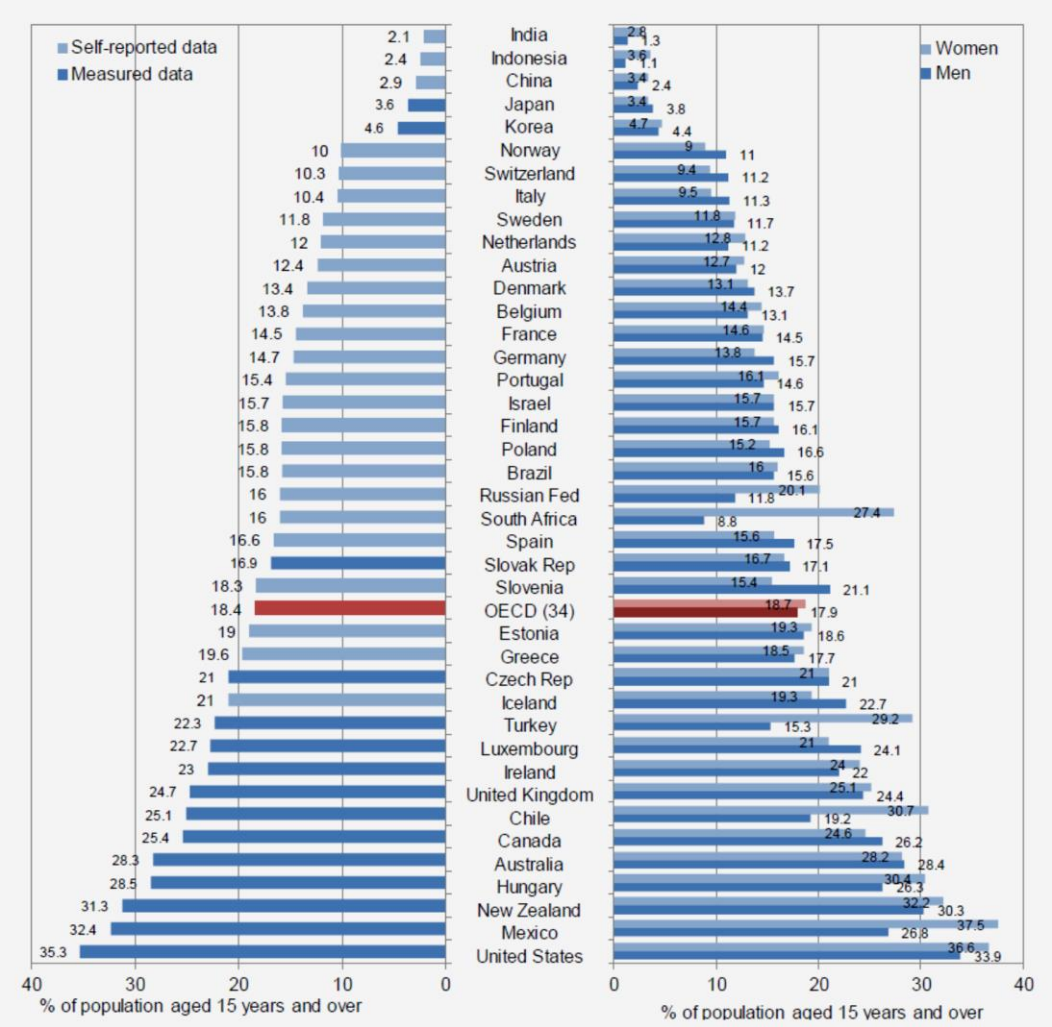


Figure 1.1. Obestiy among adults, 2012 or nearest year. *Source*: OECD (2014), *OECD Health Statistics 2014*, forthcoming, www.oecd.org/health/healthdata.

In ecology, the energy requirement of a species is a function of the number of organisms and their average body mass; accordingly global biomass of a species can be calculated. Simply based on data for one highly industrialized population which enjoys high average incomes and an abundant food supply, it emerges that the 6% of the global population that inhabits North America accounts for 34% of the human biomass, vs. 13% of the global biomass accounted for by Asia where 61% of the world's population live; if the rest of the world were to reach the same level of body mass as that found in North America, food would have to be found for an extra 0.5 billion people earth (Walpole et al., 2012), a major feat for agriculturalists and a challenge to geo-economic-political stability. Fortunately, recent data from ten Organization for Economic Cooperation & Development (OECD) countries shows that although the "obesity epidemic" has not stopped spreading, obesity rates have been slowing in the last 5 years, as compared to 20 years ago (OECD, 2014).

A multiplicity of biological and environmental factors contributes to the development of obesity. Some of the principal known factors are presented in Box 1.1.

Box 1.1. Dietary and biological, socio-economic and lifestyle causes of obesity

Increased sucrose consumption is one of the factors driving the current rise in excessive energy intake; for example, the annual per capita sucrose intake in the USA was 55.5 kg in 1970, as compared to 69.1 kg in 2000. The "sucrose temptation" started about 200 years ago when sugar plantations were introduced to the West Indies (Cordain et al., 2005). Table sugar (sucrose) comprises glucose and fructose. As already mentioned (Section 1.1.), excess glucose is converted into fat. In contrast, fructose, which is only metabolizable in the liver and cannot be directly utilized by tissues, is phosphorylated to fructose-1-phosphate, a substrate for lipid synthesis and gluconeogenesis – this results in elevated blood levels of triacylglycerol (triglycerides) and glucose (Berg et al., 2002; Bray et al., 2004). Unlike glucose, fructose does not trigger insulin signals which, besides stimulating tissue uptake of glucose, also signal satiety in the brain. Further, unlike glucose, fructose does not enter the brain and therefore cannot directly modulate appetite (Bray et al., 2004). High-fructose corn syrup (HFCS) is cheaper to produce and sweeter than sucrose, raising its use in food manufacture > 1000% between 1970 and 1990 (Bocarsly et al., 2010). HFCS actually has a slightly imbalanced mixture of 55% fructose and 42% glucose (Cordain et al., 2005); thus consumption of HFCS combined with high consumption of table sugar results in greatly increased amounts of fructose that might overwhelm endogenous food-intake regulatory mechanisms.

Increased consumption of animal fats (since the early 19th century) is also contributing to the obesity. Changes in dietary habit may be ascribed to changes in agricultural practice (e.g. mass production of animal fodder, transport etc.). As opposed to natural pastures, industrial fodders result in meats rich in saturated fatty acids (SFA) and n-6 fatty acids but low in n−3 fatty acids (Cordain et al., 2005). Notably, previous generations could only obtain meat high in SFA levels (in adipose tissue) from free-range animals during limited times of the year; thus their intake was also limited (Cordain et al., 2002; Cordain et al., 2005). Risks to human health (see 1.1.3) also come from the widespread use of refined vegetable oils and hydrogenated fats in modern human food production.

Global trade liberalization, economic growth and urbanization are other important driving forces in the world-wide spread of obesity. They are heterogeneous factors and include reductions in physical activity because of introduction of mechanization and other technologies (Malik et al., 2013) as well less exercise and growing sedentary lifestyles ("potato couch" habits) (Astrup, 2001), alcohol consumption and smoking (Hu, 2003). Further, commercial advertisements and food cues in the modern human environment are abundant, often misleading or irresponsible. However, moves by certain countries to introduce cartoons to encourage healthy eating among children (Gorn and Goldberg, 1982; Halford et al., 2008) as well as higher taxes on high-calorie foods (OECD, 2014) are proving effective in the war against overeating. Nonetheless, further significant gains will require voluntary or legislative restrictions on the food industry which has a strong political lobby and is an important driver of economies (Mialon et al., 2015).

Genetic susceptibility plays a strong part in obesity, with twin studies showing that heritable factors contribute significantly (47-90%) to body mass index (BMI, one suggested index of obesity). A few single gene mutations responsible for obesity have been identified. Most reported cases of monogenic obesity involve mutations of the melanocortin receptor 4 (*MC4R*) and proopiomelanocortin (*POMC*) genes (Perusse et al., 2005), the gene products of which lie downstream of the satiety signal leptin. In addition, genome-wide association studies (GWAS) found 32 loci that are associated with adult BMI, but these explain only 2% of the variance of BMI (Elks et al., 2012). Several single nucleotide polymorphisms (SNPs) show relatively strong association with high body mass, an interesting one being a polymorphism in the 5-hydroxy-tryptamine receptor 2A (*5-HT2A*) gene (serotonin decreases eating) associated with lower energy intake (Aubert et al., 2000); on the other hand, a polymorphism in the promoter region of the 5-HT transporter gene *SLC6A4*, whose product lowers serotonin availability in the synaptic cleft, links with adolescent and adult obesity (Sookoian et al., 2008). One of the most replicated findings is that a SNP in the *FTO (fat mass and obesity-associated)* gene (rs9939609 variant) commonly occurs in individuals with increased fat mass, predicts more daily eating episodes and preference for energy-dense food (McCaffery et al., 2012). Physical activity reduces risk of carriers of the rs9939609 variant to develop obesity by 27% (Kilpeläinen et al., 2011), indicating that environmental interventions can mitigate or potentiate genetic risk, as shown in the longitudinal Finnish Twin Cohort study (Naukkarinen et al., 2012). It is important to note that obesity is a complex trait and is unlikely to be explained by a single SNP. Thus, …/continued

Llewellyn *et al* (2013) recently introduced genome-wide complex trait analysis (GCTA) to estimate the total additive genetic effects of common SNPs. Using this analysis they could explain 30% of the variance in BMI during childhood when genetics exerts a strong influence. Early life environment influences mediate propensity for obesity in adulthood by epigenetic mechanisms. Parental over- or undernutrition not only raises the probability of obesity in the offspring, but also obesity-related conditions such as type 2 diabetes and cardiovascular disease, as demonstrated by the classical "Dutch Hunger Winter" study: children of women who were pregnant during World War II displayed reduced methylation of the insulin-like growth factor 2 (*IGF2*) gene (van Dijk et al., 2015) which is implicated in glucose homeostasis and obesity (Sandhu et al., 2002). Interestingly, intergenerational transmission of epigenetic modifications of *IGF2* may also be an important consideration in parental determination of childhood obesity (Soubry et al., 2013).

1.1.3. Pathological consequences of obesity

Persons with a body mass index > 30 (BMI, expressed as kg/m^2) are considered to be obese and at major risk for a number of non-communicable diseases, in particular, cardiovascular disease (a leading cause of death, World Health Organisation, 2015), diabetes, musculoskeletal disorders (e.g. osteoarthritis) and endometrial, breast and colon cancer (WHO, 2015). At the other extreme, a BMI < 18.5 is life-threatening (Prospective Studies Collaboration, 2009; Berrington de Gonzalez, Amy et al., 2010). It is important to note that there are controversies surrounding the relationship between BMI and morbidity; some reports indicate that overweight may actually reduce mortality (Flegal et al., 2013). However, many of the published studies have not properly accounted for other health risks such as decreased muscle mass, other chronic illness, smoking and aging (Hughes, 2013) or did not differentiate between subcutaneous (not harmful) and white adipose visceral fat depots. These issues are discussed in greater detail elsewhere (Wildman et al., 2008; Ahima and Lazar, 2013; Bigaard et al., 2005; Unger and Scherer, 2010).

Body fat reserves are maintained through dietary intake of fats, the excess of which results in larger depots. Excessive saturated FAs, n−6 poly unsaturated FAs (PUFAs) and *trans*-FAs are unhealthy because they raise the levels of total and low density lipoprotein (LDL) cholesterol (Cordain et al., 2005). Triglycerides, cholesterol and also excess carbohydrates from the food are processed into very low density lipoproteins (VLDL) in the liver (Hudgins et al., 2000) which eventually convert to LDL, the main lipoprotein fracture triggering the formation of atherosclerotic plaques. Elevated triglycerides, increased LDL and reductions in high density lipoprotein (HDL) levels which act in a counter manner to LDL, including removal and prevention of the formation of atherosclerotic plaques, accelerate atherosclerosis (Grundy, 1998; Poirier et al., 2006).

It is generally accepted that the white adipose tissue (WAT) that surrounds internal organs is harmful to health. It has also emerged that WAT cells size and turnover is an important factor: hypertrophic (small number of large) cells, rather than hyperplasic

(numerous, small cells), are associated with metabolic disturbances and other health conditions in both, lean and obese subjects (Arner and Arner, 2013). This fact has been exploited pharmacologically; for example, thiazolidinediones (TZD, e.g. rosiglitazone, pioglitazone) improve metabolic status by inducing WAT cell hyperplasia and inhibiting hypertrophy (Johnson et al., 2007) as well as by shifting WAT towards subcutaneous fat (Fonseca, 2003) and increasing tissue sensitivity to insulin.

Obesity (high blood fatty acid and triglyceride levels) is associated with insulin resistance in liver and muscle (presumably by increasing gluconeogenesis), despite compensatory hyperinsulinemia (Gastaldelli, 2008). Moreover, WAT and hypertrophic adipocytes produce large amounts of pro-inflammatory molecules (e.g. tumor necrosis factor alpha, TNFα). The state of chronic inflammation may, in turn, cause abnormal deposition of fat in the liver, heart, pancreas and skeletal muscle. Increased lipid levels induce damage to pancreatic insulin-producing cells in both *in vivo* and *in vitro* rodent models (Snel et al., 2012). Oxidative stress, subsequent lipid peroxidation, and disintegration of cell membranes are other harmful effects caused by obesity (Tangvarasittichai, 2015).

Type 2 diabetes mellitus (T2DM) is a frequent sequel to obesity. In this common chronic disease, tissue sensitivity to insulin is lost (most likely due to ectopic lipid accumulation in the liver and muscle) (Samuel and Shulman, 2016), resulting in hyperglycemia but also eventual loss of insulin-producing beta cells in the pancreas. Hyperglycemia disrupts cellular processes which, in turn, can lead to retinopathy, nephropathy and neuropathy in peripheral organs. The mechanisms of diabetes-associated organopathies are still not well understood, but the tissues affected are those that lack insulin-dependent glucose transporters, specifically GLUT4. The excess glucose taken up by such cells is aldose-reduced into sorbitol; the latter causes osmotic stress and water influx, a phenomenon ascribed to microvascular complications of diabetes. Further, glucose can trigger cell dysfunction through the formation of high levels of advanced glycosylated end products (AGEs) (Fowler, 2008). Lastly, glucose can activate the hexosamine signalling pathway, subsequently O-glycosylating serine and threonine residues that would normally be phosphorylated, and thereby disrupt and physiological processes (Marshall, 2006).

In addition, T2DM-associated disruption of the neuro-microvasculature and the ensuing hypoxia may contribute significantly to neuronal dysfunction. Further, insulin insensitivity in neurons (the so-called Type 3 diabetes) also regulates food intake and memory formation. *Post mortem ex vivo* stimulation of insulin receptors in the brains of people diagnosed with Alzheimer's disease showed reduced insulin signalling compared to controls (Umegaki, 2014b), a likely explanation for these patients' impaired cognition. Recently, blocking insulin-like growth factor I (IGF-I) in the brain of ageing mice with induced Alzheimer's pathology (*APP-PS* mice) was shown to improve age-related cognitive impairments (Gontier et al., 2015) by preventing the accumulation of amyloid β, a key pathological molecule in Alzheimer's disease. On the other hand,

improving insulin sensitivity with metformin was reported not to improve cognitive deficits in HFD-fed rats (McNeilly et al., 2012). The relationship between insulin resistance and cognitive function therefore remains an intriguing research subject.

As will be discussed later (section 1.2.3), since insulin is an important satiety signal, insulin insensitivity may contribute to excessive eating and overweight that progresses to obesity. Feeding, including food choices, is a learnt behaviour and therefore has major cognitive components (Finlayson et al., 2007). Many recent studies have linked obesity (and also T2DM) with cognitive impairments, including severe ones such as Alzheimer's disease (Umegaki, 2014). In addition, obesity has been associated with anxiety and depressed mood (Luppino et al., 2010) as well as reduced efficacy of pharmacotherapeutic interventions (Kloiber et al., 2007). While mood and cognition are reciprocally regulated, the exact mechanisms through which obesity contributes to these psychopathologies is still poorly understood and the subject of numerous ongoing investigations.

Obesity alters brain structure and function. For example, lean subjects reportedly outperform overweight/obese individuals in a battery of neurocognitive tests, including and attention and concentration tasks. Interestingly, poor food choices by obese subjects were associated with smaller orbitofrontal cortex (OFC) volume (Cohen et al., 2011). The same group of researchers found associations between obesity-mediated inflammation and structural damage in brain areas that regulate food intake, e.g. OFC and amygdala (Cazettes et al., 2011). These findings are matched by observations in rodents in which high-fat diet (HFD) has been shown to impair cognition through mechanisms involving inflammation; notably, antagonism of the interleukin-1 receptor (which mediates the actions of interleukin 1β) and/or reinstatement of standard diet reverse the cognitive deficits (Sobesky et al., 2014).

1.2. Endocrine regulation of food intake

1.2.1. Hunger signals (ghrelin, distention, glucocorticoids)

The periphery informs the central nervous system (CNS) about the energetic and nutritional needs of the organism. Hunger and satiety are conveyed to the brain via chemo-, stretch- and mechanoreceptors in the stomach and gut (Phillips and Powley, 2000); for example, chemoreceptors are less active and signal hunger when glucose and glycogen levels are low and the stomach is empty (Havel, 2001) (section 1.2.2). These signals are complemented by ghrelin, the only known circulating 'hunger hormone' (Cabral et al., 2015).

Ghrelin (MW 3370.9) is a 28-amino acid peptide synthesized mainly by cells in the gastric mucosa but also in the intestine, kidney and pancreas. The peptide triggers food intake, increases gastric motility, decreases fat utilisation and stimulates growth hormone release. Ghrelin exerts these actions by binding to growth hormone secretagogue receptor (GHS-R), a G- protein coupled receptor (GPCR); however, it is important to note that ghrelin *per se* does not activate GHS-R; the peptide has to be post-translationally octanoylated by addition of an acyl side-chain, albeit through yet unknown mechanisms. GHS-R is expressed in several peripheral tissues (Kojima and Kangawa, 2005), vagus nerve terminals and, in the CNS. In the latter, GHS-R mRNA is expressed in hypothalamic nuclei, the dorsal vagal complex, area postarema (AP), nucleus of the solitary tract (NTS), substantia nigra, ventral tegmental area (VTA) and hippocampus (Zigman et al., 2006). The downstream targets of activated ghrelin receptors are described in section 1.3.1.

Plasma ghrelin levels rise in response to fasting, and return to baseline after re-feeding. Carbohydrates as well as protein and fats, decrease ghrelin levels; glucose and insulin are also potent inhibitors of ghrelin secretion. The autonomic nervous system plays an important role in regulating ghrelin release and effects via the vagus nerve; vagotomy or vagal blockade with atropine attenuates ghrelin release and prevents ghrelin-stimulated food intake (Kojima and Kangawa, 2005; Yin et al., 2009). Ghrelin has also been ascribed a role in anticipating food (Verhagen et al., 2011), with palatable foods being stronger drivers of anticipatory increases in ghrelin secretion than standard foods (Merkestein et al., 2012). Suzanne Dickson's group has shown that intra-VTA ghrelin specifically increases consumption of rewarding but not standard food (Egecioglu et al., 2010). This observation likely reflects activation of the mesolimbic dopamine pathway (associated with reward and motivation) since ghrelin administration into the III ventricle (3V) results in dopamine overflow in the nucleus accumbens (NAc) (Jerlhag et al., 2006).

Surprisingly, ghrelin levels are low in obese subjects (Tschop et al., 2001; Greenman et al., 2004), suggesting that chronic overeating is not driven by overproduction of the peptide. Supporting this view are the observations that formation of oral or post-oral sugar and fat preferences in mice do not depend on ghrelin (Sclafani et al., 2015a). Moreover, germline knockout (KO) of the ghrelin or GHS-R genes in mice does not interfere with appetite, body weight or growth regulation (Kojima and Kangawa, 2005). However, when placed on HFD, ghrelin KO mice have a higher lean-to-fat ratio, consistent with greater utilization of dietary fats. Since HFD inhibits ghrelin levels, this switch in utilization of metabolic fuels has been interpreted as a counter-regulatory response to body weight gain when (fatty) foods are abundant (Wortley et al., 2004). A separate study involving ablation of ghrelin cells in adult mice demonstrated that prolonged fasting leads to severe hypoglycaemia in the absence of ghrelin (McFarlane et al., 2014); suggesting that ghrelin might not only be sensitive to glucose level fluctuations but might also control it. Although much is known about ghrelin, there are still gaps in our understanding of how overeating occurs. One possibility is that

overeating is primarily driven by "relief" from satiety signals/signalling (sections 1.2.3 and 1.2.3), or that cerebral processes (e.g. learning and related behaviours such as habit, hedonic stimuli, section 1.3.2) are important in the regulation of eating in the already-satiated state.

Besides the possible role of hedonic signalling in the regulation of hunger, environmental stressors, which are energy-demanding, may also trigger hunger. Stress activates the hypothalamic pituitary adrenal (HPA) axis through activation of corticotropin releasing hormone (CRH) expressing neurons in the hypothalamic paraventricular nucleus (PVN), followed by increased secretion of adrenocorticotropin (ACTH) and stimulation of ***glucocorticoid*** (GC) secretion (e.g. cortisol, corticosterone) from the adrenal cortex. The term "glucocorticoid" was originally used to embrace the fact that a primary function of GC is to stimulate the conversion of glycogen in liver and skeletal muscle into glucose as well as to stimulate the release of insulin so as to permit tissue uptake of newly-formed glucose (Smith and Vale, 2006; Patterson et al., 2013b). While GCs are known to stimulate feeding, higher GC levels suppress food intake (Maniam and Morris, 2012). In fact, ghrelin injections stimulate cortisol release in men (Kluge et al., 2011). It is also interesting to note that ghrelin is released both during acute and chronic stress (Lutter et al., 2008; Patterson et al., 2013b) and blockade of ghrelin signalling prevents stress-induced feeding (Patterson et al., 2013a). It is not clear at present whether a GC-ghrelin regulatory axis exists.

1.2.2. Energy and nutrient signals and sensing

Ingestion of food results in activation of tension- and mechanoreceptorsin the stomach and duodenum (Phillips and Powley, 2000). In addition, gastrointestinal chemoreceptors sense different nutrients (carbohydrates, FAs, amino acids, peptides) derived from digestion and transmit signals to the brainstem via vagal afferents, thus representing the first regulators of meal size. Nutrients absorbed via the hepatic portal vein may also stimulate the vagus nerve (Havel, 2001).

Whereas low glucose levels trigger food-seeking and ingestion, high blood glucose levels suppress appetite. For example, a landmark study by Smith and Epstein in 1969 demonstrated that feeding is stimulated when rats or monkeys are dosed with the synthetic glucose analogue 2-deoxy-D-glucose which limits glucose availability in cells (Smith and Epstein, 1969; Woods et al., 1984). Further, Oomura and colleagues observed that glucose increases neuronal firing rate in the ventromedial (VMH) and lateral hypothalamus (LH) (Oomura et al., 1969) and later work showed that ablation of VMH glucose-responsive neurons with gold-thio-glucose leads to obesity and disengages the glucoregulatory brake on feeding (Bergen et al., 1996). Subsequent studies have demonstrated that glucose-sensitive neurons in the dorsomedial hypothalamus (DMH),

PVN and arcuate nucleus (ARC) of the hypothalamus as well as several brainstem nuclei (dorsal vagal complex [AP, DMNX and NTS], raphe pallidus, locus coeruleus [LC], parabranchial nucleus [PBN] and the ventral part of the medulla [basolateral medulla]) also participate in the regulation of food intake (Steinbusch et al., 2015) (Fig.1.2).

It is now established that glucose sensors in various hypothalamic nuclei, the PBN and LC activate the sympathetic nervous system during states of low glycaemia; this results in the secretion of glucagon and inhibition of insulin secretion, increased thermogenesis in brown adipose tissue (BAT) and enhanced lipolysis in white adipose tissue. On the other hand, the NTS and hypothalamic nuclei which activate parasympathetic afferents that stimulate insulin secretion during hyperglycemia also activate glucagon secretion if glucose levels fall below 5 mM. Blood glucose levels sensed by the hypothalamus and brainstem are subsequently integrated in the paraventricular thalamus (PVT) from where signals are conveyed to the NAc, OFC and medial prefrontal cortex (mPFC); these latter structures are ultimately responsible for decisions to eat as well as their execution, thus fulfilling the organism's energetic needs (Figure 1.2) (Steinbusch et al., 2015). The described glucose-sensing circuit closely overlaps with the circuit that mediates the effects of other determinants of feeding, e.g. satiety peptides and taste signals (detailed review in section 1.3.1). Tanycytes, the glial-like cells lining the III ventricle which are in direct contact with the cerebrospinal fluid (CSF) and project to ARC and VMH, are glucosensitive and are perfectly positioned to sense and respond to hypoglycaemia (Bolborea and Dale, 2013).

Detection of foods that contain glucose is often based on sweet taste sensation but may also occur independently of taste signals. Anthony Sclafani's pioneering experiments that decoupled taste from gut sensations indicated that rats learned to prefer any flavour that is paired with a post-oral (intra-gastric) glucose infusion (Sclafani, 2004). Confirmation for these findings came from the work of de Araujo and colleagues who showed that mice with a deletion of the Trmp5 sweet taste receptor (*trpm5-/-* mice) retain their strong preference for sucrose, and display increased dopamine release in NAc, consistent with the earlier finding that palatable food consumption increases dopaminergic transmission in these mesolimbic areas (de Araujo et al., 2008). Glucose sensors in the gut may contribute to these post-oral effects since glucose infusions into the hepatic portal vein influence food intake and flavour conditioning more strongly than intra-jugular infusions of the nutrient (Tordoff and Friedman, 1986).

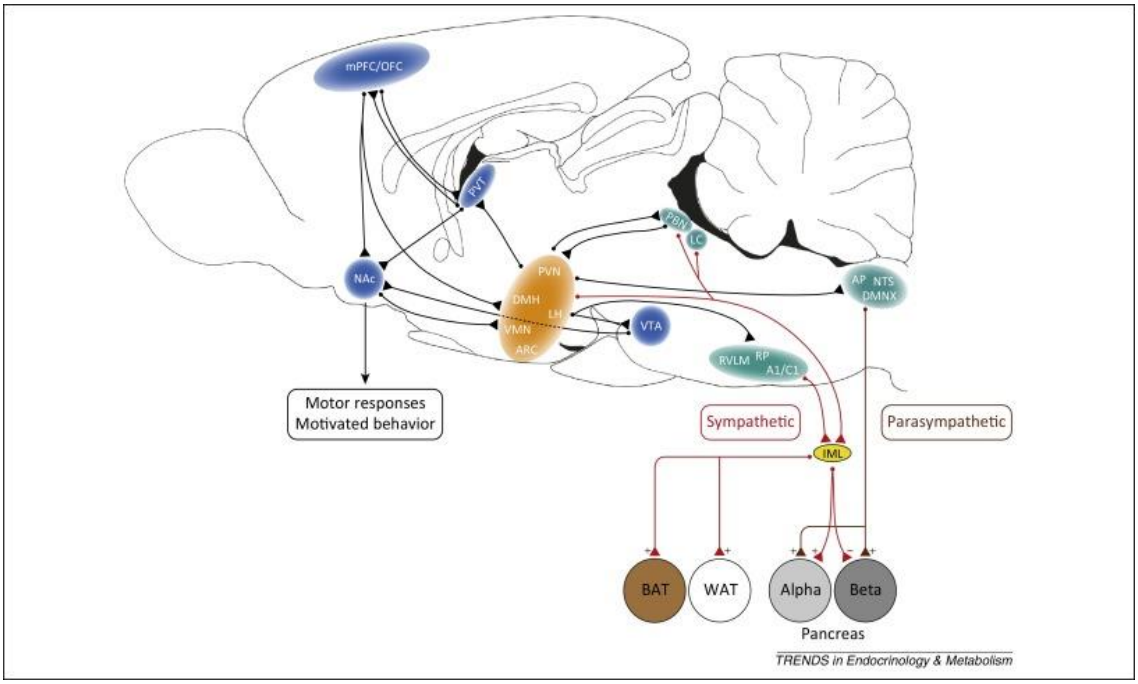


Figure 1.2. Glucose-sensing cells in homeostatic and hedonic control centers. Glucose-sensing cells, both glucose-excited (GE) and glucose-inhibited (GI), have been identified in brainstem structures (green): in the dorsal vagal complex that comprises the area postrema (AP), the nucleus of the tractus solitarius (NTS), and the dorsal motor nucleus of the vagus (DMNX); the basolateral medulla that includes the A1/C1 catecholaminergic neurons, the rostral ventrolateral medulla (RVLM), and the raphe pallidus (RP); the locus coeruleus (LC) and the parabrachial nucleus (PBN). In the hypothalamus (orange), glucose-sensing neurons are found in the arcuate nucleus (ARC) and in the ventromedial (VMN), lateral (LH), dorsomedial (DMH), and paraventricular (PVN) nuclei. The mesolimbic dopamine system, which comprises the ventral tegmental area (VTA), the major source of dopamine, the nucleus accumbens (NAc), the medial prefrontal cortex (mPFC), and orbitofrontal cortex (OFC), a system influenced by inputs from the paraventricular nucleus (PVT). This system integrates external stimuli through the mPFC and OFC, and internal metabolic cues including glucose levels, through interaction with hypothalamic and brainstem nuclei, as depicted by connecting lines. The NAc plays an important role in integrating this information to direct motor and behavior responses. Glucose-sensing cells in the hypothalamus and brainstem control the activity of peripheral metabolic organs through autonomic nervous system activity. Sympathetic efferents (red) that project to peripheral organs via the spinal intermediolateral cell column (IML, yellow) are under the control of hypothalamic nuclei, PBN, and LC, as well as of basolateral medulla glucose-sensing neurons. Parasympathetic efferents (brown) originate in the DMNX and are under the control of glucose-sensing neurons present in the NTS and several hypothalamic nuclei (Steinbusch et al., 2015).

That lipid sensing is important for the regulation of appetite has been shown in a variety of studies. For example, intravenous infusions of lipids (together with heparin that releases lipoprotein lipase which hydrolyses triglycerides to FAs and glycerol) was demonstrated to decrease appetite in baboons (Woods et al., 1984) and duodenal infusions of fat were shown to increase inter-meal intervals and decrease meal size in rodents and humans (Schwartz, 2011; Ryan et al., 2013). In contrast, inhibition of FA utilization elicits orexigenic effects (Havel, 2001), similar to those observed when glucose utilization is blocked. Further, oleic acid, a constituent of many animal and vegetable fats, provides increased signals of satiety after it is broken down into oleoylethanolamide (OEA); these actions involve vagal afferents and activation of peroxisome proliferation activating receptor alpha (PPARα) and, by binding to the GPCR

GPR119, stimulation of the release of the satiety factor glucagon-like peptide 1 (GLP-1) (DiPatrizio and Piomelli, 2015). Interestingly, both intestinal OEA synthesis and striatal dopamine release are reduced during HFD-induced obesity, whereas intraduodenal OEA administration reverses the "dopamine deficiency" in the NAc and decreases weight gain and food intake and of these obese mice (Tellez et al., 2013b). Here, it is also worth mentioning that intra-gastric fat infusions, like those of glucose, induce taste-independent dopamine release in NAc; the release is proportional to the caloric value of the infused fat emulsions (Ferreira et al., 2012; Han et al., 2016). Short-chain FAs (like acetate, propionate and butyrate) are also products of digestion in the small intestine, produced by bacterial fermentation of non-digestible carbohydrates (dietary fibre). They increase the production of the satiety peptides GLP-1, peptide tyrosine tyrosine (PYY) and leptin, and decrease body weight and appetite (Byrne et al., 2015).

Fatty acids can also be directly sensed by neuronal cells (Oomura et al., 1975; Jo et al., 2009; Le Foll et al., 2014; Magnan et al., 2015). A very interesting recent study from Serge Luquet's group (Cansell et al., 2014) showed that intra-carotid infusions of a fat emulsion decrease preference for palatable food and reward-seeking in wildtype mice; however, mice lacking lipoprotein lipase (enzyme needed to metabolize triglycerides obtained from a meal) in the NAc increased their reward intake when exposed to the same paradigm, indicating that the brain has the capacity to directly sense fats. Notably, lipoprotein lipase is detectable in other mesolimbic and hypothalamic areas, suggesting that fat sensing may also occur in these regions (Cansell et al., 2014).

Sensing of fat and sugar in the gut has generally been found to signal satiety but in some instances (like food restriction); they have also been found to increase conditioned responses (Zukerman et al., 2011). Thus, it has been proposed that gut nutrient signals may additionally serve to provide positive feedback signals on the appetite-regulatory mechanisms. Gut nutrient chemosensors are still to be identified; previous candidates, namely T1R3 (sweet receptor) and CD36 (fatty acid transporter) have since been excluded (Sclafani and Ackroff, 2012). More recent candidates include the fatty acid receptors GPR40 and GPR120; although the initial detection of fat is intact in animals lacking these receptors, they were reported to show attenuated conditioning to intragastric fat (Sclafani et al., 2015b). Other studies have reported that, in addition to the above-mentioned oleic acid, two other long-chain unsaturated FA derivates (arachidonoylethanolamide [anandamide] and 2-arachidonoyl-*sn*-glycerol [2-AG], which are both agonists of cannabinoid receptors [CB1, CB2] (DiPatrizio and Piomelli, 2015), may contribute to the regulation of eating since CB1 activation increases appetite and injections of anandamide into Nac "hedonic hotspots" (reviewed in 1.3.2) enhance liking reactions to palatable food (Berridge and Robinson, 2003). In the periphery, endocannabinoids induce lipogenesis, decrease peristalsis and increase food consumption. Interestingly, the taste of long-chain unsaturated FAs seems to be

sufficient to induce an orexigenic response through vagus nerve-mediated increased endocannabinoid content and signalling in the gut (DiPatrizio and Piomelli, 2015).

Dietary proteins also induce satiety; in fact, proteins are considered to be the most satiating of all macronutrients (> carbohydrates > fats) (Porrini et al., 1997; Soenen and Westerterp-Plantenga, 2008). However, a recent study reported that intraduodenal lipid or protein result in comparable reductions in food intake (Ryan et al., 2013). Proteins induce satiety by regulating the production of various gut signals (discussed in section 1.2.3), probably involving amino acid detection in the gut (Sclafani and Ackroff, 2012). In addition, protein regulates blood glucose levels by stimulating insulin and glucagon secretion (Ryan et al., 2013). It is also worth noting that protein-deficient diets increase appetite for protein-containing foods (Gibson et al., 1995); this is not surprising because, in contrast to carbohydrates and fat, excess protein is not stored. Proteins also have post-oral actions; this was demonstrated by the fact that intragastric infusions of the protein casein (like that reported for glucose and fat infusions) elicit flavour preferences towards the taste they are paired with (Sclafani and Ackroff, 2012).

1.2.3. Endocrine signals of satiety to the brain

The terms “satiety” and “satiation” are often used interchangeably since they are both involved in “satisfying hunger”. One key researcher in the field, John Blundell, has nevertheless proposed the following distinctions between the two terms. “Satiation” is defined as the end of *desire* to eat after a meal, a phenomenon that can occur at any time after the onset of eating. “Satiety,” on the other hand, is a physical feeling of fullness that terminates eating for a while; ideally, satiety dwindles as nutrients diminish (Blundell & MacDiarmid, 1997; www.foodprocessing.com/articles/2014/understanding-satiation-and-satiety/).

Signals of fullness during/following a meal are transmitted to the brain through mechano- and nutrient-sensors located in the stomach and gut. In addition to the nutrient signals described in the previous section, various enzymes and gut hormones involved in digestion and absorption also serve as satiation signals; evidence for this is based on the fact that their administration just before a meal reduces food intake (Begg and Woods, 2013). Anorexigenic gastrointestinal peptides generally have short half-lives and are responsible for the acute sensing of satiation; these hormones do not influence body weight in a significant manner. These gut peptides include: cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), peptide YY (PYY), oxyntomodulin (OXM, formerly known as enteroglucagon) pancreatic polypeptide (PP) and amylin (Havel, 2001; Hussain and Bloom, 2013). Three of the most prominent of gut peptides are described in Box 1.2.

Box 1.2. Gastrointestinal peptides

Cholecystokinin (CCK) (MW 1063.2) was the first satiety hormone to be identified by Gibbs & Smith who were searching for a mechanism to explain the termination of eating episodes. These researchers observed that CCK is anorexigenic and decreases meal size without inducing malaise (Gibbs et al., 1973). By now it has long been established that CCK inhibits gastric emptying and stimulates pancreatic and gall bladder enzyme release. Fat, protein and carbohydrates all relatively rapidly (within 15 min in humans) stimulate the release of CCK from I-cells of proximal small intestine (Liddle et al., 1985). CCK modulates food intake via CCK1 receptors on vagal afferents and also acts directly in the CNS via CCK1 receptors in the brainstem and hypothalamus. Administration of CCK during a meal decreases meal size, an effect that is compensated by increasing the frequency of meals (Figlewicz et al., 1996); thus CCK treatment is not an effective long-term weight loss strategy (Hussain and Bloom, 2013). Interestingly, CCK is also expressed in the CNS where it exerts effects on sleep, reproduction and several emotional behaviours (Crawley and Corwin, 1994).

Glucagon-like peptide 1 (GLP-1) (MW 4111.5) is cleaved from preproglucagon in L-cells in the gut, in the brain and pancreas. Bioactive GLP-1 in the circulation represents N-terminal truncation of GLP-$1_{1\text{-}36}$ amide into GLP-$1_{7\text{–}36}$ and GLP-$1_{7\text{–}37}$ amide (Mojsov et al., 1986). Other cleavage products of preproglucagon include GLP-2 and oxyntomodulin (in the brain and intestine) and glucagon (in pancreas) (Kieffer, 1999). GLP-1 is secreted in response to nutrients in the gut and its release is also stimulated by enteric neuronal signals (Drucker, 2006). GLP-1 inhibits gastric acid secretion and gastric emptying and, like CCK, suppresses appetite. The peptide is a potent stimulant of glucose-independent insulin release, its actions involving reduced glucagon secretion and increased pancreatic β-cell mass. Clinical studies have shown GLP-1 analogues to be effective in managing otherwise poorly-controlled T2DM (Hussain and Bloom, 2013).

Receptors for GLP-1 (GLP-1R) are relatively widespread in the gastrointestinal tract, pancreas, kidney, lung, heart (Pyke et al., 2014), vagal afferents and CNS, especially within the brain stem, hypothalamus and a number of forebrain areas (Merchenthaler et al., 1999). The release of GLP-1 from ileal L-cells in response to fat is abolished after subdiaphragmatic vagotomy, so CNS input is required for its release from the gut (Rocca and Brubaker, 1999). GLP-1 is also synthesized in the brainstem (Jin et al., 1988). Gastric distention activates brainstem neurons to produce GLP-1 and vagal afferent denervation blocks the effects of GLP-1 on gastric emptying. Since GLP-1 is degraded very rapidly in the circulation, it is likely that many of its effects depend on the ascending vagal afferents (Drucker, 2006) or GLP-1 of brain origin.

Peripheral injection of GLP-1 decreases food intake and increases immediate early gene *c-fos* expression in the rodent brainstem, PVN and central amygdala (CeA) (Tang-Christensen et al., 2001; Yamamoto et al., 2003). In humans, GLP-1 dose-dependently decreases feeding (Verdich et al., 2001), although physiological levels of GLP-1 do not influence food intake (Flint et al., 2001), again pointing to the potentially greater importance of vagal stimulation and/or central GLP-1 in the regulation of food intake than peripheral GLP-1. In fact, acute intra-cerebroventricular (ICV) and intrathecal PVN injections of GLP-1 reduce food consumption (Tang-Christensen et al., 2001). At the same time, recent evidence suggests that GLP-1 acts in NAc core to inhibit the amount of palatable solution intake, possibly reflecting nutrient responses elicited in the gut (Dossat et al., 2013). The widespread distribution of GLP1R in the brain suggests that GLP-1 may have functions other than satiety induction, with evidence pointing to its role in enhancing hippocampus-dependent cognitive behaviours such as associative and spatial learning (During et al., 2003).

Peptide tyrosine tyrosine (PYY) (MW 4049.5) belongs to the pancreatic polypeptide (PP)-fold family, along with neuropeptide Y (NPY) and PP. All these peptides share a PP-fold tertiary structure and bind to the Y family of receptors. $PYY_{3\text{–}36}$ is the active form of PYY and binds preferentially to the Y2 receptor (Fuhlendorff et al., 1990). PYY is mainly produced in the distal enteroendocrine L-cells, together with GLP-1 and oxyntomodulin (OXM) (Chaudhri et al., 2006). PYY release correlates with caloric intake and plasma levels rise within 30 min after the start of eating, plateau 1-2 h after a meal, and remain to be elevated for up to 6 h (Ba-tterham et al., 2003). Thus, as compared to other satiety-related gut peptides, PYY exerts its actions for an extended period of time. PYY responds strongly to both protein and fat in the intestinal tract (Lin and Chey, 2003; Batterham et al., 2006); FAs are thought to be sensed via the short-chain FA receptors GPR43 and GPR41 (Tazoe et al., 2009).

Peripheral administration of PYY reduces food intake and body weight gain in both, rodents and humans. At least in rodents, the actions of PYY are highly sensitive to stress; the effects of PYY can be …/continued

masked by even mild stressors (Sam et al., 2012). Although overweight subjects show impaired PYY release following eating, exogenous PYY is equipotent in obese and lean subjects (Batterham et al., 2003). Post-prandial PYY levels are strongly increased in subjects that have undergone bariatric surgery, the currently most effective weight-loss intervention (Stanley et al., 2005; Vincent and le Roux, 2008); such effects are also seen in mice with bariatric surgery and the acute effects of the surgery on body weight are not displayed when the *PYY* gene is deleted (PYY KO mice) (Chandarana et al., 2011). Besides directly inhibiting food intake, PYY appears to decrease appetite by increasing gastric distention through inhibition of gastric motility and delaying gastric emptying (Sam et al., 2012). Interestingly, central injections of PYY_{1-36} reportedly stimulate appetite. This has been hypothesized to hinge on the orexigenic effects mediated by Y1 and Y5 receptors (PVN and other brain areas); on the other hand, the anorexigenic effects are mediated by Y2 receptors on neuropeptide Y (NPY) neurons in the ARC (the ARC is easily accessible to circulating PYY because of its location in the mediobasal hypothalamus). Since Y2 receptors function as autoreceptors on orexigenic NPY neurons (see 1.3.1), activation of NPY neurons is inhibited by PYY (Batterham et al., 2002; Stanley et al., 2005). Other studies point to a similar role for Y2 receptors in the vagus nerve since the anorectic actions of PYY are blocked after sub-diaphragmatic vagotomy or lesions of the brainstem–hypothalamic pathway (Abbott et al., 2005; Sam et al., 2012).

In contrast to the above short-acting satiety signals, insulin and leptin are released in proportion to adipose tissue stores and exert regulatory effects on levels of adiposity and body weight; two other peptides, adiponectin and amylin, are also linked to fat mass (Begg and Woods, 2013). Below, the main characteristics and actions of insulin and leptin are annotated since these peptides rank among the best-studied adiposity-related hormones.

Insulin (MW 5808), a peptide produced by pancreatic β-cells, helps maintain normoglycemia by stimulating the uptake of glucose into liver, skeletal muscle and adipose tissue and perhaps more importantly, plays a critical role in suppressing hepatic glucose production (gluconeogenesis), as well as proteolysis and lipolysis in general (Sonksen and Sonksen, 2000; Biddinger and Kahn, 2006). The importance of insulin in preventing hyperglycemia cannot be overemphasized: patients with type 2 diabetes (T2DM) suffer from a host of conditions, including psychopathologies such as depression and dementia, neuropathies that can lead to blindness, impotence and wasting of soft tissues (gangrene); all of these conditions reflect tissue glucoprivation (Section 1.1.3).

From a historical perspective, it is interesting that Claude Bernard, at the end of 19th century, proposed that the brain is the main regulator of glucose homeostasis. Since the discovery of insulin in 1921 by Banting and Best, the evidence that diabetes is caused by deficiency in insulin signalling is unquestionable and has been under thourough investigation (http://www.nobelprize.org/educational/medicine/insulin/discovery-insulin.html, Schwartz et al., 2013). Meanwhile, Barnard's suggestion of the role of the brain in glucose homeostasis has generated attention only relatively recently. Key findings from these recent studies demonstrate that intra-hypothalamic insulin injections increase insulin sensitivity in liver and restore normal blood glucose levels (Obici et al., 2002b; Lam et al., 2005).

Insulin secretion occurs rapidly in response to elevated blood glucose levels, but T2DM patients are resistant to the actions of the hormone for reasons that are incompletely understood (Cusi et al., 2000; Morton et al., 2014). The failure of adequate delivery of glucose to tissues (Kahn et al., 1991) triggers a vicious cycle that leads to events that resemble those seen during food restriction, namely, compensatory rises in glucagon, catecholamine, glucocorticoid secretion, reduced thyroid hormone release, increased sympathetic outflow and reduced thermogenesis in brown adipose tissue (BAT), all of which elevate blood glucose levels (Morton et al., 2014). Despite the further elevation of blood glucose, however, the tissue uptake of glucose is still sub-optimal due to insulin resistance, and the vicious cycle continues.

The insulin receptor is a membrane-bound tyrosine kinase receptor. Insulin binding leads to auto-phosphorylation of tyrosine residues on the intracellular side of the receptor and of insulin receptor substrate proteins (IRS). Phospho-IRS activates a signalling cascade involving phosphatidylinositol-3 kinase (PI3K) and AKT (also known as protein kinase B) which regulates the transcription of downstream genes. In addition, insulin can activate the mitogen-activated protein kinase (MAPK) pathway. Further, the insulin signalling pathway converges with that of leptin (Figure 1.3) (Konner and Bruning, 2012). Thus, the insulin signalling cascade is highly complex, with an estimated 228 known combinations of IRS and AKT isoforms and PI3K heterodimers (Biddinger and Kahn, 2006). The inter-relationship between insulin sensitivity and adiposity (as obesity predisposes individuals to T2DM (Gastaldelli, 2008)) is largely based on the fact that excess glucose gets converted into fat (see Section 1.1.1). Interestingly, deletion of the insulin receptor gene in fat improves glucose and lipid homeostasis and decreases body weight without changing food intake (Biddinger and Kahn, 2006).

Insulin can penetrate the blood-brain-barrier where it interacts with insulin receptors in the brain to suppress appetite (Woods et al., 1979) and eventually, body weight - in addition to its above-mentioned glucoregulatory actions. That insulin acts in the brain as a satiety factor has been amply demonstrated in a variety of species, most clearly in mice in which insulin receptors were specifically knocked down in the brain (Bruning, 2000; Obici et al., 2002a). Other studies have shown that the anorexigenic effects of insulin are strongest in the mediobasal hypothalamus (greater in males than females) and that the anorexigenic effects of insulin are lost in HFD-fed obese animals (Woods and Begg, 2015). The actions of insulin resemble those of leptin (see below) in at least two ways: first, insulin signals satiety, and second, insulin signals adiposity – blood insulin levels correlate strongly with the size of fat depots (Begg and Woods, 2013; Woods and Begg, 2015).

Leptin (MW 16240) is a major adipokine released by adipocytes. In addition to adipocytes, leptin is synthesized in the gastrointestinal tract (Peters et al., 2005). The protein was cloned from mice homozygous for spontaneous mutations in the mouse

obese (*ob*) gene (Ingalls et al., 1950) in Jeffrey Friedman's laboratory (Zhang et al., 1994). The *ob* mouse, sourced as outbred mice from The Jackson Laboratory, is characterized by excessive eating, high body weight and insulin resistance (Friedman et al., 1991; Bahary et al., 1993). *Ob* codes the hormone leptin that it is a recessive gene on chromosome 6. Douglas Coleman and Katharine Hummel conducted a classical parabiosis experiment in which they surgically joined the vascular systems of an *ob/ob* mouse with that of either a wild-type (WT) or *db/db* mouse; the *db/db* mouse has a phenotype similar to that of the *ob/ob* mouse (*db* was later identified as the leptin receptor gene) including the diabetes-like features hence the name *diabetes* or *db*. The aim of these authors' work was to test the hypothesis that the metabolic phenotype of the *ob/ob* mice is determined by the lack of a circulating factor (i.e. a hormone). *Ob/ob* parabionts from *ob-db pairs* fed less and had lower body weights than the ones from *ob-WT* pairs; on the other hand, *db/db* mice did not show any improvement when paired with either WT or *ob/ob* mice, while the WT parabionts from *db-WT* pairs lost body weight and eventually died of starvation. Coleman proposed that an unknown circulating satiety factor was lacking in *ob/ob* mice and was working inefficiently in *db/db* mice (Coleman and Hummel, 1969; Coleman, 1973; Friedman, 2014). Friedman's group later identified this hypothesized factor to be the product of the *ob* gene and it was named leptin, after the Greek word "*leptos"* for "thin". The leptin receptor was cloned one year later, and as predicted, coded by *db* locus (Tartaglia et al., 1995). Various studies thereafter showed that recombinant leptin can reverse the usual phenotype of *ob/ob* mice (Halaas et al., 1995; Pelleymounter et al., 1995; Weigle et al., 1995).

Like those of insulin, blood levels of leptin are proportional to body fat levels in rodents and humans; adipocyte levels of leptin mRNA also display proportionality to adiposity levels (Frederich et al., 1995a; Maffei et al., 1995; Considine et al., 1996). Thus, leptin mRNA is upregulated and the peptide is released at times of positive energy balance. Ingestion of all macronutrient groups stimulates leptin secretion (lipids, branch chained amino acids and carbohydrates – most probably hexoses and lactate, rather than glucose) (Lee and Fried, 2009). Interestingly, leptin secretion is inhibited within 2 h of increased non-esterified fatty acid (free FA) availability (Shintani et al., 2000); since free FAs are produced from the breakdown of triacylglycerides during periods of energy deficiency, downregulation of satiety signal would be expected. Other positive regulators of leptin include insulin, oestrogens, glucocorticoids and melanin-concentrating hormone (MCH). Activation of β-adrenergic receptors, elevated TNFα, IL-6, testosterone and NPY, on the other hand, decrease leptin amounts (Ahima et al., 2000; Lee and Fried, 2009). Acute and chronic administration of leptin inhibits feeding and stimulates energy expenditure in many mammals (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Oral et al., 2002; Friedman, 2009). The body weight-reducing actions of leptin are, at least partly, exerted via its lipolytic actions that

originate in the hypothalamus and involve sympathetic (noradrenergic) innervation of adipose tissue, and subsequently, phosphorylation of local lipases (Zeng et al., 2015).

Leptin has rather diverse physiological effects. It exerts positive effects on angiogenesis and sympathetic tone - effects that might explain cardiovascular co-morbidities of obesity due to insufficient leptin (signalling). In fact, leptin increases sympathetic tone in many tissues, including the adrenals, kidney, heart and the brown adipose tissue (Mark et al., 2002; Ren, 2004; da Silva et al., 2006). In addition, leptin deficiency or insensitivity to its actions (see later) has been associated with disturbances in pubertal development and fertility, increased glucocorticoid levels, and impaired growth hormone secretion (Mantzoros et al., 1997; Clement et al., 1998; Ahima et al., 2000). Strikingly, weight loss through caloric restriction does not compensate for leptin deficiency in mice, pointing to a critical role of the hormone itself (Ahima et al., 2000).

The leptin receptor (LepRb) gene encodes several alternatively-spliced isoforms, but it is the long isoform that is responsible for the physiological effects of the hormone (Uotani et al., 1999). The LepRb is a transmembrane protein that belongs to the class I cytokine receptor family. Upon ligand binding, LepRb binds (non-covalently) to cytoplasmic Janus kinase-2 (JAK2) tyrosine kinase which, and after multiple autophosphorylation events phosphorylates LepRb at Tyr1138. This results in recruitment of signal transducer and activator of transcription 3 (STAT3) protein to the LepRb/JAK2 complex and phosphorylation (Tyr705) of STAT3. Subsequently, pSTAT3 is translocated to the nucleus where it functions as a transcription factor (Figure 1.3). Important targets of pSTAT3 that are upregulated include proopiomelanocortin (POMC) and suppressor of cytokine signalling 3 (SOCS-3); the latter is a negative feedback regulator of the JAK/STAT pathway (Banks et al., 2000; Fruhbeck, 2006). The key role of Tyr1138-LepRb (STAT3 binding site) was demonstrated by the fact that substitution of Tyr with Ser1138 blocks the effects of leptin on energy balance and food intake; mice with this mutation are leptin resistant, hyperphagic and obese (Bates et al., 2003). Another signalling cascade activated by leptin (and insulin, as discussed earlier) is the phosphatidylinositol-3 kinase (PI3K) pathway (Konner and Bruning, 2012) (Figure 1.3). Pharmacological inhibition of PI3K abolishes the anorexigenic effects of leptin (Niswender et al., 2001). Interestingly, however, PI3K appears essential for the acute effects of leptin on body weight regulation, whereas it is not required for long-term body weight regulation (Hill et al., 2008).

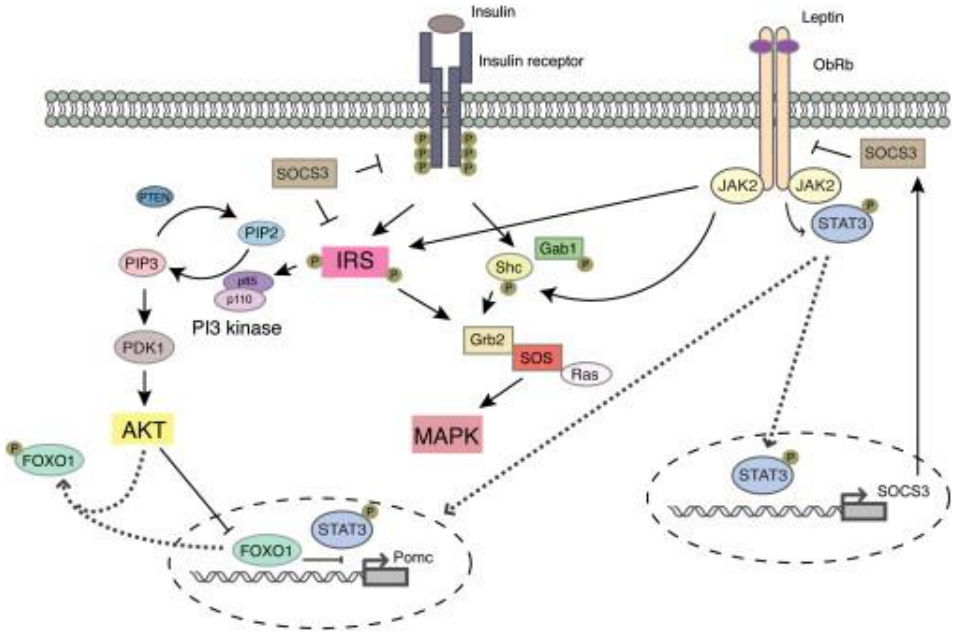


Figure 1.3. Convergence of Insulin and Leptin Signaling. Binding of leptin to the long form of the leptin receptor (ObRb/LepRb) leads to activation of Janus kinase (JAK) 2 and, subsequently, to JAK2-mediated phosphorylation of the receptor and activation of signal transducer and activator of transcription (STAT)3 molecules. Activated STAT3 forms dimers, which translocate to the nucleus and activate target genes including suppressor of cytokine signaling (SOCS)3. Increased STAT3-mediated SOCS3 expression leads to the combined feedback inhibition of leptin and insulin signaling. Phosphorylated JAK2 also directly activates the IRS/PI3 kinase signaling pathway, initiating downstream signals such as activation of serine/threonine kinases downstream of PIP3. Moreover, leptin activates the MAPK pathway by inducing tyrosine phosphorylation of Shc and its association with Grb2. In POMC cells, insulin-mediated activation of AKT leads to phosphorylation of FOXO1 and its exclusion from the nucleus, thereby allowing for transcription of Pomc. Pomc transcription is also increased by binding of STAT3 to its promoter. (Konner and Bruning, 2012)

Leptin is detectable in the cerebrospinal fluid (CSF) and was initially thought to be transported into the brain via passive diffusion through the circumventricular organs (mediobasal hypothalamus, subfornical organ and area postrema) whose capillaries are fenestrated and thus, do not have an intact blood-brain barrier (BBB) (Banks et al., 1999; Ahima et al., 2000; Banks and Farrell, 2003a). It was also proposed that the short form of leptin receptor could function as a transporter on BBB (Bjorbaek et al., 1998; Boado et al., 1998). However, recent evidence suggests that leptin uptake occurs through the median eminence via LepRb on tanycytes that deliver it to the III ventricle and from where it arrives at its central targets (Langlet et al., 2013; Balland et al., 2014).

The hypothalamus displays the greatest abundance of LepRb, and is also the major site of leptin action (further discussed in section 1.3.1). Particularly strong LepRb immunoreactivity is found in the supraoptic nucleus (SON), paraventricular nucleus (PVN), periventricular nucleus, arcuate nucleus (ARC), and lateral hypothalamus (LH). Weaker LepRb signal is present in the lateral and medial preoptic nuclei, suprachiasmatic nucleus (SCN), ventromedial (VMH) and dorsomedial (DMH) hypothalamus, and in the tuberomammillary nucleus (Hakansson et al., 1998). LepRb are

expressed at lower levels in other brain areas, including the pontine parabranchial nucleus (PBN), Edinger-Westphal nucleus, VTA and the nucleus of the solitary tract (NTS) (Hakansson et al., 1998; Scott et al., 2009; Garfield et al., 2012) as well as in the periphery (in intestine, adrenals, fat, heart, lymph nodes, lungs and spleen) (Hoggard et al., 1997; Lollmann et al., 1997). Leptin targets in the mouse brain have been mapped at both protein and mRNA levels. In all of the brain regions mentioned, leptin contributes to the regulation of feeding and energy homeostasis (Hommel et al., 2006b; Xu et al., 2011a; Garfield et al., 2012; Alhadeff et al., 2014). In contrast, although LepRb have also been mapped in the thalamus, choroid plexus, cerebral cortex, insular cortex, lateral septal nucleus and hippocampus (Hakansson et al., 1998; Scott et al., 2009), their role in feeding and energy metabolism is unclear; rather, they have been shown to be involved in cognitive and emotional processes (Farr et al., 2006; Garza et al., 2012; Guo et al., 2012; Licinio et al., 2014).

Mutations in the leptin gene are very rare, but when they do occur, they result in obesity and very severe insulin resistance, a phenotype rescuable with leptin therapy (Farooqi et al., 1999). While these findings suggested that leptin might be useful in treating obesity, the strategy proved successful in only a small subset of patients because most obese indivduals have high endogneous levels of the hormone and are therefore insensitive to its actions. However, leptin has proved valuable in the treatment of lipodystrophies and other conditions associated with low blood leptin levels (Friedman, 2014).

Humans and mice with elevated leptin levels do not show reductions in food intake, and are thus highly likely to be leptin resistant (Frederich et al., 1995b; Maffei et al., 1995; Halaas et al., 1997; Friedman, 2009; Myers et al., 2010). Available data suggests that, on the one hand, leptin resistance can trigger the development of obesity by gradually switching the “defended level of body weight” to a higher one (Velloso and Schwartz, 2011; Morton et al., 2014). On the other hand, obesity itself is considered to be responsible, at least partly, for the development of leptin resistance. For example, after mice were a fed high fat diet (HFD) to induce obesity, central leptin resistance was observed in the ARC (reduced pSTAT3 and increased SOCS3 levels) already within 6 days of the HFD regime (Münzberg et al., 2004). In addition, other studies in mice have implicated impaired PI3K signalling in the ARC, VMH, DMH and ventral pre-mammillary area after 4 weeks of HFD (Metlakunta et al., 2008). Interestingly, research on rats not only demonstrated HFD-induced reductions in STAT3 phosphorylation in the ARC but also in the ventral tegmental area (VTA), an area that regulates motivated behaviour (Matheny et al., 2011).

The exact mechanism leading to leptin resistance and obesity are still being uncovered (Velloso and Schwartz, 2011), but numerous plausible suggestions exist in the literature. Proposals include the idea that inadequate amounts of leptin reach its sites of action

because high triacylglyceride levels in the blood hinder leptin transport across the BBB (free FA do not exert such an effect) (Banks et al., 2004). Yet other researchers have suggested that leptin resistance results from saturation of BBB-located LepRb by high circulating levels of leptin (Burguera et al., 2000; Banks and Farrell, 2003b). Another mechanism proposed as a possible cause of obesity-driven leptin (but also insulin) resistance is chronic low-grade inflammation. Various authors showed that obesity triggers cellular (endoplasmic reticulum, ER) stress in muscle, fat and liver and therefore, an increase in pro-inflammatory molecules (e.g. tumour necrosis factor-α, TNF-α) which contribute to insulin resistance and very likely to leptin insensitivity. ER stress and inflammatory signalling in the hypothalamus interrupt central leptin signalling via SOCS3 and a phosphatase PTP1B (Zhang et al., 2008; Ozcan et al., 2009; Myers et al., 2010; Thaler et al., 2012). Lastly, saturated fat intake increases circulating FAs (e.g. palmitate) which have been shown to impair both leptin and insulin signalling in the hypothalamus via activation of Toll-like receptor 4 on microglia (Benoit et al., 2009; Kleinridders et al., 2009).

States of insulin deficiency (see 1.1.3) and leptin deficiency often occur together. Both states share several common features (energy deficiency and, consequently, neuroglucopenia as well as hyperglycaemia, ketosis and hyperphagia). Curiously, both obese mice and mice lacking adipose tissue show insulin resistance. The diabetes phenotype seen in the latter could be reversed by transplanting fat from healthy mice to lipoatrophic mice (Gavrilova et al., 2000); however, fat transplants from *ob/ob* mice failed to restore insulin sensitivity (Colombo et al., 2002), indicating that leptin is required for normal glucose tolerance – a conclusion consistent with the fact that leptin promotes glucose disposal even in the absence of insulin. In fact, insulin deficiency was shown to be rescuable with exogenous leptin given peripherally, albeit at supraphysiological doses (da Silva, Alexandre A et al., 2006; Fujikawa and Coppari, 2015), but also after central administration (German et al., 2010; German et al., 2011; Morton et al., 2014).

1.3. From neural circuits and behavioural mechanisms to metabolic homeostasis

Ultimately, the brain is the “master regulator” of feeding, and thus body weight. It is able to detect satiety signals (gut peptides) and information regarding energy stores “online” and directs behaviours (to eat or not to eat) by integrating these homeostatic signals with emotional state and other psychological modulators of feeding such as taste hedonics and preferences, memories and habits. The hypothalamus is the hub at which information from the periphery (via the brainstem) and limbic and cortical brain areas is

integrated and processed, and from where commands to eat (or not) are distributed to centres regulating motivation, decision-making and the (motor) act of feeding (Figure 1.4). Identification and understanding of the components and their myriad interactions that together form the neurocircuitry of feeding is still an active area of research and discovery. Here, an attempt is made to summarize the key knowledge available so as to provide the backdrop against which the questions addressed in this thesis were developed.

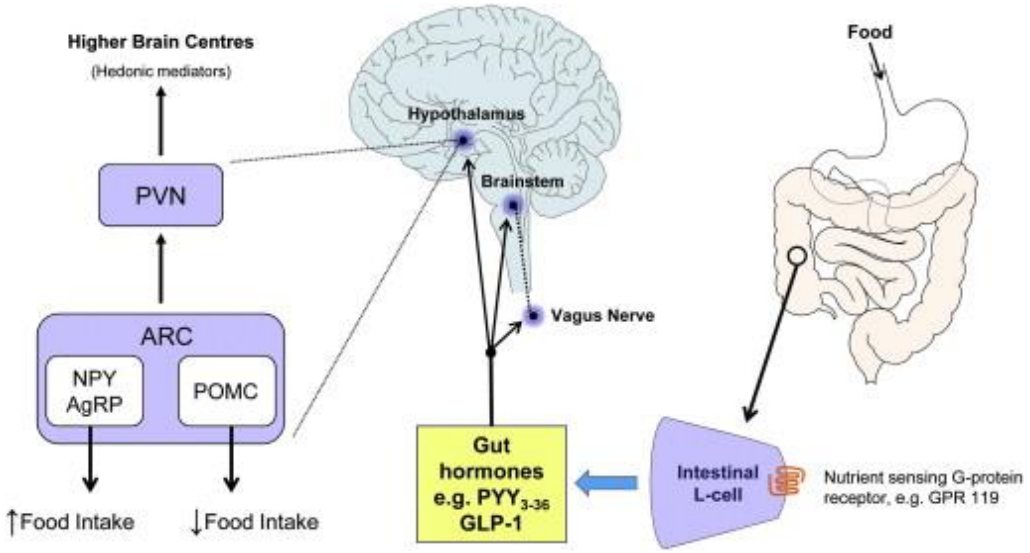


Figure 1.4. Gut-brain axis: regulation of food intake (Sam et al., 2012). Post-digestive nutrients activate G-protein coupled receptors on the luminal side of entroendocrine cells, stimulating the release of gut hormones which, in turn, may influence food intake at three sites: the vagus nerve, brainstem and hypothalamus. Two neuronal populations within the arcuate nucleus of the hypothalamus (orexigenic NPY/AgRP neurons and anorexigenic POMC neurons) are thought to be critical conduits through which peripheral signals are integrated to alter the drive to eat. There are also other connections between hypothalamic nuclei and higher brain centres that control the hedonic aspects of food ingestion. *ARC (arcuate nucleus), AgRP (agouti related peptide), GLP-1 (glucagon like peptide-1), NPY (neuropeptide Y), POMC (pro-opiomelanocortin), PVN (paraventricular nucleus), PYY (peptide YY)* (Sam et al., 2012).

1.3.1. Central reception and integration of peripheral signals

The ***nucleus tractus solitarius (NTS***) serves as a point where gustatory signals (from taste buds) as well as information about distention, nutrients and gut peptides from the gastrointestinal tract via vagal afferents (see 1.2.2 and 1.2.3) converge. The NTS also senses circulating adiposity signals (e.g. leptin) and nutrients (e.g. glucose); these signals reach the NTS via the area postrema (AP; a circumventricular organ located in the roof of the IV ventricle) from where they are relayed to the parabrachial nucleus (PBN) of the pons and other hindbrain regions, as well as to the POA, ARC and DMH (energy balance) and PVN (neuroendocrine-related); satiation signals are additionally relayed to the motivation and reward-related areas (VTA and NAc)(Ahima and Antwi, 2008; Grill and

Hayes, 2012; DiPatrizio and Piomelli, 2015). The integration of peripheral signals in the vagus nerve has been extensively reviewed by Schwartz and Moran (Schwartz et al., 1993), Huo et al (Huo et al., 2007) and Wang et al (Wang et al., 2000). Interestingly, the NTS also receives reciprocal (modulatory) innervation from the hypothalamus (Figure 1.5).

Lesioning of the ventromedial (VMH) and lateral (LH) hypothalamus in the first half of the last century was found to result in obesity (lesions in VMH) and extreme leanness (in LH) (Hetherington and Ranson, 1942; Brobeck et al., 1943; Anand et al., 1955); these findings pointed to the key role of the ***hypothalamus*** in the central control of energy balance. Notably, only modest effects of ARC lesions were reported (Tanaka et al., 1978), possibly reflecting the fact that this nucleus is composed of neurons with opposing actions (Velloso and Schwartz, 2011).

The ARC is now recognized as one of the most important areas in the regulation of feeding (Hussain and Bloom, 2013). Given its proximity to the median eminence which has fenestrated capilliaries and β-tanycytes, the ARC is easily accessible to signals circulating in the general bloodstream (Langlet, 2014). There are two well-characterized populations of cells in the ARC: orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons and anorexigenic pro-opiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) neurons. Both populations express receptors for a variety of peripheral (e.g., leptin, insulin, PYY and ghrelin) and central (e.g., α-MSH, NPY, GABA, and serotonin) signals involved in feeding regulation (Sohn et al., 2013), as shown in Figure 1.5.

Anorexigenic leptin, acting through leptin reeptors (LepRb), activates POMC/CART neurons (Cheung et al., 1997; Elias et al., 1998a; Cowley et al., 2001). Exogneous leptin upregulates POMC mRNA, an effect also seen in *ob/ob* mice and food-restricted rodents with low levels of POMC mRNA (Thornton et al., 1997). On the other hand, NPY and AgRP mRNA are upregulated after fasting (Sanacora et al., 1990; Swart et al., 2002); in fact, NPY/AgRP neurons have been shown to be required for the orexigenic effects of ghrelin (Chen et al., 2004). Reciprocity within this microcircuit is illustrated by the fact that ghrelin decreases the spontaneous activity of anorexigenic POMC neurons (Chen et al., 2004), while leptin suppresses NPY/AgRP mRNA expression (Schwartz et al., 1996) as well as NPY/AgRP neuron activity through hyperpolarization via either PI3K-dependent activation of an ATP-sensitive potassium channel (Spanswick et al., 2000; Cowley et al., 2001) or by suppressing activation of a subpopulation of NPY neurons that otherwise inhibit POMC cells through the mediation of γ-aminobutyric acid (GABA) (Atasoy et al., 2012). It is important to note that, although the ARC circuit was initially characterized by studying the effects of leptin and ghrelin, other factors such as glucose, insulin, serotonin, PYY, glucocorticoids and FAs are now known to be directly sensed by (sub-populations of) NPY and POMC neurons (Batterham et al., 2002; Savontaus et al., 2002;

Williams et al., 2011; Sohn et al., 2013; Magnan et al., 2015). Lastly, in light of the role of the ARC as a integrative hub, it is important to note that POMC and NPY neurons receive (largely overlapping) inputs from many other brain areas, including the forebrain, brainstem and other hypothalamic nuclei (PVN, DMH, VMH, LH) (Wang et al., 2015).

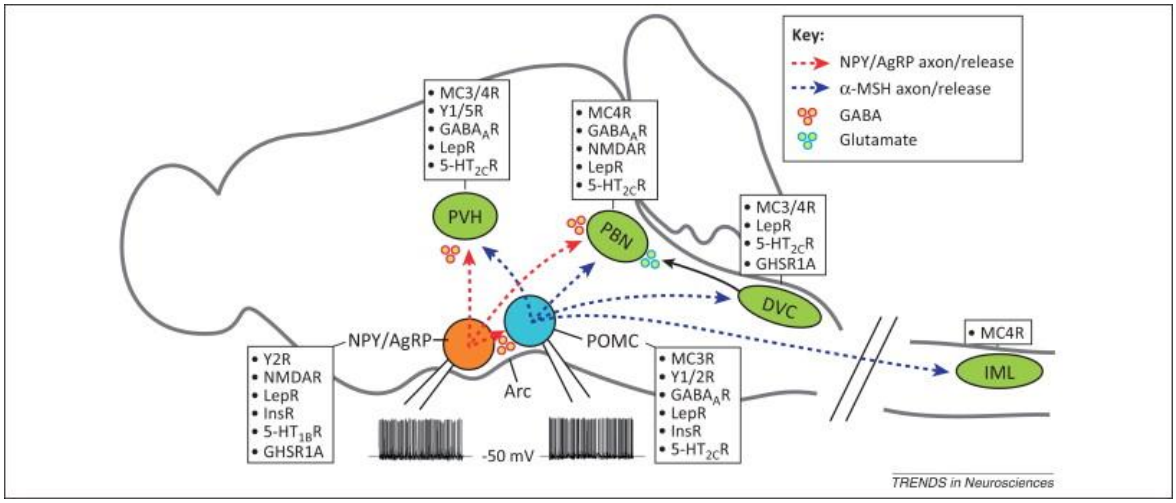


Figure 1.5. **Central neuronal circuits that regulate energy and glucose homeostasis**. (Sohn et al., 2013). The hypothalamic arcuate nucleus (ARC) contains two neuron populations that are involved in food intake and energy regulation: pro-opiomelanocortin (POMC) and neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons. POMC neurons release the anorexigenic peptide α-melanocyte stimulating hormone (α-MSH), whereas NPY/AgRP neurons release the orexigenic peptides NPY/AgRP and GABA at their target nuclei in various brain regions and the spinal cord. Arcuate POMC and NPY/AgRP neurons express receptors for peripheral and central signals. POMC neurons receive GABAergic (inhibitory) input from NPY/AgRP neurons. The central melanocortin pathway includes POMC neurons and melanocortin receptors (MC3R and MC4R).The peptides α-MSH (agonist) and AgRP (inverse agonist) act at anorectic MC4R expressed by neurons in the hypothalamic paraventricular nucleus (PVH), parabrachial nucleus (PBN), dorsal vagal complex (DVC), and intermediolateral column of spinal cord (IML). Notably, MC4R in the PVH mediate anorexigenic actions, whereas those in the autonomic preganglionic neurons in the DVC and IML regulate energy expenditure and glucose homeostasis. It is thought that the orexigenic effects of NPY/AgRP neurons in the ARC first activate NPY receptors (NPY1R and NPY5R) and then inhibit melanocortin receptors. An important role is also played by GABAergic neurotransmission in the orexigenic circuit in the PVH and PBN**.** Note that PBN neurons receive glutamatergic (excitatory) input from the nucleus tractus solitarius (NTS) within DVC; this may mediate satiety signals from the gastrointestinal tract and act to antagonize GABA-mediated hunger signals relayed by NPY/AgRP neurons. Neurons within PVH, PBN, and DVC are also directly regulated by central and peripheral signals such as leptin (via LepR), insulin (via InsR), serotonin (via 5-HT$_{1B}$R and 5-HT$_{2C}$R), and ghrelin (via GHSR).

Pre-POMC products include adrenocorticotropic hormone (ACTH), β-lipotropin, corticotropin-like intermediate lobe peptide (CLIP), γ-lipotropin, β-endorphin, and melanocortins (α-, β-, γ-MSH) (Uhler and Herbert, 1983). Interestingly, high concentrations of α-melanocyte-stimulating hormone (α-MSH) are found in the ARC (Eskay et al., 1979) and increased serum leptin leads to both an increase in POMC mRNA expression and post-translational α-MSH peptide cleavage in the ARC (Schwartz et al., 1997; Thornton et al., 1997; Mizuno et al., 1998). Neuropeptide Y (NPY), which is co-expressed with AgRP in ARC neurons, represents an important orexigenic signal (Chen et al., 2004). NPY acts by binding to Y-receptors (Y1, Y2, Y4 and Y5) that are widely

expressed in the hypothalamus and other brain areas (Dumont et al., 1998; van Swieten et al., 2014). Antisense studies showed that Y5 receptors are important for the food ingestion-inducing effects of NPY (Schaffhauser et al., 1997) whereas Y2 receptors modulate appetite acting as presynaptic autoreceptors that inhibit NPY release (King et al., 2000). Interestingly, peptide YY (PYY) also activates Y receptors, with PYY3-36 binding selectivity to Y5 and Y2 receptors; moreover, PYY produces anorectic effects by disinhibiting POMC neurons (via Y2 receptors) in the ARC (Chaudhri et al., 2006).

Five melanocortin receptors (MCR) were cloned by Roger Cone and collagues (Mountjoy et al., 1992; Adan and Gispen, 1997). The MC1R is responsible for skin/hair pigmentation; MC3R and MC4R regulate feeding and energy homeostasis and are widely expressed in the brain (Williams et al., 2011). MC3Rs are expressed in parts of the brainstem, and various hypothalamic nuclei, including the ARC. MC4Rs show more widespread distribution in the brain, including the cortex, thalamus and hypothalamus (PVN, DMH and LH), and brainstem (Mountjoy et al., 1994). The natural ligands of the MC1R, MC3R, MC4R are melanocortin α-MSH (agonist) and AgRP (inverse agonist/antagonist) (Yang, 2011). Yellow agouti mice A(Y) that overexpress agouti protein, a MC4R antagonist, indeed display the same phenotype as MC4R KO mice (adult onset obesity, leptin resistantance) (Fan et al., 1997; Butler et al., 2001). Additionally, intracerebroventricular (ICV) infusions of α-MSH or other MC4R agonists produce satiety, an effect blocked by AgRP induced hyperphagia (Mizuno et al., 1998; Rossi et al., 1998; Benoit et al., 2000); these findings demonstrate that the melanocortin system is a major regulator of energy homeostasis. The importance of MC4R in the PVN in the regulation of feeding is attested to by the fact that its genetic ablation in mice results in obesity due to increased food intake without a change in energy expenditure; this phenotype is rescuable by re-expression of MC4R in the PVN (Balthasar et al., 2005).

Arcuate POMC and NPY/AgRP neurons send projections to second order neurons in the PVN, LH, VMH, and DMH, where they exert anorexigenic and orexigenic effects, respectively (Elias et al., 1998b; Stanley et al., 2005; van Swieten et al., 2014). As shown in Figure 1.6, POMC and NPY/AgRP neurons project to the PVN (Cowley et al., 1999). Whereas POMC neurons activate corticotrophin releasing hormone (CRH), thyrotropin releasing hormone (TRH) (via MC3R/4R) and oxytocin (OXY) neurons (possibly indirectly via effects on Y5 receptors) (Kim et al., 2002; Atasoy et al., 2012; Sabatier et al., 2013), the NPY/AgRP projections inhibit PVN neurons and increase feeding (Kim et al., 2000; Atasoy et al., 2012). Although not shown in the scheme, leptin from the periphery also activates CRH neurons in the PVN (Schwartz et al., 1996).

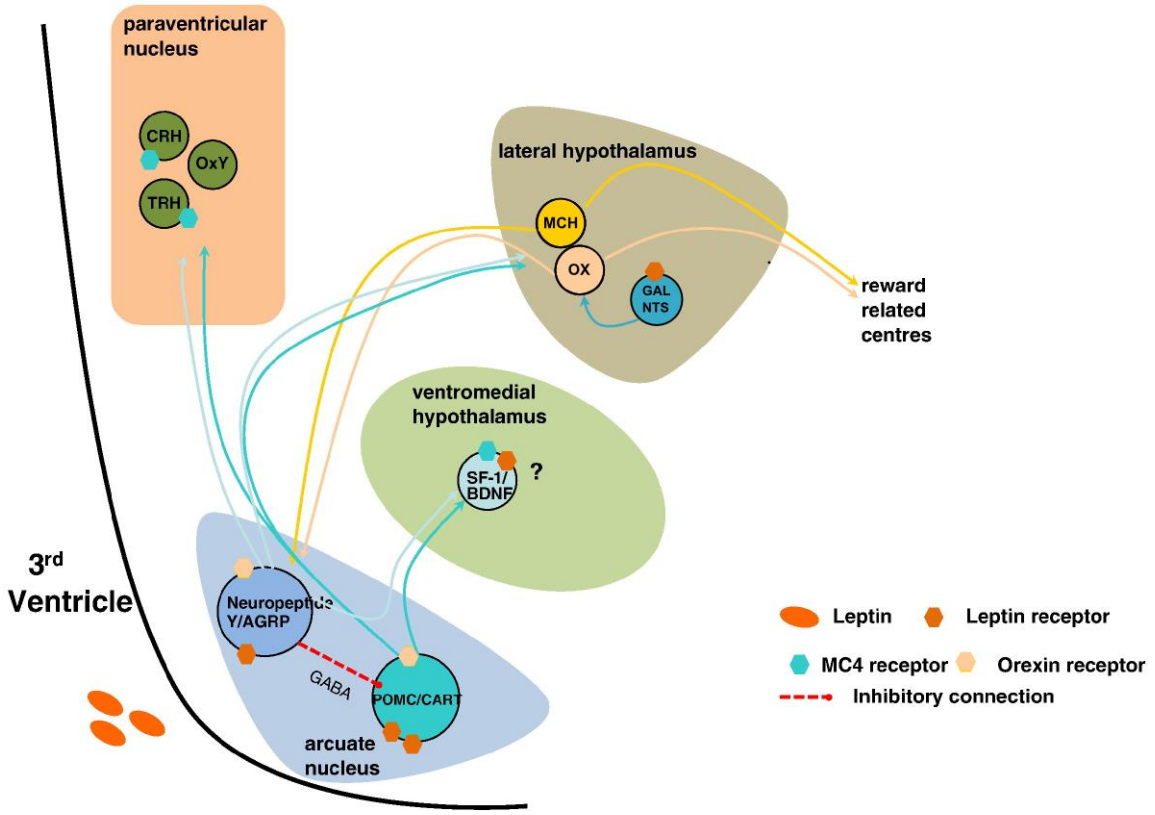


Figure 1.6. Hypothalamic circuits regulating energy homeostasis (Based on Pandit et al., 2011, modified according to van Swieten et al., 2014). Note, the original scheme showed the presence of leptin receptors on orexin and MCH neurons, an assumption that has proven to be incorrect. In addition, the original scheme did not indicate that leptin receptors are expressed galanin/neurotensin/GABA-ergic neurons in the LH. Abbreviations: NTS: neurotensin, GAL: galanin, OX: orexin, MCH: melanocyte concentrating hormone, CRH: corticotropin releasing hormone, TRH: thyrotropin releasing hormone, OxY: Oxytocin, SF-1: orphan nuclear receptor SF-1, BDNF: brain derived neurotrophic factor, AGRP: agouti-related protein, POMC: proopiomelanocortin, CART: cocaine and amphetamine related transcript.

Lesion studies have shown that the LH is an important driver of feeding (see above 1.3.1.). This area receives NPY/AgRP inputs from the ARC and the strongest pro-feeding responses to exogenous NPY are elicited in the perifornical area of the LH (Stanley et al., 1993). The LH contains two populations of orexigenic neurons which express the peptides orexin (OX) and melanin-concentrating hormone (MCH) (see Table 1.1), and both sets of neurons display synaptic terminals containing NPY/AgRP and POMC (Broberger et al., 1998; Elias et al., 1998b). It is also interesting to note that, OX neurons in the LH back-project to the ARC, making direct excitatory synaptic contact with NPY neurons; through this pathway, the LH exerts inhibitory actions on POMC neurons, thus potentiating the pro-feeding effects of NPY (Horvath et al., 1999). As mentioned above, POMC and NPY/AgRP neurons in the ARC (as well as PVH, PBN, and DVC neurons) are additionally subject to the modulatory effects of central and peripheral signals, including leptin, insulin, serotonin, and ghrelin whose actions are mediated by LepR, InsR, 5-HT_{1B}R and 5-HT_{2C}R and GHSR, respectively (Sohn et al., 2013) (Figure 1.5).

TABLE 1.1. Characteristics of Orexin (OX) and Melanin-concentrating hormone (MCH) that are relevant to feeding behaviour, including energy/nutrient sensing and hedonic pathways.

Characteristic	Orexin (OX) (hypocretin)[1]	Melanin-concentrating hormone (MCH)[2]
Molecular weight (KDa)	OX-A: 3561.1, OX-B: 2936.4	2386.8
Distribution	LH, DMH[3]	LH, DMH, ZI[4]
Co-expressed neuropeptides/transmitters	Dynorphin, prolactin, glutamate[5]	Ach, glutamate, CART[6]
Surface receptor expression	5HT1R, glutamate receptors, CRH1R, NPY1R, $GABA_aR$, Gal1R[7]	5HT1R, MC3R[8]
Known inputs (afferents)	NAc: GABA Basal forebrain: Ach; LH: Gal Hypothalamus: GABA; ARC: NPY, AgRP, α-MSH; Raphe nuclei: 5HT[9]	LH: GABA, OX ARC: NPY, AgRP, α-MSH Pons, tegmentum: Ach[10]
Known outputs (efferents)	Cortex (OFC, Cg, AI), septal nuclei, VP, BNST, amygdala, thalamus, hippocampus, hypothalamus (LH, VMH, DMH, ARC), ZI, VTA, SN, LC, Raphe nuclei, medulla oblongata[11]	Cortex (OFC, Cg, AI, Pir), septal nuclei, NAc, BNST amygdala, hippocampus, thalamus, hypothalamus (PVN, LH, ARC), ZI, VTA, LC, Raphe nuclei, medulla oblongata [12]
Effect on feeding	Induces appetite[13]	Induces appetite[14]
Other related functions	Induces wakefulness, arousal and alertness, activates HPA axis, sympathetic NS activity, thermo-genesis, reward, drug abuse[15]	Induces sleep, enhances cognition[16]

References [1](Sakurai et al., 1998); (Lecea et al., 1998); [2](Qu et al., 1996); [3](Peyron et al., 1998); [4](Qu et al., 1996); [5](Chou et al., 2001), (Risold et al., 1999); (Schöne and Burdakov, 2012); [6](Lecea and Sutcliffe, 2005); [7](Johnson et al., 2012); (Laque et al., 2015), [8](Lecea and Sutcliffe, 2005); [9](Sakurai et al., 2005); (Elias et al., 1998) (Laque et al., 2015); [10](Louis et al., 2010); (van den Pol, Anthony N et al., 2004); (Elias et al., 1998); (Bayer et al., 1999); [11](Peyron et al., 1998); (Marcus et al., 2001); [12](Kilduff and Lecea, 2001); (Saito et al., 2001); (Gutierrez et al., 2011); [13](Sakurai et al., 1998); (Sakurai, 2014); [14](Qu et al., 1996), (Edwards et al., 1999); [15](Hagan et al., 1999); (Yamanaka et al., 2003); (Owens and Nemeroff, 1991); (Harris and Aston-Jones, 2006); (Sakurai, 2014); [16](Hassani et al., 2009); (Adamantidis and Lecea, 2009) Gutierrez et al. (2011)

Abbreviations: 5-HT: serotonin, 5HT1R: serotonin receptor 1, α-MSH: alpha melanocyte stimulating hormone, Ach: acetylcholine, AgRP: Agouti related peptide, ARC: arcuate nucleus, AI- anterior insular cortex, BNST: bed nucleus of stria terminalis, CART: cocaine- and amphetamine-regulated transcript, Cg: Cingulate cortex, CRH1R: cortiotropin releasing hormone 1 receptor, DM: dorsomedial hypothalamic nucleus, GABA: gamma-amiobutyric acid, Gal: galanin, Gal1R: galanin receptor 1, HPA: hypothalamic pituitary adrenal, LC: locus coeruleus, LH: lateral hypothalamus, MC3R: melanocorting receptor 3, NAc-nucleus accumbens, NPY1R- neuropeptide 1 receptor, NS- nervous system, OFC- orbitofrontal cortex, Pir: piriform cortex, VP: ventral pallidum, VTA: ventral tegmental area, SN: substantia nigra, ZI: zona incerta.

Several extra-hypothalmic sites also receive POMC afferents from the ARC; these include the dorsal vagal complex (DVC) of the brainstem and intermediolateral cell column (IML) of the spinal cord which are implicated in the regulation of energy metabolism and glucose homeostasis (Figure 1.5). The latter areas are also activated by leptin (via sympathetic preganglionic neurons) which contributes to their role in regulating glucose

homeostasis, thermogenesis (increased), energy expenditure (increased) and body weight (decreased) (Elias et al., 1998a; Sohn et al., 2013).

Besides the ARC, the NTS is the only other brain area that contains POMC neurons; melanocortins derived from these neurons appear to be responsible for regulating energy homeostasis. These neurons are activated by the gut hormone cholecystokinin (CCK; see Box 1.1.) upon satiety; indeed, MC4R are necessary for CCK to terminate eating (Fan et al., 2004). The parabranchial nucleus (PBN), which is innervated by the NTS, also plays a role in integrating physiological signals of hunder and fullness; it does so by responding to inhibitory input from NPY/AgRP neurons (Wu et al., 2012).

The above represents an attempt to summarize the most important signalling molecules and neural connections that serve to regulate feeding in such a way as to maintain metabolic balance. The picture would be incomplete without mentioning that these circuits are additionally modulated by other factors acting on the hypothalamus, midbrain and brainstem. These modulators include FA-derived molecules such as the endocannabinoids (2-AG and anandamide) which promote appetite via CB1 receptor activation (as discussed in section 1.2.2), opioids (e.g. β-endorphin which is involved in the rewarding aspects of feeding through its activation of μ opioid receptors MOR) (see section 1.3.2), and neurotransmitters (e.g. serotonin, GABA and dopamine). Dopamine is most likely involved in motivation to obtain food and, as hypothesized by some authors, to contribute to the addiction-like traits related to overeating (section 1.3.2). Serotonin (5-HT) has an anorectic effect and notably, deficiency of its 5-HT2C or 5-HT1B receptors leads to hyperphagia and obesity. Serotonin is a strong regulator of feeding; 5-HT2C receptors activate POMC neurons, whereas 5-HT1B receptors inhibit NPY/AgRP neurons (Xu et al., 2011b; Sohn et al., 2013). Even with the mention of these additional players, these descriptions of the complex interactions that help regulate feeding behaviour remain over-simplified and yet, new players and pathways are continually being discovered.

1.3.2. Sensory and behavioural circuits and mechanisms regulating food ingestion

Eating is a highly regulated behaviour, aimed at fulfilling the organism's energy requirements. This regulation includes fine integration and matching of internal needs with environmental signals of food (availability, palatability, reward value) as well as improving the chances of survival until the next meal. These processes are further subject to modulation by other factors such as learning, stress, social influences, habits and ease of access to food; when food is abundant, nutritional and/or energetic content may become less of a determinant and this can lead to overeating. One other important aspect that needs consideration concerns the additional factors (e.g. pleasure, past-

experience) that may determine selection of a particular food when multiple choices are possible (Begg and Woods, 2013).

Integration of sensory inputs

Food selection is based on the ability to detect specific nutrient classes and to adjust their intake according to physiological need (DiPatrizio and Piomelli, 2015). Foods are endowed with multisensory cues (visual, olfactory, gustatory and tactile signals); the relative importance of each cue may differ according to species but also momentary states of hunger and emotion.

In addition to the common oral taste sensations (sweet, salty, sour, bitter, savory), food perception depends strongly on retronasal-mediated stimuli (volatiles from the mouth enter the nasal cavity via the nasopharynx); some authors suggest that these signals are more potent than "taste" (Murphy and Cain, 1980). The multimodal experiences triggered by food (taste, smell and texture) are integrated into a single one when different sensations occur simultaneously or in close succession; although multimodal sensation is often subjectively perceived as "taste", a more precise term would be "flavour" (Rozin, 1982)[1]. Multi-modal perceptions are formed through the activity of different sets of neurons that respond to inputs from distinct sensory receptors; their receptive fields overlap across modalities and the responses are "supra-additive". The convergence of these signals happens at almost every level of the neuroaxis, but the initial integration of sensory "flavour" signals occurs in the anterior insular cortex (Small, 2012).

Taste receptors are located in the taste bud cells of the tongue, palate and throat. Sweet, bitter and umami are sensed by G-protein coupled receptors (GPCRs) (e.g. heterodimeric receptors consisting of T1R2 and T1R3 for sweet detection) (Breslin and Spector, 2008), whereas salty and sour tastes are sensed by ion channels. Although the taste of "fat" is not considered to be a basic one, oral (but not olfactory) fat receptors are necessary for the detection of fat (Fukuwatari et al., 2003; Pittman et al., 2007). The fatty acid transporter CD36 and the GPCR GPR120 and GPR40, that are expressed in taste buds have high affinity for long-chain FAs and are involved in the detection of diets high in fat (Laugerette et al., 2005; Cartoni et al., 2010).

Taste receptors transform chemical information into electrical signals that are, in turn, relayed to the rostral nucleus of the solitary tract (NTS) by fibres of the cranial nerve VII (chorda tympani) and the trigeminal, glossopharyngeal and vagal nerves (Breslin and Spector, 2008). In rodents, the NTS integrates taste information with satiety signals from the gut and transmits this to the parabrachial nucleus of the pons (PBN)**,** which then sends (and receives) signals to forebrain regions that control food reward and energy

[1] The words "flavour" and "taste" are usually used synonymously in English and other languages (Rozin, 1982).

homeostasis (Grill and Hayes, 2012; DiPatrizio and Piomelli, 2015). Information about taste is then transmitted from the PBN to the thalamus, lateral hypothalamic area, central amygdala (CeA), dorsolateral bed nucleus of the stria terminalis (BNST), and frontal cortex (insula, orbitofrontal cortex) (Norgren, 1976, 1978; Zampini and Spence, 2004). It should be noted that in primates, the NTS sends signals directly to the thalamus (the PBN relay hub has only been described in rodents) (Small, 2012).

There are numerous olfactory receptors, encoded by an estimated 400 functional genes in humans and 800-1200 genes in other species. All the known receptors are GPCRs and are primarily expressed by olfactory sensory neurons on the epithelium of the nasal cavity (Niimura and Nei, 2007; Rinaldi, 2007). Both orthonasal (inhalation through the nose) and retronasal olfactory signals reach the olfactory bulb via cranial nerve I, from where projections are sent to various brain areas, including the anterior olfactory nucleus, olfactory tubercle, piriform cortex, several amygdaloid subnuclei, the rostral entorhinal cortex and thalamus. These areas, in turn, project to additional amygdalar subnuclei, the lateral hypothalamus and the entorhinal, insular, orbitofrontal (OFC) cortex, as well as to the locus coeruleus and medial raphe nuclei (Olmos et al., 1978; Turner et al., 1978). In the field of olfactory research, the cortical targets are jointly referred to as the primary olfactory cortex (piriform cortex, insular cortex, the olfactory tubercle, entorhinal cortex, anterior cortical amygdaloid nucleus and periamygdaloid cortex); however, in the broader field of neuroscience, these areas belong to the association cortices because they integrate olfactory information with information from other sensory modalities, as well as cognitive and contextual signals (Weiss and Sobel, 2012).

Besides olfactory and taste inputs, visual, thermal, texture, viscosity and even auditory sensations (e.g. crispiness, crunchiness) contribute to the overall perception of the quality of a given food. Further, visual information may provide clues (e.g. colour) about “flavour” (Zampini and Spence, 2012). Likewise, perceived crispiness offers subjective clues of “pleasantness” of the food (Zampini and Spence, 2004; Zampini and Spence, 2012).

Gustatory, olfactory and oral somatosensory information is relayed in the brain by distinct pathways which do not overlap with each other; ultimately, however, primary somatosensory cortex inputs synapse on higher-order cortical regions. The integration of experiences of “flavour” involving gustatory, olfactory and tactile pathways is thought to first converge in the anterior ventral insular cortex (as mentioned before) (Small, 2012); in this respect, the insula differs from the NTS and thalamic ventroposterior medial nucleus (Small, 2012). Functional magnetic resonance (fMRI) in humans revealed an overlap between taste (sucrose and monosodium glutamate) and odours (strawberry aroma and cooked chicken flavour) in the anterior ventral insula (de Araujo et al., 2003). The same authors also demonstrated a supra-additive response in the anterior

orbitofrontal cortex (OFC), suggesting that this area lies downstream of the insula (de Araujo et al., 2003). Similarly, studies in the monkey have identified multi-modal neurons that respond to multiple cues (e.g. visual–taste–odour neurons) in the OFC; these neurons are intermingled with unimodal sensory neurons (Rolls and Baylis, 1994). Other fMRI studies demonstrated that the OFC plays an important role in the evaluation of pleasant flavours and odours during human feeding (Gottfried et al., 2003; Kringelbach et al., 2003; Small et al., 2003; Small, 2012); in fact the OFC is activated by both, pleasant and aversive stimuli (O'Doherty et al., 2001), corresponding to its known role in decision-making and value attribution (Cai and Padoa-Schioppa, 2012; Rudebeck and Murray, 2014). In considering the overall complexity of the sensory control of feeding, it is interesting to note that a higher level of activation of the insular and orbitofrontal cortex predicts further eating in the fed state; in contrast, the hypothalamus shows higher activation in the hungry state (Batterham et al., 2007; Haase et al., 2009).

Learning about foods

Taste is the major determinant of pleasure derived from eating. Sweet and fat taste, especially their combinations, are innately and highly preferred by humans and animals, as discussed in section 1.1.1. In addition to caloric content, taste critically participates in satiety feelings. Thus, oral consumption of foods reduces appetite ratings more than naso-gastric infusion of nutrients, although gastric infusion of calories increases insulin and CCK and decreases ghrelin more than orally-ingested food (Spetter et al., 2014). In fact, the mere sensation of taste has been shown to decrease appetite (Smeets and Westerterp-Plantenga, 2006). This implies that oral consumption (flavour and probably mastication) favours behavioural satiety and thus is involved in learning about foods. The pleasure from flavour (influenced by the innate preference towards certain tastes), together with post-oral nutrient signals, are thought to form a positive association (flavour-nutrient association) with any cues co-occurring with the experience, e.g. smell, sight, sound, mouth feel, context (Mehiel and Bolles, 1988; Yeomans, 1998; Lundy, 2008; de Araujo, 2011; Zukerman et al., 2011; Wald and Myers, 2015). This process fits the description of classical conditioning (Pavlovian conditioning) in which the subsequent presentation of an unconditioned stimulus (e.g. food) with a conditioned stimulus (e.g. a tone) leads to the pairing of an initial unconditioned response (salivation) to the new conditioned stimulus (salivating after hearing the tone). This process is important for learning about foods and conditioned stimuli have been even shown to enhance feeding in satiated states (Weingarten, 1983; Cornell et al., 1989; Petrovich et al., 2007). The brain areas mediating the association between learned cues and feeding include the amygdala, hypothalamus and, ventromedial prefrontal cortex (vmPFC), with vmPFC projections to the LH being particularly important (Petrovich et al., 2002; Petrovich et al., 2007b).

In addition to learning about which flavours and contexts associate with nutrients, animals seem to "learn" to physiologically prepare themselves for the arrival of food by secreting hormones (e.g. ghrelin) in anticipation of a meal and, in addition, environmental cues or habit may help prediction of food arrival. Interestingly, some satiety signals such as GLP-1 and insulin are secreted in advance of meals (Begg and Woods, 2013). Conditioning experiments in rats have shown anticipatory secretion of insulin (Woods et al., 1977), a phenomenon called „cephalic secretion", because the process is triggered in the brain and is conveyed to the pancreas via vagal inputs a few minutes before food availability. In addition, the cephalic signals also stimulate the secretion of orexigenic ghrelin (Begg and Woods, 2013). Cephalic insulin release, which likely occurs in response to prior signalling by GLP-1, reduces the post-prandial increase of glucose and other nutrients, thus helping maintenance of normoglycemia (Teff, 2011). In summary, habit formation occurs at both, the psychological and endocrine levels.

Initiating food intake depends on many factors other than nutrient-associated cues. Feeding is strictly controlled by circadian mechanisms, with rodents eating most in the beginning of the daily dark phase (Silver et al., 2011). The suprachiasmatic nucleus (SCN) is the master endogenous circadian clock; its ablation results in loss of rhythmicity in most physiological and behavioural systems (reviewed in Sookoian et al., 2008). Surprisingly, the circadian oscillation in food anticipation (manifested as increased locomotor activity, 1-3 h before food presentation) is not altered after SCN ablation. Rather, feeding behaviour is thought to be controlled by food-entrainable oscillators (FEOs) which predict the availability of food. Food anticipatory behaviour continues over several days after acquisition, even if meals do not arrive, indicating the persistent effects of entrainment. FEOs also enable the anticipatory preprandial secretion of hormones (insulin, GLP-1, ghrelin). The anatomical substrate(s) of FEOs is/are unknown, but likely involve a distributed circuit which is activated when food-derived, learned and photic cues are synchronized with the animal's activity (Silver et al., 2011).

There is evidence from rodent studies that post-oral mechanisms play a more important role in developing long-term flavour preferences and controlling feeding than palatability (Sclafani, 2004; de Araujo et al., 2008; de Araujo, 2011) (discussed in Section 1.2.2). This is supported by the observation that obesity-prone rats seem to be more sensitive to post-oral nutrient reward than to taste *per se*, and hence form stronger preference for flavours paired with intra-gastric-delivered glucose compared to obesity-resistant rats (Wald and Myers, 2015). In addition, nutritional value is a better predictor of sweet preference than sweetness itself (measured by chorda tympani activation and consumption in lick tests) (Glendinning et al., 2010). On the other hand, rats readily consume corn oil mixtures even in the absence of postingestive signals (in sham feeding experiments) (Greenberg and Smith, 1996), indicating that flavour alone can drive ingestion. Naim and collagues conducted several pioneering experiments to find out whether adding preferred flavours to a normal diet would increase consumption

compared to taste-neutral diet. Interestingly, enhanced intake of the preferred flavours was observed only during the initial 5 days with no change in body weight. In contrast, feeding high-fat high-carbohydrate diets which, in addition to a variety of pleasant flavours also contain greater caloric density, results in increased food intake and body weight gain as compared to a nutritionally balanced diet (Naim et al., 1985). In fact, gastrointestinal signals of satiety (e.g. CCK) which are released in proportion to nutrient intake are implicated in conditioning flavour preferences in rats (Vanderweele et al., 1985; Perez and Sclafani, 1991). It is thus proposed that oral sensory mechanisms enable the rapid recognition of nutrients and promote acute consumption via affective mechanisms; on the other hand, while palatability *per se* is not sufficient for the formation of long-term food preferences, post-ingestive effects of nutrients are necessary for determining long-term consumption (de Araujo, 2011).

Dopamine (DA) is the strongest candidate for the neural signal that triggers motivated behaviour; therefore, DA also plays an important role in reinforcing food intake. Dopamine elevations in the nucleus accumbens (NAc) correlate with the intake of palatable food (Hernandez and Hoebel, 1988; Wilson et al., 1995; Small et al., 2003; Norgren et al., 2006). In addition, increased DA levels in NAc stimulate intake of sucrose solutions (Hajnal and Norgren, 2001). The NAc is part of the ventral striatum and belongs to the mesolimbic motivation and reward circuit that receives innervation from VTA (Wise, 2005). Palatable foods activate DA cell bodies in the VTA (Kosobud et al., 1994) and VTA integrity has been shown to be required for the consumption of normally-preferred sucrose solutions (Shimura et al., 2002). Additionally, optogenetic activation of DA receptor 1 neurons in mPFC (another projection area of VTA) stimulates palatable food intake (Land et al., 2014).

In the 1950s, Olds and Milner discovered that animals would press a lever to receive an electrical stimulation in the medial forebrain bundle that connects the VTA with the basal forebrain (includes NAc) (Olds and Milner, 1954). Interestingly, stimulation of the medial forebrain bundle also initiates reward-seeking consummatory responses or provides reward to the instrumental acts that follow the behavioural responses (Wise, 2005). Injections of indirect DA receptor agonists (e.g. amphetamine, cocaine) or drugs that stimulate the DA system, enhance the rewarding effects of electrical stimulation (Wise, 1996). Indeed, both, the presence of palatable food in the mouth without entry into the stomach (sham feeding) and delivery of calorie-dense solutions directly into the stomach (by-passing oral sensory stimulation) elevate DA in NAc (Hajnal et al., 2004; Liang et al., 2006; de Araujo et al., 2008; Tellez et al., 2013a; Ferreira et al., 2012; Han et al., 2016). The DA surge in the VTA and NAc has been shown to meditate learning about foods, presumably by strengthening the associative link between flavour and post-ingestive effects (Di Chiara and Bassareo, 2007; Touzani et al., 2008; Adamantidis et al., 2011). Indeed, intake of the non-nutritive sweetener saccharine produces a DA response that is similar to that found after sucrose ingestion; however, when the taste of

saccharine was associated with a toxin, there was no DA response (Mark et al., 1991). Importantly, neither nutrient learning nor DA release in the NAc requires concomitant flavour sensing (Hajnal et al., 2004; Liang et al., 2006; de Araujo et al., 2008). It should be noted that the literature also includes contrasting views regarding the role of DA in reward, with some studies suggesting that increased DA release in the NAc is not necessary for rats to learn to recognise palatable food or to mediate predictive associations (Berridge and Robinson, 1998; Aberman and Salamone, 1999); this view was supported by the results of a recent study in which optogenetic stimulation of DA neurons in the VTA facilitated instrumental responses in a food seeking operant task, but did not increase the amount of food consumed (Adamantidis et al., 2011). In fact, other substances that do not directly influence DAergic transmission (μ and δ opiates, glutamate, nicotine, and cannabinoids) have actions that summate with rewarding electrical stimulation of the medial forebrain bundle (Wise, 1996), and may thus participate in reward learning.

The effect of gustatory input on DA release in the NAc appears to be mediated by PBN in rodents; lesions of the PBN-limbic pathway blunt DA release in response to palatable solutions, but do not markedly change sensitivity to different tastes (Hajnal and Norgren, 2005). Gustatory signals received in the PBN are relayed to limbic structures and targets (CeA, LH, BNST), all of which project to both, the VTA and NAc; these structures can potentially modulate DA release from the VTA or alternatively, the BNST and CeA may engage neurons in the NAc to modulate dopamine (DA) signalling and motivate eating behaviour (Norgren et al., 2006; Grill and Hayes, 2012).

An interesting question concerns what drives post-ingestive signals to stimulate DA release (and therefore, to stimulate food consumption). Current evidence favours the theory of postabsorptive effects in establishing reinforcement of certain food choices (de Araujo et al., 2008). Leptin and insulin receptors have been detected on DA neurons in the substantia nigra and VTA, where they act to decrease the DA response to food intake (Figlewicz et al., 2003; Figlewicz et al., 2004; Hommel et al., 2006a; Morton et al., 2009; Liu et al., 2013); these peripheral hormones could be the link between postingestive state and DA release. Phosphorylation signals generated by oxidative processes in the liver also have flavour-conditioning potential (de Araujo et al., 2008). The fast DA response in the NAc, however, cannot be easily explained by post-absorptive satiety factors (e.g. leptin) because these are not rapidly released in response to food consumption. The possibility that DA neurons in the VTA act as nutrient sensors (e.g. sensitive to glucose) would seem to be a more likely (fast) mechanism (de Araujo, 2011). To date, no glucose sensors have been found in the VTA, but glucosensing areas such as the hypothalamus and hindbrain innervate the VTA (Steinbusch et al., 2015) and could thus influence VTA activation. Interestingly, the possibility that post-absorptive feeding signals can affect DA release in NAc warrants testing of the hypothesis that, as all the mentioned post-ingestive signals function to inhibit feeding, the DA increase in the NAc

may also serve to decrease food intake, independently of its food-seeking motivational function. Supporting this, Fulton *et al* showed that the lower dopamine tone in the VTA of obese mice (*ob/ob*) can be reversed by leptin infusions (Fulton et al., 2006). Although these results are not conclusive as a developmental KO model was used and the results diverge from acute effects of leptin (Hommel et al., 2006a), additionally, the profile of *ob/ob* may simply reflect long-term leptin insensitivity. On the other hand, the hypothesis that dopamine might also have satiating effects is supported by the observation that another satiety signal, PYY, also increases dopamine levels in NAc (Stadlbauer et al., 2014).

Kent Berridge has proposed that taste-driven hedonia is reflected in the orofacial "liking" reactions of human infants, primates and rats (Figure 1.7) (Berridge, 2000); these "liking" responses are elicited faster than any gastric signals can be generated. The "liking" response is not only evoked by innate taste reactions, but also by previously-formed Pavlovian type associations, and will depend on the physiological state of the animal. In fact, different taste sensations (e.g. sucrose or sodium chloride solution) can trigger similar facial "liking" reactions in rats, depending on the state of the animal; the same tastant can, on the other hand, elicit different responses, depending on the physiological needs (e.g. sodium depletion); this suggests that "liking" expressions are not just sensory reflexes (Steiner et al., 2001; Castro et al., 2015). When rats are conditioned to associate sweet taste with nausea, they change their facial "liking" reaction to a "disgust" expression (Spector et al., 1988). Berridge and colleagues argue that many tests that attempt to estimate palatable food intake (working for food, voluntary intake or appetitive behaviour) rely upon the mechanisms of incentive motivation, in addition to hedonic liking. In many tests, a divergence has been recorded between "liking" reactions in taste reactivity tests that do not depend on dopamine and intake/lickometer/choice measures that rely on DA; all of these can be attenuated or abrogated after dopamine depletion or DA receptor blockade. Responses that depend on incentive motivation are referred to as "wanting" of food reward (Castro et al., 2015).

The basic orofacial reactions to food are generated by the brainstem circuitry of taste processing (Grill and Norgren, 1978). In contrast, hedonic responses involve psychological and physiological factors and thus require forebrain control. Manipulations in forebrain areas such as the NAc, ventral pallidum (VP) and cortex can strongly increase or decrease "liking" reactions to palatable foods (Castro et al., 2015). The "hedonic hotspots" or brain areas that generate "liking" expressions and increase of palatable food intake were identified by injecting μ-opioid agonists (e.g. morphine) into candidate regions (Figure 1.7) (Berridge and Kringelbach, 2013). Morphine injections into the shell of the NAc of rats were shown to trigger food intake and to increase affective "liking" reactions, while not changing the aversive response to quinine (Pecina and Berridge, 2000). It is important to note that these opioid effects might be mediated

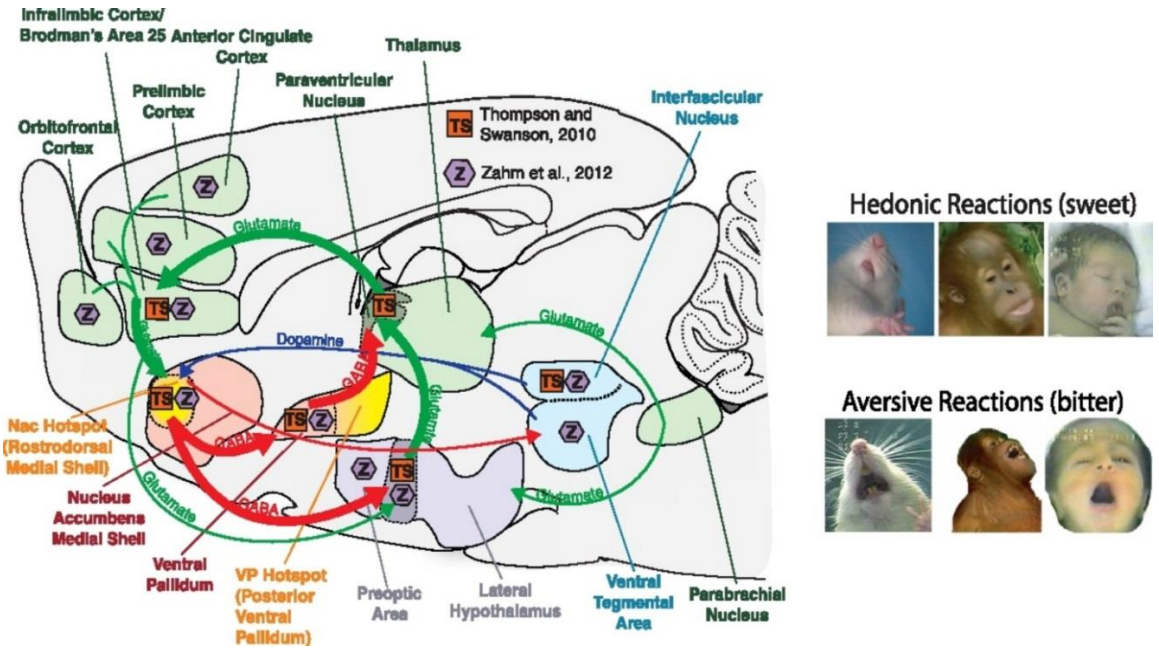


Figure 1.7. Hedonic hotspots and anatomical circuits that distinguish the nucleus accumbens hotspot in rostrodorsal medial shell as a unique site (anatomy based on Thompson and Swanson (TS symbol in orange boxes; 2010) and on Zahm and colleagues (Z symbol in purple hexagons; 2012). Thompson and Swanson (Thompson and Swanson, 2010) reported that the nucleus accumbens hotspot of rostrodorsal medial shell is uniquely embedded in its own closed-circuit corticolimbicpallidal-thalamocortical loop, connecting discrete input subregions and output subregions, and segregated from other parallel loops passing through other regions of medial shell. Zahm and colleagues suggested additional unique connections for the rostrodorsal hotspot (Zahm et al., 2013). GABAergic projections are indicated in red, hedonic hotspots are marked in yellow, glutamatergic projections are green, and dopaminergic projections are marked in blue. Figure by Daniel Castro, modified from Richard et al., 2013 (Berridge and Kringelbach, 2013).

by other transmitters since GABA-A and GABA-B receptor agonists and antagonists of AMPA and kainate glutamate receptors are, like opiates, much more potent stimulators of food intake when injected into the NAc shell *vs.* the NAc core (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1997). Upon more detailed investigation of the NAc "hedonic hotspot" in the rat, using microinjections of DAMGO, a synthetic enkephalin analog that selectively activates μ-opioid receptors, it emerged that hedonic responses are elicited from just a 1 mm^3 site within the rostrodorsal quadrant of the medial NAc shell; in contrast, μ-opioid stimulation of the caudal half of the NAc shell reduced hedonic reactions. Notably, DAMGO injections to any site in the NAc shell increased food intake ("wanting" response) (Pecina and Berridge, 2005; Castro and Berridge, 2014).

A second opioid-responding "hedonic hotspot" (around 0.8 mm^3) was found in the posterior VP; lesions or muscimol (GABA-A receptor agonist) injections to this area induced intense disgust reactions. Opposite to what was found for the NAc, opioid infusions into the anterior VP decreased and injections to the caudal VP increased liking reactions (Smith and Berridge, 2005; Ho and Berridge, 2014). Interestingly, orofacial "liking" expressions could also be elicited by injections of orexin-A into the caudal VP "hedonic hotspot" (Ho and Berridge, 2013), suggesting that this hotspot may allow hunger signals form the LH to enhance sensations of palatability to promote eating.

1.3.3 The apparent conflict between hedonic and homeostatic mechansims

The homeostatic control of food intake (see Section 1.2) is strongly influenced by so-called non-homeostatic factors, including habits, learned preferences (see 1.3.2), stress, social influences, exercise and hedonic modulators. This thesis focuses on the interplay between peripheral homeostatic and the energy/sensory signals derived from food rich in fat and carbohydrate content. There are a number of plausible mechanisms through which homeostatic signals can be overridden and lead to the development of obesity.

The body weight "set-point" theory addresses how an individual's weight is maintained within a relatively narrow range throughout adulthood. It proposes that a deviation from an individual's set-point will trigger a compensatory physiological response. However, evidence suggests that this set-point can be re-set upwards without difficulty (often referred to as a "settling-point") (Wirtshafter and Davis, 1977; Levin, 2005; Levitsky, 2005; Tam et al., 2009). Further, it is thought that body weight is regulated in an asymmetric manner, responding more strongly to weight loss than to weight gain (Müller et al., 2010). The latter is exemplified by the fact that intervention-induced weight loss in people is difficult to maintain because of a tendency to return to their original or "defended" body weight (Svetkey et al., 2008); this phenomenon is referred to as the "yo-yo effect".

Leptin is thought to be a key feedback signal that helps regulate adiposity (see 1.2.3) and interestingly, it exerts biphasic effects (Müller et al., 2010): while leptin is best known for its ability to decrease food intake and increase energy expenditure, low leptin levels trigger feeding and energy conservation. Indeed, the correlation between leptin levels and energy expenditure is only apparent when leptin levels are low, leading to the suggestion that the high leptin levels seen during weight gain are ineffective in eliciting appropriate metabolic adaptations (Haas et al., 2010). It can be extrapolated from this that low levels of leptin that serve as a proxy of an energy deficit, serve a more prominent role than high levels of leptin. Tam et al. (2009) incorporated this interpretation, together with the theory of increasing body weight "settling points" into a mathematical model in an attempt to explain how diet-induced obesity develops. Their model was consistent with the theory that mechanisms evolved to *promote, rather than suppress,* feeding, given that free-range animals (and ancestral humans) do not have readily-available (and predictable) sources of energy-dense foods (see 1.1.1). Thus, the body weight set-point is only maintained during fasting or under conditions in which balanced diets are available (but not when there are abundant supplies of palatable and calorie-rich food available for consumption).

The mechanisms through which body weight set-points are upwardly adjusted remain unknown. Importantly, however, the body weight rheostat can be re-set: diet-induced

obesity can be gradually reversed when animals are returned to a balanced (standard) diet (Parekh et al., 1998) and when humans are subjected to caloric restriction (Svetkey et al., 2008). As previously suggested by Wirtschafter and Davis in 1977, the sensory inputs (high palatability) of foods available to humans appear to play a more dominant role in the regulation of eating than initial body weight set-points or fat mass-signalling molecules (Wirtshafter and Davis, 1977). Taking an evolutionary perspective, Berridge and Kringelbach proposed that hedonic (pleasurable) processes facilitate adaptation and survival (Berridge and Kringelbach, 2013). In other words, they suggested that hedonia must have an evolutionary advantage; in particular, they noted that the neural substrates and processes that mediate the rewarding effects of food (and other stimuli) are highly conserved across mammalian species.

Calorific content may also contribute to hedonic sensations. In fact, both taste and post-oral nutrients induce the release of dopamine (DA) in the nucleus accumbens (NAc); moreover, the brain areas related to pleasure and motivation to obtain food overlap to some extent (see 1.3.2). Thus, hedonia may be viewed as a drive to execute an evolutionarily beneficial behaviour (cf. reproduction is driven by the pleasure from coitus). Both, oronasal sensations ("liking") and post-oral nutrient signals reinforce eating and enhance the motivation to obtain the food ("wanting"), and eventually reward-related learning (Berridge and Kringelbach, 2013). In addition to studies suggesting that post-ingestive reward signals might be prominent in the obese state (see section 1.3.2, Wald and Myers, 2015), there are reports that obese humans are more sensitive to oral hedonic stimuli (e.g. the taste of fat) (Greenberg and Smith, 1996), with some stating that obese people 'live in different orosensory and orohedonic worlds' as compared to the non-obese, i.e. that obese subjects assign greater value to tasteful rewards than to other hedonic experiences (Bartoshuk et al., 2006). The possibility that overweight subjects are less sensitive to the satiating effects of a pleasant taste or become habituated to it more slowly, and thus inadvertently consume more calories, should also be considered (Epstein et al., 2008; Pepino and Mennella, 2012).

Other interesting findings relate to the greater activation of reward-processing areas in the brains of obese *vs.* lean subjects after tasting or, even just viewing pictures of palatable food (DelParigi et al., 2004; Rothemund et al., 2007; Stoeckel et al., 2008; Stoeckel et al., 2009; Rudenga et al., 2013) (albeit with some contradictory findings) (Stice et al., 2008). Another important observation was that of lower dopamine receptor 2 (D2R) binding in obese (vs. lean) individuals; this led to the introduction of the reward-deficiency hypothesis of obesity, namely that obese subjects overeat to "self-medicate" or compensate for reduced activation of reward circuits (Wang et al., 2001). Similarity of this response to the neurochemical correlates of drug addiction (Volkow et al., 2008; Gipson et al., 2013) have since led to the popular concept of "food addiction". Recently this view has been revised by some authors to state that one cannot become addicted to a specific type of food (e.g. fat or sugar) but can rather be addicted to eating (Hebebrand

et al., 2014). Here it should be mentioned that, although the DA theory of addiction has gained strength over the years, one main criticism against it is that association does not prove causation. Secondly, although DA release in the NAc is involved in motivational processes and is related to certain features of drug abuse, it participates in many processes beyond reward; for example, DA mediates appetitive as well as aversive motivational processes, behavioural activation, exertion of effort to gain a reward, task engagement etc. (Salamone and Correa, 2012). In addition, although stimulants acutely increase DA levels, similar DA profiles are not found in subjects that are dependent on (addicted to) amphetamine, alcohol, cannabinoids, opiates, or nicotine (Nutt et al., 2015). Lastly, the synaptic changes that are linked to cocaine addiction (rapid transient increase in dendritic spine size and synaptic strength in NAc) are not seen during cue-induced sucrose seeking (Gipson et al., 2013).

Some authors have suggested that obese people eat more because of increased "wanting" rather than "liking", and that "wanting" stems from the post-ingestive effects of eating, rather than oronasal stimulation (Berridge, 2004; Mela, 2006). Others have related over-eating to habit since animals trained to "work" to obtain food (in operant conditioning paradigms) will persist with this instrumental behaviour even if food is freely available and also, in the absence of food (Jensen, 1963; Morgan, 1974). This compulsive behaviour depends on striatal regions, that mediate habitual responses, and on decreased activation of prefrontal areas; importantly, these habitual aspects of eating behaviour do have similarities to those seen in compulsive drug seeking (Everitt and Robbins, 2005).

The lateral hypothalamus (LH) is an important hub where peripheral and central signals converge to generate a behavioural feeding response (Saper et al., 1979; Kelley et al., 2005; Hussain and Bloom, 2013) (Figure 1.8, Berthoud and Münzberg, 2011). The LH influences both feeding and reward. Lesion studies by Anand and Brobeck showed that the LH is crucial for appetite and body weight regulation (see 1.3.1), a finding confirmed by the demonstrations that electrical stimulation of the LH increases feeding and drinking (Delgado and Anand, 1953; Mogenson and Stevenson, 1967), and that LH neurons fire during eating and drinking (Burton et al., 1976; Schwartzbaum, 1988 Tabuchi et al., 2002). Moreover, Olds and co-workers demonstrated that animals robustly press levers to receive electrical stimulation of the LH region; these self-stimulation sites overlap with those that elicit feeding (Olds, 1958) and, interestingly, LH self-stimulation (LHSS) is dependent upon nutritional/energy status (Margules and Olds, 1962; Blundell and Herberg, 1973). Hypoglycemia and decreased insulin levels increase LHSS, whereas glucagon (which increases blood glucose) reduces LHSS. Indeed, neurons in LH respond to blood glucose levels, supporting the hypothesis that the LH is a glucose-sensing region (Oomura et al., 1969; Steinbusch et al., 2015). Notably, however, NPY which responds to ghrelin to stimulate feeding, does not modulate LHSS when injected into the LH (Fulton et al., 2002). Finally, leptin, like insulin, attenuates LHSS after food

restriction (Carr et al., 2000; Fulton et al., 2000), suggesting that metabolic need drives reward-related behaviours, including the motivation to eat.

Neurons in the LH respond to multimodal sensory inputs (Norgren, 1970, 1976), reward-associated cues (Saper et al., 1979; Nakamura et al., 1987), and peripheral metabolic signals (Hoebel and Teitelbaum, 1962; Berthoud and Münzberg, 2011) (Figure 1.8). Interestingly, and contrary to the view that the LH enhances positive hedonic reactions to taste, stimulation of the LH does not affect orofacial "liking" reactions; rather, it appears that the LH modulates the assignment of salience to rewarding stimuli and

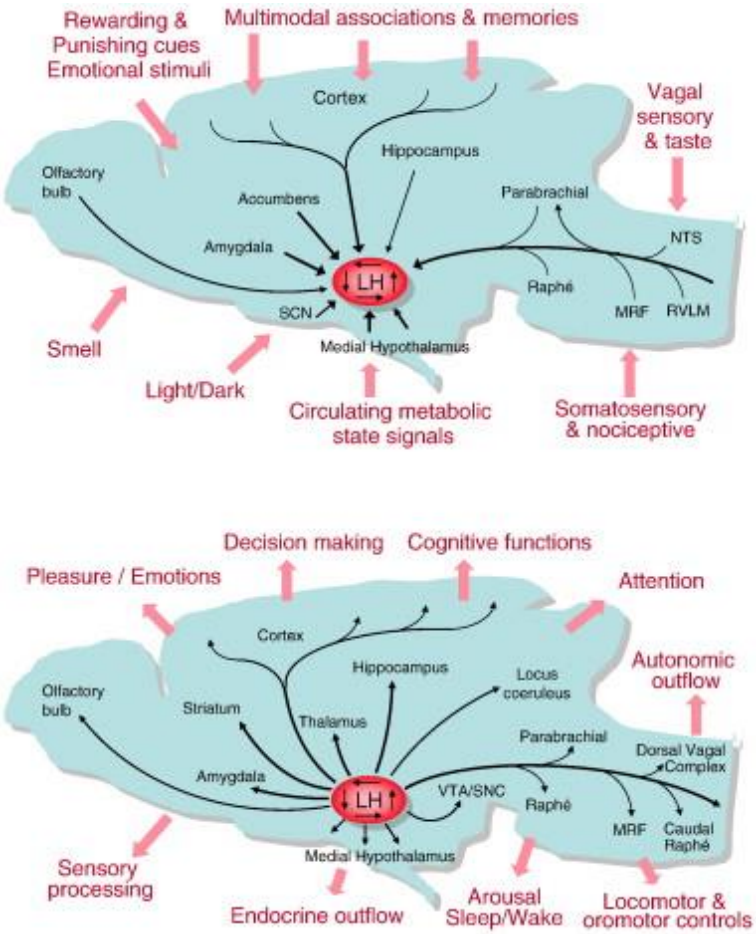


Figure 1.8. Connectivity of the lateral hypothalamic area (LH). The LH receives inputs (*top*) from both peripheral and central areas involved in the processing of feeding related information. Outputs from the LH are sent to many cortical and brainstem areas (*bottom*). Figure from Berthoud and Münzberg, 2011.

executes feeding behaviour, depending on the internal and external environments (Berridge and Valenstein, 1991). This interpretation is supported by the observations that LH lesions block the preference for (strong) salty solutions when animals are sodium-depleted (Wolf and Quartermain, 1967), and that consumption of sucrose solutions is increased by both, LHSS and stimulus-coupled drinking (Norgren, 1970). As

shown in Fig. 1.8, the LH receives information from multiple areas sensitive to the organism's internal and external milieu and also from taste and other sensory processing and integratory areas. The LH projects to, and receives reciprocal inputs from, both the VTA and NAc, and thus participates in the execution of eating behaviour. In addition, the LH has reciprocal connections with emotional and cognitive centres (amygdala, hippocampus), motivational and motor areas (VTA, substantia nigra, ventral pallidum), and areas known to integrate and regulate central and peripheral signals regarding satiety and energy status (parabrachial nucleus, Raphe nucleus and nucleus tractus solitarius) (Simon et al., 1979; Barone et al., 1981; Berthoud and Münzberg, 2011; Watabe-Uchida et al., 2012; Hurley and Johnson, 2014).

Only a few studies have addressed the question of how hedonic signals interfere with the homeostatic control of food intake; most of these were done in subjects that were already obese and are thus not informative about events in the early phases of altered eating behaviour. Among such studies are those done in overweight subjects with insulin and leptin resistance (Myers et al., 2010; Morton et al., 2014) or in leptin deficient (and obese) humans receiving leptin replacement therapy (Farooqi et al., 2007). Using functional magnetic resonance (fMRI), the latter study showed altered activity in the ventral striatum together with decreased food intake and reduced valuation of food images after leptin therapy. Some attention has been paid to orexigenic signals such as ghrelin in the context of acute overeating and reward-related hedonic feeding (Perelló and Zigman, 2012). The pro-feeding effects of ghrelin become more pronounced when a palatable reward is presented in free-choice and operant conditioning paradigms (Egecioglu et al., 2010; Skibicka et al., 2012).

Hedonic effects of energy-dense food may be mediated via interactions between opioid signalling in the NAc (increased by palatable food via unknown mechanisms) and hypothalamic orexinergic projections to the VTA (Zheng et al., 2007). In addition, the satiety hormone leptin was shown to suppress orexin neuron activation as well as decrease LHSS after food restriction (Fulton et al., 2000; Yamanaka et al., 2003). Leptin is found to be a potent anorexigenic factor even when highly palatable and energy-dense foods are present (Figlewicz et al., 2001; Figlewicz et al., 2004; Domingos et al., 2011). On the other hand, *in vitro* studies indicated that saturated fatty acids disrupt leptin signalling (STAT3 phosphorylation) (Kleinridders et al., 2009), therefore pathways lying downstream of leptin signalling could be a "victim" of hedonic signalling.

While the main goal of the present work was to understand the initiation (pathways and mechanisms) of hedonic overdrive of palatable food consumption, the lack of an adequate background literature to this complex (and highly dynamic) phenomenon necessitated the use of simple test paradigms. Thus, the work described here only focused on a few selected homeostatic factors (ghrelin, leptin) and did not attempt to

distinguish between the different components of hedonic affect (liking, wanting, learning).

1.4. Aims of this thesis

- To establish paradigms (*Chapter 2*) that facilitate understanding of how extraneous food cues override physiological satiety signals that result in development of food preferences and overeating (*Chapter 2 and 3*)

- To identify brain areas involved in hedonic eating (*Chapter 3*)

- To explore the circuits and molecules that allow food preferences to prevail over physiological signals of satiety (*Chapter 3*)

- To test pharmacological reinforcment of physiological signals of satiety as a means to block hedonia-driven eating processes (*Chapter 4*)

CHAPTER 2

FOOD CHOICE UNDER DIFFERENT PHYSIOLOGICAL STATES

Abstract

Obesity endangers health and is a socio-economic burden. Obesity occurs when energy intake exceeds expenditure; this imbalance may result from sedentary life-style or overeating, in particular palatable, energy-rich foods. Overeating most likely occurs from an overriding of satiety signals by components of food that confer hedonic value. To mimic this situation we exposed mice to a choice paradigm consisting of foods with different nutrient and taste compositions (high-fat/high-carbohydrate, low-fat/high-carbohydrate and standard food) under different physiological and pharmacologically induced satiety states. We observed that mice strongly preferred high-fat/high-carbohydrate food to other diet options even in a state of satiety. This palatable food (PF) option was thereafter used as a hedonically-weighted food; consumption of PF was compared to standard chow (SC) intake in a control condition (absence of PF option) to examine how hedonic stimuli modulate homeostatic feeding. Injection of leptin (3 or 5 mg/kg), a potent anorexigenic hormone, to fasted mice did not suppress PF intake but decreased SC intake in a control condition. It is thus proposed that the hedonic properties of PF might override the effects of leptin. This interpretation is supported by our observation that injection of the orexigenic peptide ghrelin (0.3 mg/kg) in satiated animals also increases the ingestion of PF relative to SC. In addition, prolonged exposure of mice to PF (6 days) results in decreased daily PF consumption, albeit without any negative impact on body mass gain; thus, even just an acute exposure to PF can alter the ratio between energy intake, utilization and storage, and might be important for initiating the development of obesity.

Highlights

- High fat (HF)-containing foods are highly preferred over standard chow (SC) and low-fat (LF)-containing foods and HF food is accordingly designated as a highly palatable food (PF). Importantly, SC and LF are completely neglected in food choice paradigms in which a PF is included.
- Exogenous ghrelin and leptin do not exert their normal physiological regulatory effects on PF consumption in choice paradigms. Ghrelin potentiates PF (but not SC) eating even when animals are in a state of satiation and the feeding-restraining actions of leptin are abolished when PF is available.
- The “hedonic” properties of PF appear to succumb to endogenous satiety signals over time; nevertheless, the body weight of mice exposed to PF for 6 days continues to rise despite a reduction in caloric intake.

2.1 Introduction

The question of what determines food choice and, in particular how preferences for specific food types are initiated and develop, is complex; this is because a variety of factors, including sensory inputs, post-ingestive value, reward signals, habits and memories contribute to the regulation of feeding behaviour (see Sections 1.2.3, 1.3.2). Based on the „hedonia hypothesis of over-eating", the present work considered the initial sensory and post-oral signals to be the primary drivers of reward learning and dysregulated eating. In the long-term, such behavioural modifications can be expected to be accompanied by compensatory physiological and metabolic adaptations that will promote excessive eating and increased body weight.

Here, a simple food choice paradigm was established to help estimate how foods with different nutrient composition, caloric value and taste/smell/texture (comparison of bland, standard chow [SC] with iso-caloric low-fat [LF] and high-fat food [HF] that differ in taste/smell/texture) are consumed during states of natural or pharmacologically-induced hunger and satiety. The pharmacological manipulations were used in order to directly test the hypothesis that the *hedonic appeal of a food is enhanced by a combination of flavour/energy/texture/novelty, resulting in excessive consumption of a given food type due to interference with the homeostatic mechanisms that normally serve to restrain food intake* (Section 1.3.3). The effect of obesity on satiety peptides and feeding in rodents has been the subject of many studies (e.g. Cusi et al., 2000; Gastaldelli, 2008; Friedman, 2009; Myers et al., 2010; Morton et al., 2014). In contrast, there is a paucity of reports in humans, the most notable being ones that compared the processing of satiety signals in reward-related areas of the brains of lean and obese subjects (Del Parigi et al., 2002; Stoeckel et al., 2009; Rudenga et al., 2013). An extensive literature survey did not yield substantial reports on how physiological signals modulate food choices by lean individuals.

2.2 Materials and Methods

Animals

Experiments were performed on male C57BL/6 mice purchased at the age of 2 months (Charles River Laboratories, Sulzfeld, Germany); animals were used after at least 2 weeks of habituation to the animal facility of the Max Planck Institute of Psychiatry (Munich, Germany). Mice were single-housed in transparent type-2 polycarbonate cages (macrolone, 25.5 cm x 19.5 cm x 13.8 cm) under constant temperature (21 ± 2 °C) and humidity (50 ± 10%) and under a daily 12h light-dark cycle (lights on: 07:30, Zeitgeber time (ZT 0); lights off: 19:30 (ZT 12)). Food and water were available *ad libitum* throughout, unless specified otherwise. Animal experiments were approved

by the local commission for the Care and Use of Laboratory Animals of the State Government of Upper Bavaria and complied with the EU Directive 2010/63/*EU*.

Test foods

During acclimation and unless specified otherwise, animals were maintained on standard chow (SC, 1320, 2.84 kcal/g, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany). To evaluate food preference, mice were given simultaneous access to SC, high-fat food (HF, D12451, 4.73 kcal/g, ResearchDiets Inc., New Brunswick, NJ, USA) and low-fat food (LF, D12450B, 4.06 kcal/g, ResearchDiets Inc., New Brunswick, NJ, USA).

The HF and LF food had similar (high) caloric values. The HF and LF foods contained 45% kcal and 10% kcal, respectively, from fat (Table 2.1). Carbohydrate content of the LF food provided 70% of energy kcal. Note: although HF and SC provided similar kcal/g from carbohydrate, most of the energy in the HF food was provided by sucrose, whereas the carbohydrates in the SC food comprised mainly polysaccharides; as such, the sweetness of the three foods varied, with LF > HF > SC foods.

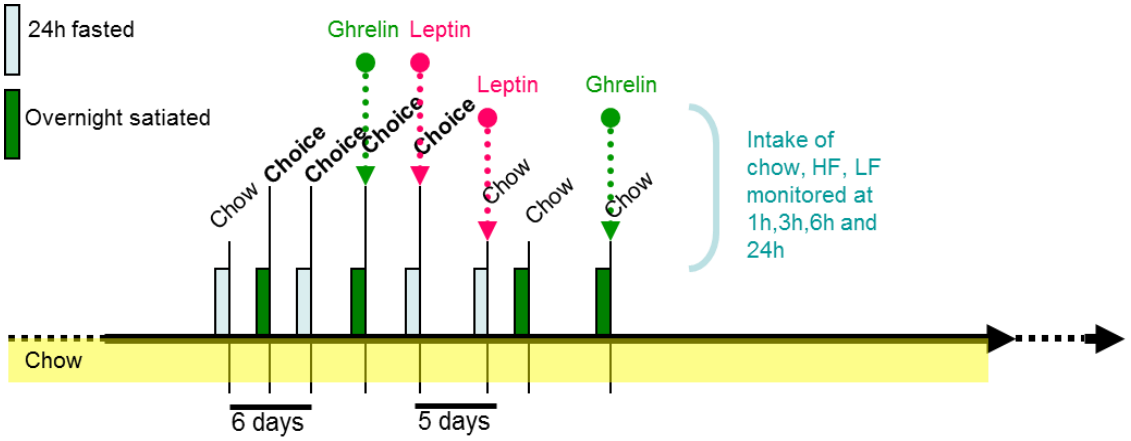
TABLE 2.1. Macronutrient composition of animal diets used

	% kCal from individual macronutrient		
	High-fat food (HF, D123451)	**Low-fat food (LF, D123450)**	**Standard chow (SC, 1320)**
Protein	20 %	20 %	24 %
Carbohydrate	35 % (17% sucrose)	70 % (35% from sucrose)	65 % (4.9% sucrose)
Fat	45 %	10 %	11 %
Total kcal/g	4.73	4.06	2.84

Experiment 1: Pilot study to establish a food preference test paradigm under different states of satiety

Mice (n = 18) used in the experiments were aged 2.5-3.5 months (body weight range: 24- 27 g). Animals were single-housed to allow monitoring of individual food intake. On 4 consecutive days, prior to actual testing, baseline body weight and food intake were recorded, and the animals were habituated to 0.9% saline intraperitoneal (i.p.) injections.

Food choices (standard chow, *SC*; low fat food, *LF*; high fat food, *HF*) and control food (*SC*) intake were studied in a sequential, non-randomized manner (Scheme 2.1). This design allowed within-subject comparison (each subject in every condition), thus decreasing the effects of inter-individual variability and increasing the statistical power of the study. On the other hand, possible carry-over effects from one condition to another could be a confound despite the appropriate interspersed „wash-out“ days. This initial experimental design was considered suitable for setting up the food choice paradigm; the test paradigm was further validated using a between-subject design (each subject in only one condition) in the next experiment (Section 2.1.2).

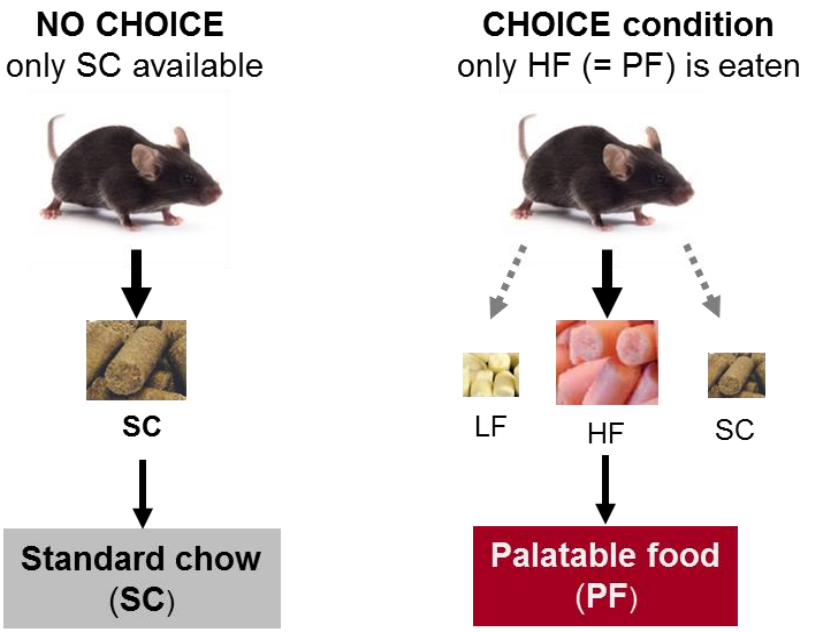


Scheme 2.1. Experimental design used in Experiment 1. Food intake during exposure to standard chow control condition (chow) and during exposure to a choice between high-fat (HF), low-fat (LF) and standard chow (SC) was measured on different test days over 24 h, following a 24 h fast (light-blue bar) or when animals were satiated (green bar). Ghrelin (0.3 mg/kg) was administered (i.p.) when mice were in a fed state; i.p. leptin (3 mg/kg) was administered after mice had been fasted.

Food preference was measured by simultaneously presenting mice with ad libitum HF, LF and SC (choice condition) for 24 h, starting at ZT 3. For comparison, animals received *ad libitum* SC for 24 h on a different occasion (control condition). Each exposure was separated by at least 2 days of "wash-out". Testing was done in animals that were either in the satiated or fasted (24 h fast) state; when fasting was involved, animals were allowed to recover for at least 5 days before being entered into a subsequent fasting period. In addition, on some test days, animals that were fasted, received an i.p. injection of leptin (3mg/kg; # LEP-6, Protein Laboratories Rehovot Ltd., Rehovot, Israel) 30 min before choice or SC presentation; on others, food preferences by animals that were in the fed state were presented with the food choices immediately after an i.p. injection of the hunger-signaling gut hormone ghrelin (0.3mg/kg octanylated rat ghrelin, # 1465, Tocris, Bristol, UK). Both, leptin and ghrelin were diluted in 0.9% saline; this vehicle was used in control experiments (injected immediately before presentation of the food choice or SC).

Consumption of the different foods (SC, HF and LF) was gravimetrically monitored at 1, 3, 6 and 24 h from the time of their presentation. We chose to monitor food consumption during the daily resting (light) phase since a main objective of this work was to investigate how palatable food interferes with the normal physiological regulation (e.g. circadian-entrained endogenous signals of hunger) of food intake.

Since the initial results from the above experimental design revealed that mice neglect SC and LF foods when HF food is also available (choice paradigm), the data obtained in the repeated measures food choice design in Scheme 2.1 was subsequently analysed to allow comparisons between HF consumption in the food choice paradigm and SC consumption in the absence of any other food option (Scheme 2.2); from this point on, HF food is considered to be the highly **palatable food (PF)** in the food choice paradigm.



Scheme 2.2. Shift in data analysis paradigm was used in all the figures starting from Figure 2.3. Results obtained in the choice paradigm, where HF was clearly the palatable food (PF) preferred over SC and LF food, are compared against SC consumption under a "no-choice" condition.

Measurements of food consumption at 1 and 24 h after ghrelin administration (repeated measures design) was computed as follows:

$$\% \text{ Food intake (FI) stimulated} = (100 - (FI_t \text{ saline}/FI_t \text{ ghrelin} * 100),$$
where t = 1 or 24 h

To evaluate the extent of leptin-induced suppression of food intake (repeated measures design), the following formula was used to calculate cumulative consumption at 1 and 24 h post-leptin injection

$$\% \text{ Suppression of food intake (FI)} = (100 - (FI_t \text{ leptin}/FI_t \text{ saline} * 100),$$
where t = 1 or 24 h

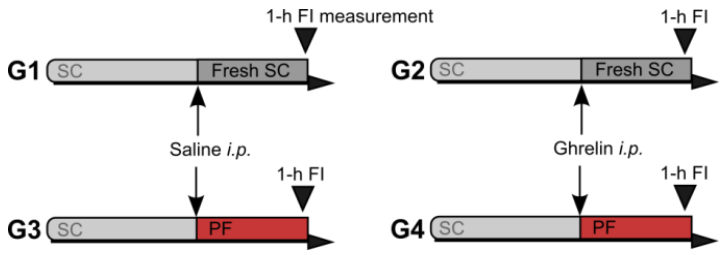
Experiment 2: Validation of pilot study findings, using an improved experimental design with reduced risk of confounds

Results obtained using the choice paradigm in Experiment 1 suggested that the paradigm can be a valuable approach for assessing the mechanisms underlying the excessive consumption of foods with a given set of properties (energy, sensory stimulation). Experiment 1 followed a non-randomized treatment/within-subject design; although potentially powerful, this design could lead to the results in successive treatments being subject to confounds from preceding treatments. Thus, Experiment 2 sought to validate the preliminary finding that mice overconsume PF compared to SC despite the presence of physiological control signals. Since animals ate minimal amounts of SC and no LF when PF was available in Experiment 1, the food choice consisted of only SC and PF. Food comparisons following administration of ghrelin (0.3

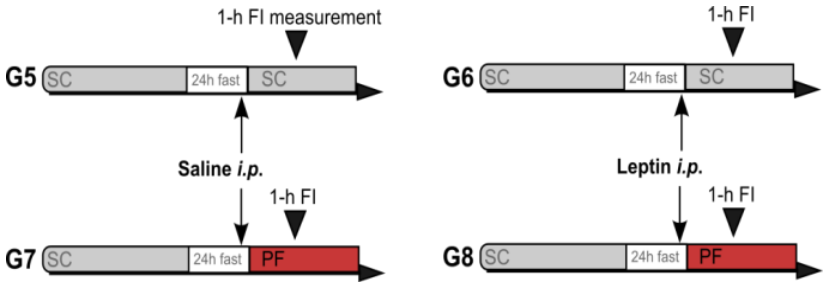
mg/kg) immediately before test food presentation (in a fed state), or leptin (5 mg/kg) 1 h before test food presentation (following a fast), were made. Specifically, a between-subjects design was

Scheme 2.3. Design used in Experiment 2

Satiety state	Pharmacological treatment (ZT 3)	Test food	n	Time of tests
Fed	Saline, 0.2 ml	SC	10	ZT 3
	Ghrelin, 0.3 mg/kg	SC	10	
	Saline, 0.2 ml	PF	10	
	Ghrelin, 0.3 mg/kg	PF	10	



Satiety state	Pharmacological treatment (ZT 16)	Test food	n	Time of tests
Fasted	Saline,0.2 ml	SC	10	ZT 15
	Leptin, 5 mg/kg	SC	10	
	Saline,0.2 ml	PF	10	
	Leptin, 5 mg/kg	PF	10	



used in which separate groups of animals were exposed to only one experimental treatment per group. Male mice (n = 80), aged 2.5-3.5 months and weighing 25-28 g were used. Animals were habituated to handling and injection procedures (0.2 ml saline i.p), and their BW and food intake (FI) were measured daily for 4 days before the experiment; based on their BW and FI, mice were assigned to one of each of the treatment groups shown in Scheme 2.3.

In light of results presented under Experiment 1 (most significant drug-induced changes in eating occurred within the first hour), food (SC and PF) consumption was monitored at a single time-point (1 h after test-food presentation). Note that a slightly higher dose of leptin (5 mg/kg) was used here, as compared to that used in experiment 1 (3 mg/kg), in an attempt to reduce inter-individual differences in response. In addition, in contrast to Experiment 1, leptin was injected 1 h prior to food presentation at the beginning of the dark-phase (leptin injection at ZT 14, food given at ZT 15). Dark-phase is the main activity and feeding period of mice, thus the effects of a satiety signal leptin were probed during the circadian phase in which animals are conditioned to

consume food actively. Food intake was measured at 1, 3, 6 and 24 h from the time of presentation.

Experiment 3: Does hedonic eating prevail over time?

In this experiment changes PF consumption were monitored during a week of *ad libitum* PF exposure. A between-subjects design with repeated measures was applied to male mice (n = 20), aged 2.5-3.5 months and weighing 25-28 g at the start of the experiments. Animals were habituated to handling and their BW and food intake (FI) were measured daily for 4 days before the experiment; based on their BW and FI, mice were assigned to two treatment groups: saline-SC, saline-PF (PF available for 6 d). Before PF and SC presentation at ZT 15 on day 1 and day 7, mice were fasted for 24h and injected with saline 1h before food presentation (to ensure that measurements are comparable to Experiments 1 and 3); food consumption and body weight were measured after 24 h.

Statistics

Data (in text given as mean ± SD; in figures shown as mean ± SD or ± SEM) were analyzed for statistical differences using Prism 6 software (GraphPad, San Diego, CA) after checking for normality. Student's *t*-test (normally distributed data) was used to compare data from two groups. To analyse more than two experimental groups two factor analysis of variance (2-ANOVA), followed by Sidak's or Tukey's multiple comparison (*post hoc*) tests, was used. The level of significance was set at $P \leq 0.05$.

Data in Experiment 2 were analyzed so as to exclude the possibility that leptin effects were masked by large differences in the calorific contents of PF and SC. Specifically, the magnitude of food intake suppression by leptin was estimated by z-scores (allows comparison of populations with different absolute values) using the following formula:

$$z = x - \text{Mean}_{\text{respective cotrol}} / \text{Standard deviation}_{\text{respective control}},$$

where x is an individual measurement

To this end, z scores for the SC-leptin-treated group injected was calculated by normalizing to data obtained in the SC-saline-treated group.

2.3 Results

High preference for HF food, irrespective of natural or pharmacologically-mimicked states of satiety (Experiment 1)

Using the experimental design depicted in Scheme 2.1, strong preference was found for HF food over LF and SC options under both, satiated and fasted states, as evident from the cumulative food intake profiles shown in Figure 2.1. Note that when HF was present, the intake of SC and LF was negligible, irrespective of satiety states or an iso-caloric food option (LF: high carbohydrate from sucrose and low fat content). Note that satiated mice consumed HF at almost the same rate (marginally lower) as fasted animals.

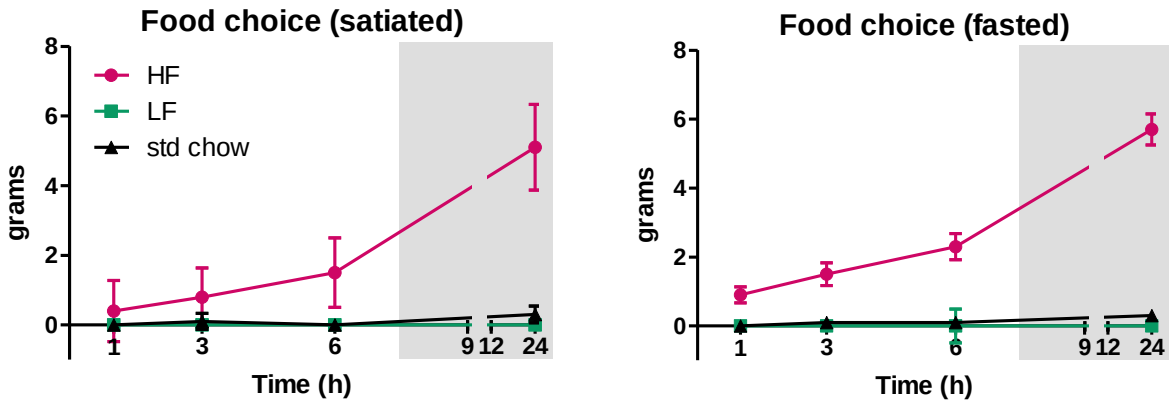


Figure 2.1. Food preference by mice in a choice paradigm in fed and fasted states. High-fat (HF) food is preferred over low-fat (LF) and standard chow in satiated (*left panel*) and fasted *(right panel*) conditions. All food types were presented simultaneously (choice) and were available *ad libitum*. Data are shown as mean ± SD (n = 18) for each time-point in this repeated-measures design experiment. The shaded area depicts the dark phase of the daily light-dark cycle.

In a subsequent step, we examined food type preference in animals receiving ghrelin in the fed state or leptin in the fasted state; this design allowed the study of these dominant hunger and satiety signals (respectively), thus avoiding the potentially confounding effects of other peptides/factors. Neither exogenous ghrelin nor leptin altered animals' overt preference for HF food over LF and SC (Figure 2.2). Also, neither hormone affected the relative amount of the different food types consumed. Comparison of Figures 2.1 and 2.2 reveals that the food preferences displayed under natural states of satiety or hunger (Figure 2.1), or after pharmacological mimicry of these physiological states, are superimposable.

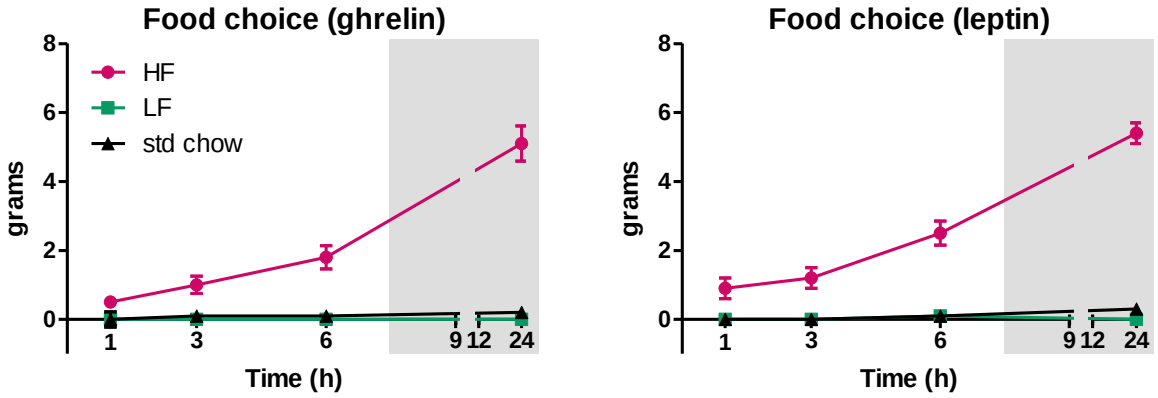


Figure 2.2. **Food preference in a choice paradigm after exogenous ghrelin or leptin administration**. Animals showed high preference for high-fat (HF) food (purple), consuming negligible amounts of low-fat (LF) food (green) and standard chow (black). All three food types were presented simultaneously. *Left panel* shows effects of exogenous (i.p.) ghrelin (0.3 mg/kg, immediately before provision of food-type choices); *right panel* depicts food choices made following exogenous (i.p.) leptin (3 mg/kg, 30 min before provision of food-type choices). Ghrelin and leptin were administered to animals in the fasted and satiated states, respectively. Values shown are mean ± SD (n = 18). The daily period of darkness is depicted by the grey-shaded bar.

Given the observation that mice neglect SC and LF foods when HF food *(henceforth referred to as palatable food, PF)* is also available (choice paradigm), we next sought to confirm that the greater palatability of HF food leads to its overconsumption. Demonstration of this was considered to be essential in light of the leading hypothesis that sensory and energy properties (i.e. hedonic properties) of a food cause an overriding of physiological controls (e.g. leptin) of feeding behaviour. To this end, only HF food intake (from now on PF) data from repeated measures food choice design was analysed (see Scheme 2.1) and compared to SC consumption in a no-choice condition (shift in analysis approach presented on Scheme 2.2).

Under conditions of satiety, the 24 h caloric intake of PF (0.9 ± SD 10.14 kcal/g BW) exceeded that of SC (0.46 ± SD 0.07 kcal/g BW) when mice were consuming either PF (in choice condition) or given only SC (no-choice paradigm) (Figure 2.3). The same pattern was observed when animals were fasted before presentation with either PF or SC (PF: 1.12 ± SD 0.11; SC: 0.7 ± SD 0.9 kcal/g). Thus, PF is over-consumed in comparison to SC, even when physiological signals of satiety are present. Two-way ANOVA (repeated measures) over 24 h revealed significant main effects of food type ($F_{1,17}$ = 251.9, P < 0.0001) and satiety state ($F_{1,17}$ = 392.8, P < 0.0001); the two factors did not interact significantly ($F_{1,17}$ = 0.0847, P = 0.77) but there were overall significant differences between all conditions (Sidak's multiple comparison test: P < 0.05).

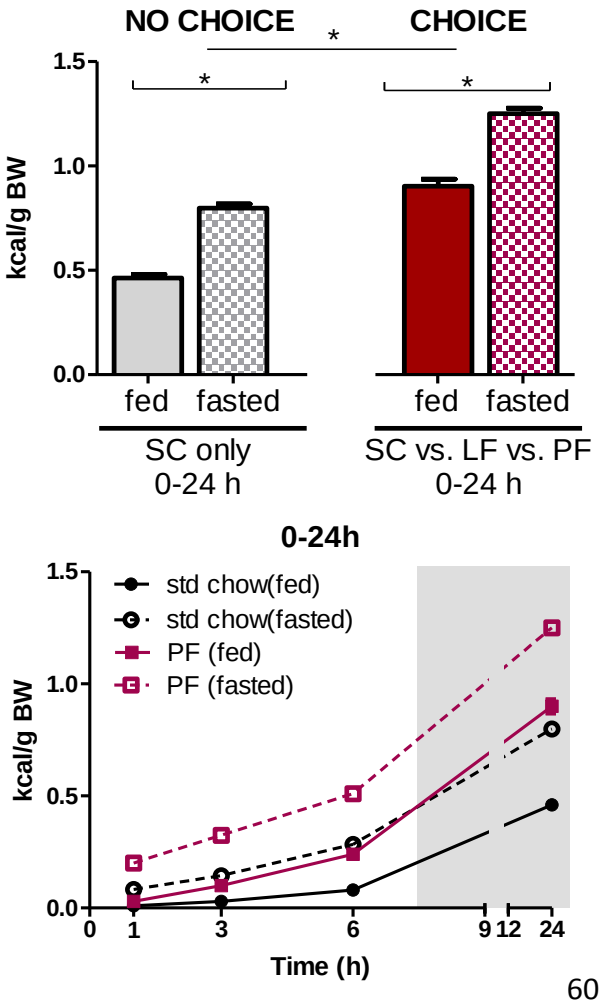


Figure 2.3. Energy (kcal) acquired per unit body weight (BW) from palatable food (PF) exceeds that obtained from standard chow (SC) in both, satiated and fasted states. Food/energy intake was monitored over 24 h (*upper panel*) but also at intervals (1, 3 and 6 h) after presentation of SC or PF (*lower panel*). Multiple comparison tests showed that the factors food type and satiety/hunger state contributed significantly to the overall result. Data are depicted as mean ± SEM (n = 18). * denote P ± 0.05

The PF intake data from when exogenous ghrelin is administered in the fed state (already shown in Figure 2.2) was next compared to SC consumption from the separate control condition (analysis as indicated in Scheme 2.2). PF consumption in "choice" paradigm in the fed state or after ghrelin injection was compared to SC intake ("chow" condition in experimental design shown in Scheme 2.1 in the fed state and after ghrelin administration (Figure 2.4).

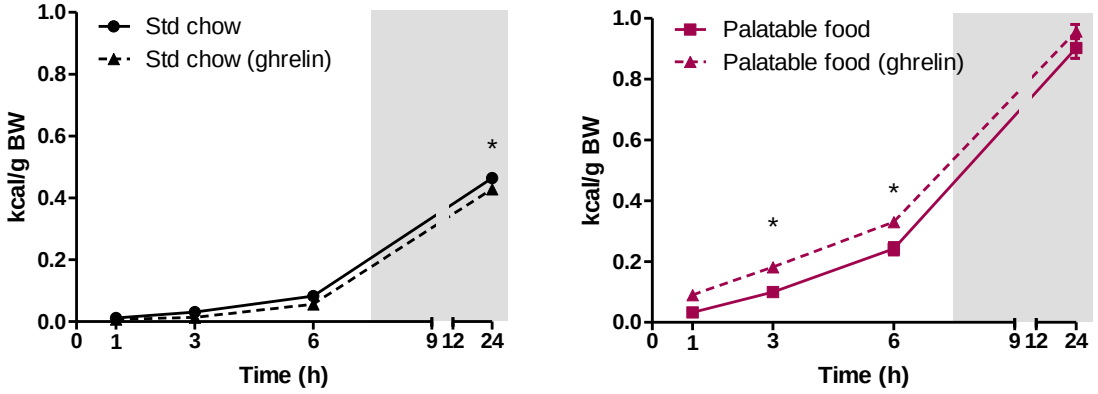


Figure 2.4. **Exogenous ghrelin stimulates PF, but not SC, consumption over a 24 h period.** Ghrelin (0.3 mg/kg, i.p). was administered to satiated mice. Ghrelin had its most pronounced effect on PF ingestion at 3 and 6 h (cumulative) (*right panel*). Data are depicted as mean ± SEM (n = 18). * denote $P \leq 0.05$. The shaded areas represent the dark phase of the diurnal cycle of illumination.

A 2-way ANOVA of PF consumption revealed a main effect of time ($F_{3, 68} = 790.6$, $P < 0.0001$) and of treatment (ghrelin, $F_{1, 68} = 34.42$, $P < 0.0001$), without any between-factor interactions ($F_{3, 68} = 0.4957$, $P = 0.6865$). Sidak's post-hoc analysis revealed that ghrelin significantly increased PF intake at 3 and 6 h ($P < 0.05$). Main effect of time ($F_{3, 68} = 1083$, $P < 0.0001$) and ghrelin ($F_{1, 68} = 16.03$, $P = 0.0002$) were detected when SC consumption was analysed by 2-way ANOVA, but other than for the measurement at 24 h ($P < 0.05$), no between-group differences were detectable by post-hoc tests; the former difference was due to the unexpected decreased in food intake observed at 24 h after exposure to SC.

Active ghrelin (octanylated ghrelin) has a half-life of 30 -105 min in mice (Dornonville de la Cour, C et al., 2005; Hillman et al., 2011). Thus, measurements of the "cumulative" effects of a single injection most likely reflect subsequent physiological and behavioural adaptations, not the actions of the peptide itself, thus the most informative measurement of the effects of ghrelin might be seen at the 1 h time-point. The response of individual animals to ghrelin in the repeated measures design used here was computed as percentage increased food intake relative to individual intakes of SC (baseline) as described in *Materials and Method (Experiment 1).* The results (Figure 2.5) were subsequently analysed using Wilcoxon matched-pairs signed rank (non-parametric) test. During the first hour, ghrelin-stimulated PF intake was significantly greater than

that of SC intake (PF: 64.8 ± SD 49.4; SC: 17.9 ± SD 53.1; W = -88, P < 0.05). Over 24 h, ghrelin did not have significantly different effects on the ingestion of PF (5 ± SD 17.9) *vs.* SC (-2.9 ± SD 32.7) (W = -77, P = 0.10).

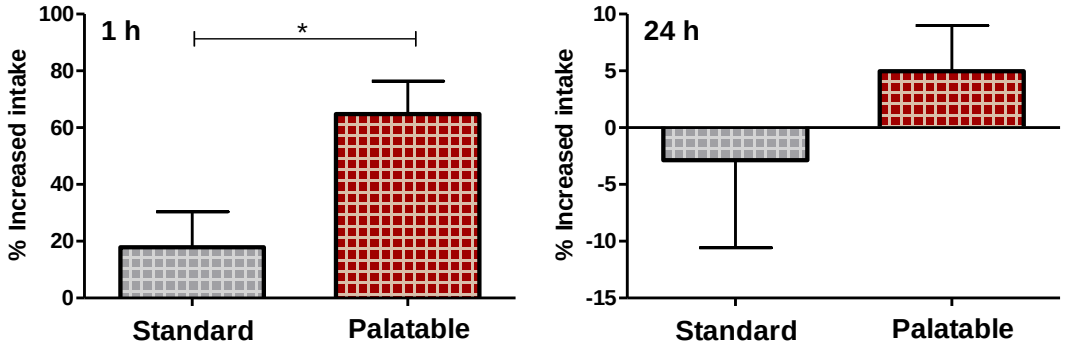


Figure 2.5. **An acute injection of ghrelin (0.3 mg/kg) stimulates PF ingestion at 1 h after administration (*vs.* SC), but does not significantly influence the cumulative consumption of SC or PF after 24 h.** Results shown are mean ± SEM (n = 18). * Represents P < 0.05.

The half-life of leptin is approximately 71-180 min in rodents (Ahima et al., 1996; Hill et al., 1998). As shown in Figure 2.6 (*left panel*), exogenous leptin, administered i.p. at 3 mg/kg 30 min before the start of SC intake monitoring, expectedly suppressed the cumulative ingestion of SC (no-choice condition), as compared to SC consumption in fasted mice. Two-way ANOVA on the 24 h SC intake revealed main effects of time ($F_{3, 68}$ = 1182, P < 0.0001) and leptin treatment ($F_{1, 68}$ = 104.4, P < 0.0001), with these factors showing a significant interaction ($F_{3, 68}$ = 12.91, P < 0.0001). Sidak's post-hoc multiple comparison tests confirmed that leptin significantly suppressed cumulative SC intake at all time points (P < 0.05), except for 0-1 h. In contrast, a similar leptin treatment regimen in mice given a choice between SC, LF and PF, only slightly reduced PF consumption (SC and LF options were neglected by animals, as discussed before), as compared to PF consumption in fasted mice Figure 2.6 (*right panel*). ANOVA showed main effects of time ($F_{3, 68}$ = 974.1, P < 0.0001) and treatment ($F_{1, 68}$ = 28.36, P < 0.0001), and revealed an interaction between time and treatment ($F_{3, 68}$ = 0.6096, P < 0.0001); post-hoc multiple comparison analysis confirmed significant effects of leptin on the cumulative ingestion of PF at 3 and 24 h (Sidak; P < 0.05).

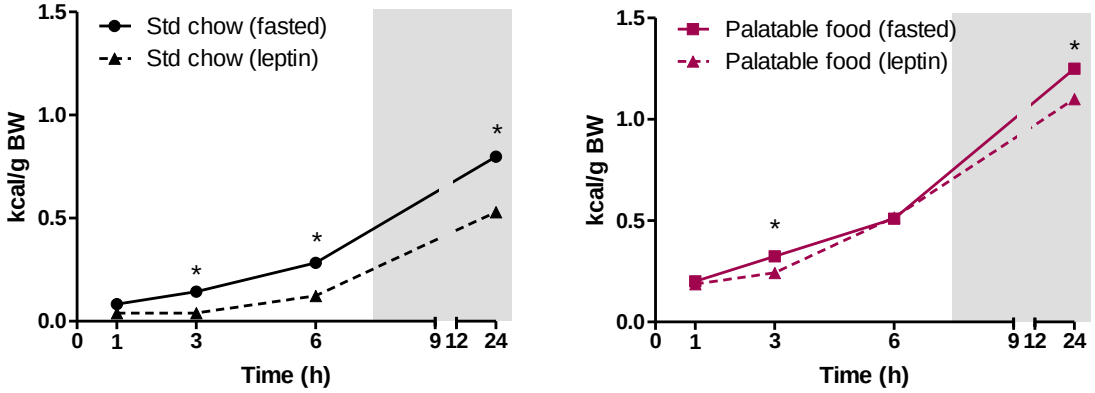


Figure 2.6. **The effect of leptin on standard chow and palatable food intake.** Leptin (3 mg/kg i.p., injected after 24h fast) significantly suppresses cumulative SC consumption at 3, 6 and 24h (*left panel*), but reduces PF intake only during 3 h and 24 h time-point (*right panel*). N = 18, *P < 0.05.

Leptin-induced suppression of food intake was calculated as described in *Materials and Methods (Section 2.2, Experiment 1)*; this provided a measure of cumulative SC and PF consumption at 1 and 24 h (Figure 2.7). The analysis of individual time-points was done to unmask possible effects at early-time points (e.g. 1 h) that were not discernible by overall analysis of cumulative 24-h food intake. This analysis showed a significant decrease in SC intake (40.9 ± SD 29.3), but not PF intake (1.6 ± SD 37.9), during the first hour after leptin administration. Student's *t*-tests revealed the difference in the efficacy of leptin in suppressing SC *vs*. PF consumption during 1 h to be statistically significant (t = 2.6, P = 0.0187). Leptin also more potently suppressed SC intake (25.3 ± SD 14.7) than PF intake (12.2 ± SD 7.9) over 24 h (t = 2.7, P = 0.0197).

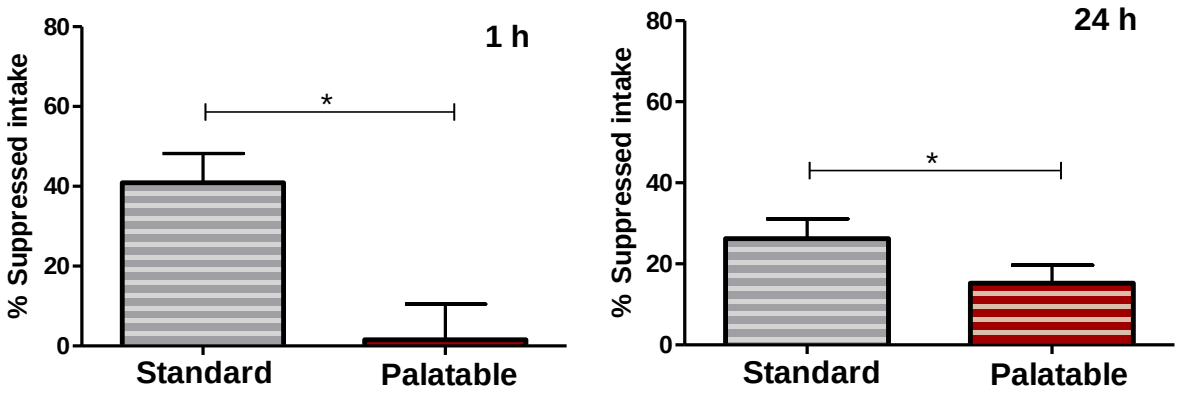


Figure 2.7. Leptin suppresses consumption of standard chow (SC) more effectively than that of palatable food (PF). Degrees of suppression of SC and PF consumption 1 h (*left panel*) and 24 h (*right panel*) after leptin injection (3mg/kg after 24 h fasting) are shown. Numerical values represent means ± SEM (n=18); * indicates P < 0.05.

Ghrelin potentiates PF intake but not SC consumption in a satiated state (Experiment 2)

A between-subjects experimental design (Scheme 2.3) was used to confirm the results obtained in Experiment 1 using the approach described in Materials and Methods (Section 2.2.). The results from Experiment 1 were confirmed insofar that animals consumed significantly more PF than SC (control condition) in a state of satiety; the consumption of PF after ghrelin (0.22, SD ±0.07) (during 1 h) was greater than that after saline (M=0.15, SD ±0.04), whereas there was only a mild effect of ghrelin on SC intake in the control groups (saline +SC= 0.021 ±0.01; ghrelin +SC= 0.037 ±0.02) (Fig. 2.8). Statistical analysis revealed main effects of food type ($F_{1,36}$ = 135.8, P < 0.0001) and treatment ($F_{1,36}$ = 9.667, P = 0.0037) that were not inter-related ($F_{1,36}$ = 3.787, P = 0.0595). Tukey post-hoc multiple comparison tests showed significant differences between all groups, except SC-saline- and SC-ghrelin-treated groups (P < 0.05).

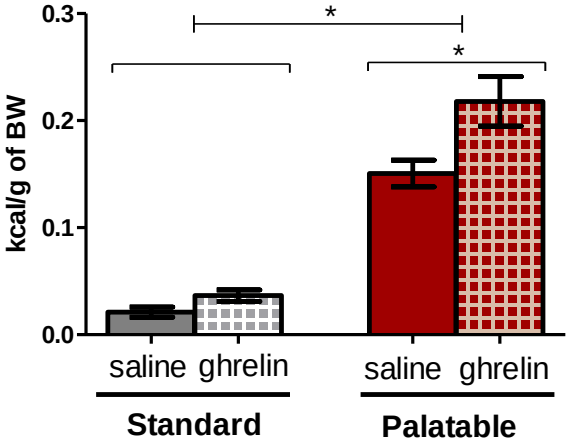


Figure 2.8. Ghrelin did not affect SC consumption but increased PF intake (confirming the results form Exp1, Fig 2.4). Ghrelin (0.3 mg/kg) was injected in a satiated state (n=10 per group). Food intake was measured 1 h after injections. Numerical values represent means ± SEM (n = 10 per group); * indicates P < 0.05.

Leptin suppression of SC consumption is greater than that of PF ingestion (Experiment 2)

Leptin significantly suppressed the cumulative intake of SC over 24 h. Two-way ANOVA detected a main effect of time ($F_{3, 54}$ = 1582, P < 0.0001) and treatment ($F_{1, 18}$= 4.552, P = 0.0469), but no interaction between these factors ($F_{3, 54}$ = 0.9421, P = 0.4268). No effect of leptin treatment was seen on PF intake ($F_{1, 18}$ = 2.442, P = 0.135); there was a significant main effect of time ($F_{3, 54}$ = 1238, P < 0.0001), but no treatment X time-point interactions were detectable ($F_{3, 54}$ = 1.071, P = 0.3693). Moreover, leptin did not significantly influence the intake of either food type at any of the individual time points measured during 24 h according to two-way ANOVA analysis (P > 0.05, Sidak's multiple comparison test, data not shown).

Individual time-bins were subsequently analysed (here and henceforth) to rule out the possible masking of significant effects in analyses of 24 h (cumulative) data. Analysis of the eating patterns at single time-points identified the first hour of food presentation as the only one during which leptin exerted significant effects on SC intake relative to PF

intake (Fig 2.9, left panel). Main effects of food type ($F_{1, 81}$ = 71,86, P <0.0001) and treatment ($F_{1, 36}$ = 4.930, P = 0.0328) were found by 2-way ANOVA, but there were no significant food type X treatment interactions ($F_{1, 36}$ = 0.2097, P = 0.65). Tukey post-hoc multiple comparison test revealed significant differences in the consumption of SC and PF, but failed to detect any effects of leptin on the ingestion of either food type.

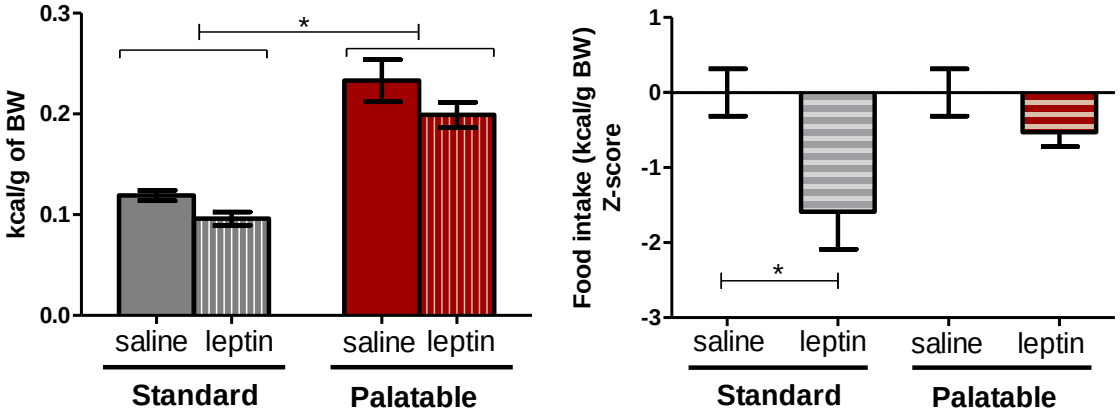


Figure 2.9. Leptin suppresses SC, but not PF, intake during the first hour of food exposure (confirming the results form Exp1, Fig 2.6). Leptin (5 mg/kg, i.p.) or saline were injected to fasted mice (n=10 per group) at ZT 14; 1 h after these injections, food was presented. Absolute energy consumed per g BW is shown as mean ± SEM (*left panel*) and energy consumed per g BW by each group is shown as z-scores (*right panel*). * indicates P < 0.05.

To exclude the possibility that leptin effects were masked by large differences in the calorific contents of PF and SC, the magnitude of food intake suppression by leptin was estimated by z-scores (standard scores), as described in *Materials and Methods (Statistics)* and shown in Fig 2.9 (right panel). These z-scores were subequently used in a 2-way ANOVA which revealed main effects due to treatment ($F_{1, 36}$ = 9.150, P = 0.0046). Tukey's post-hoc test confirmed the significant difference between saline and leptin treatment on SC intake and that leptin did not exert a significant effect on PF intake. These results are consistent with those reported for Experiment 1.

Novelty and body mass status modulate rate and magnitude of high-fat diet consumption in the presence of leptin (Experiment 3)

The previous experiments showed that PF is robustly overconsumed, presumably in excess of real physiological needs when considered in light of SC intake. In order to study if this overconsumption prevails even when PF is continuously availabile, animals were given access to PF for 6 d; 24 h food intake was compared after the first and sixth days of PF availability.

As compared to SC, PF is overconsumed on the first day of availability, i.e. when PF is novel; however, the "novelty effect" attenuates after 6 d of continuous exposure to PF,

after which mice decrease their intake of calories from PF to a level similar to that from SC in the control condition (Figure 2.10, *left panel*). Two-way ANOVA showed that the results were significantly affected by time ($F_{1, 18}$ = 13.36, P = 0.0018), and that food type also exerted a significant influence on the overall results ($F_{1, 18}$ = 14.19, P = 0.0014); moreover, there was a significant interaction between the factors time and treatment ($F_{1, 18}$ = 53.45, P < 0.0001). Post-hoc tests confirmed differences between the SC and PF groups when PF was novel, but not when subjects were exposed to PF for 6 days (P < 0.05).

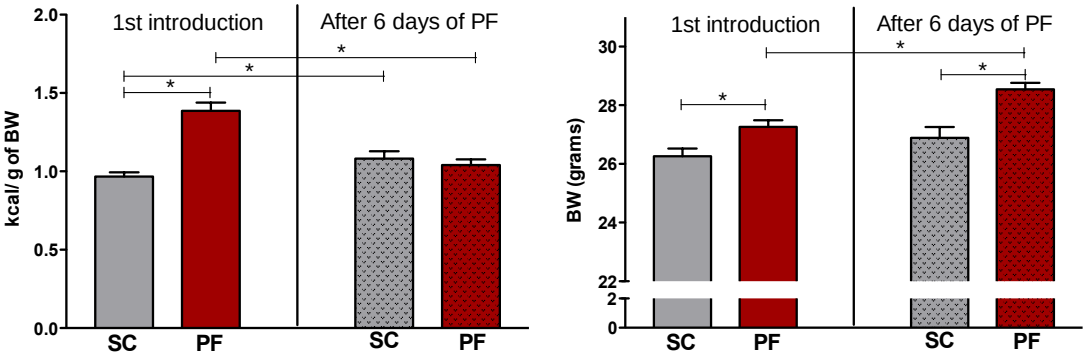


Figure 2.10. Overconsumption of palatable food (PF) does not prevail over time. Mice (n=10 per group) initiated to (d1) and maintained on PF (for 6 d) reduced their daily caloric intake to control (Standard chow, SC) levels *(left panel)*. Nevertheless, the PF-fed mice showed a significant increase in body weight between d1 and d6 of the experiment (*right panel*). Data are shown as means ± SEM.

Interestingly, body weights of animals consuming PF were increased after 6 d exposure to PF despite the reduction in the total calorie intake, suggesting that even short periods of over-eating may lead to lasting metabolic changes (Figure 2.10*, right panel*). Two-way ANOVA analysis of the body weights (BW) indicated main effects of time ($F_{1, 18}$ = 26.74, P < 0.0001) as well as of food type ($F_{1, 18}$ = 14.82, P = 0.0012) and no interaction between the factors ($F_{1, 18}$ = 3.097, P = 0.0954). Post-hoc tests confirmed that there was an increase of BW after 6 d of PF but not after SC.

2.4. Discussion

Hedonic mechanisms are thought to be responsible for overeating of foods with high palatability; the latter results from properties such as smell, taste, texture and possibly, post-oral nutrient sensing, all of which reinforce the consumption of calorie-dense foods (Section 1.2.2). The hypothesis that hedonic mechanisms prevail over the normal physiological controls of eating (Section 1.3.3) is a plausible one that may explain excessive food intake to the point that overweight and obesity set in. The work in this chapter was undertaken to test this hypothesis in the mouse, an organism commonly

used to study the physiology and neurobiological basis of feeding behaviour. The experiments involved a number of paradigms, a particular consideration being that the events occurring during initial exposures to hedonically-weighted foods will determine subsequent behaviour and body weight trajectories. The experimental designs used here included testing under satiated and fasted conditions as well as after pharmacological mimicking of these states by administering either ghrelin (hunger-signalling hormone) or leptin (satiety hormone) to mice that had been either satiated or fasted, respectively.

One observation, replicated in different experimental settings was that high fat-containing (HF) food (an energy-rich diet, comprising both fat and sugars) is highly preferred over low fat-containing (LF) food and standard chow (SC) when all three food types are presented simultaneously (food choice paradigm). This finding is consistent with previous observations that foods which are fatty and sweet are more palatable than foods containing just one of these components (Drewnowski and Greenwood, 1983; Takeda et al., 2001; van de Giessen et al., 2012); as was found here, the before-mentioned authors also reported that HF foods are consumed even when animals are satiated by their normal (SC) diet.

Interestingly, mice in the present experiment neglected LF food, even though the food mixture was isocaloric with the HF food used. This suggests that taste and texture play more dominant roles than energy *per se* in driving HF vs. LF food consumption. One might speculate that the high intake of the HF food reflects its post-oral effects; such effects have been ascribed to cannabinoid receptor-mediated actions, resulting in the stimulation of food intake (DiPatrizio and Piomelli, 2015). It is also plausible that HF diets pass through the gastrointestinal tract more rapidly than LF and SC, thus reducing their relative satiety-inducing efficacy. This hypothesis will need further testing, in view of a report that infusion of fat into the duodenum to slow down gastric emptying and be more satiating (Heddle et al., 1989) and other studies reporting that chronic exposure to HF food disinhibits gastric emptying and thus reduces the satiating effects of fat (Rolls et al., 1994; Covasa and Ritter, 2000). Another important point to be mentioned is that, because LF and SC were completely discounted in the choice paradigm used here, HF was later designated a “palatable food” (PF); to facilitate preference comparisons, PF ingestion was subsequently compared to the ingestion of SC under non-choice conditions, i.e. when only SC was provided.

Results showed that exogenous ghrelin treatment of satiated mice not only increased the intake of SC, but also that of PF; importantly, however, PF consumption was increased to a much greater extent than SC in response to ghrelin injections, indicating that the hedonic properties of PF are perceived even under conditions of energy equilibrium. This observation matches other reports that ghrelin infusion into the ventral tegmental area (VTA) stimulates the prefrential consumption of a rewarding HF food vs. SC in a free choice paradigm (Egecioglu et al., 2010) and that ghrelin appears to

increase the reward salience of HF food in both, conditioned place preference and operant conditioning paradigms (Perelló and Zigman, 2012); in the latter study, orexin neurons were suggested to mediate the actions of ghrelin. Interestingly, ghrelin levels were shown to increase in anticipation of a palatable (chocolate) food vs. SC (Merkestein et al., 2012).

Palatability was found to have an important influence on the physiological response to leptin. This was particularly evident during the first hour of presentation of a calorie-dense, high fat (HF)-containing food[2], also referred to as palatable food (PF) in this work; briefly, using different experimental designs, leptin, administered either during the daily period of activity (dark phase) or quiescence (light phase), proved less effective than expected at constraining PF intake.

To our knowledge, the present data represent the first report of the failure of leptin to suppress acute PF consumption; accordingly, it also provides clear support for the hypothesis that hedonic mechanisms can override physiological signals of satiety. Previous studies on leptin in the context of overeating focused on decreased leptin efficacy and leptin resistance in subjects chronically exposed to a HF diet (Myers et al., 2010; Morton et al., 2014). Short-term (8 days) high-fat/high-carbohydrate food availability has been also shown to induce peripheral and arcuate leptin resistance, however, food intake response to central leptin remains intact (van den Heuvel et al., 2014). In other studies that are indirectly related to the present work, Figlewicz *et al* reported that leptin decreases food consumption as well as the reward salience of sucrose and HF food in the conditioned place preference test (Figlewicz et al., 2001; Figlewicz et al., 2004). Additionally, Hommel *et al* found that leptin decreases the firing rate of reward-mediating dopaminergic neurons (Hommel et al., 2006). Along the same lines, Domingos *et al* recently described the ability of leptin to reverse preference of sucrose over a non-nutritive sweetener (sucralose) paired with optogenetic stimulation of dopaminergic neurons in the VTA in fasted mice (Domingos et al., 2011). It should be noted, however, that in contrast to our own, the studies on how leptin acutely influences eating focused on consumption of a specific food component (e.g. fat or carbohydrate), rather than palatability derived from a mixture of these components.

Although not previously mentioned (and never considered in animal experiments hitherto), novelty of a food was here considered as a potential contributor to the hedonic properties of PF. In this study we demonstrated that novelty might indeed add to the hedonia experienced upon presentation of PF for the first time. We found here that the consumption of PF dwindled over time. Unexpectedly, however, despite the progressive reduction in PF intake, there was a progressive net gain of body weight

[2] As mentioned before, the so-called high-fat diets used in research (palatable food, PF) in fact have not only a high content of fat (45 %) but also a high content of sucrose (17 %), compared to standard chow (SC) (11% and 4.9% respectively).

among the mice that had consumed PF for several days (6 d). Presently, the reasons for this discordant finding can only be speculative. The results suggest that the initial experience of PF sets into a series of behavioural and physiological adjustments that are not coordinated in the usual way. Following the first exposure to PF, an imbalance between energy intake and expenditure (reflected in body weight) emerges, probably reflecting the apparent ability of energy-dense foods to re-set the defended body weight set-point and the rate of energy metabolism (Levin, 2005; Müller et al., 2010). PF might also trigger the increase in the efficiency of nutrient absorption (more energy retrieved from a smaller amount of consumed calories) together with increased secretion of the gut peptide CCK (Spannagel et al., 1996) and therefore, decrease appetite, which may help explain our findings. We propose that dys-synchrony between central and peripheral signals, triggered at the time of first exposure to a PF, may be a critical event in the pathway to obesity.

CHAPTER 3

MECHANISMS RESPONSIBLE FOR HEDONIC OVERRIDING OF LEPTIN SIGNALS

Abstract

Feeding behaviour depends on the integration of peripheral and central signals; the latter include sensory, motivational and affective domains. This study was undertaken to gain further insights into the mechanisms responsible for our previous observation that foods with greater hedonic value are consumed in excess of need, probably by subjugating homeostatic signals that normally act to curtail feeding. Of the several brain areas activated by ingestion of a hedonic food during a state of satiety, we focused on the lateral hypothalamus (LH) because appetite-inducing orexin neurons therein are normally suppressed by the anorexigenic hormone leptin when satiety is reached. Our results show that in satiated, as well as in previously-fasted mice, exposure to a palatable food (PF) for 1 h selectively upregulates orexin expression, without inducing any changes in the expression of another orexigenic peptide, melanin-concentrating hormone (MCH). Further, we show that exogenous leptin does not suppress orexin neuron activity and fails to restrain overconsumption of PF; importantly, our analysis showed that this phenomenon cannot be explained by failure of proximal leptin signalling cascades. These results suggest that hedonic foods generate activity in yet unidentified neurons that either stimulate orexin neurons in the LH which, in turn, become refractory to the inhibitory actions of leptin, or inhibit neurons that normally function to decrease orexin neuron activity in response to leptin.

Highlights

- Consumption of a palatable food (PF) in the satiated state activates brain areas that contribute to the physiological and behavioural homeostatic regulation of feeding control; one of these is the lateral hypothalamus (LH), an area normally activated by hunger.
- Exogenous leptin fails to suppress PF ingestion due to its inability to inhibit the activity of orexin neurons in the LH.

3.1. Introduction

Much knowledge has been acquired in recent decades about the neuroendocrine and behavioural neurocircuitry that controls feeding. This circuitry normally functions to maintain a balance between energy intake and endogenous energy depots; as a result, body mass is maintained at a level that matches environmental demands (e.g. seasonal changes in food availability, pregnancy) and ensures healthy functioning of tissues and organs. With the abundance of readily-available foods, as well as industrially-introduced sensory enhancers (tastants, odours, textures and colouring) modern humans are increasingly consuming more calories than they expend and are therefore developing overweight and obesity, conditions that are causally linked to a host of somatic and mental disorders, ranging from diabetes and hypertension to mood and cognitive disturbances (see Section 1.1.3). While there is an abundant literature on the peripheral and central adaptations that result from chronic overeating (Gastaldelli, 2008; Friedman, 2009; Berrington de Gonzalez, Amy et al., 2010; Myers et al., 2010; Snel et al., 2012; Morton et al., 2014), little is known about the proximal mechanisms that initiate overeating in the first place. Sensory enhancers clearly are a main incentive to eat more of a particular food, but why do the physiological mechanisms that should normally curtail eating by signalling fullness or energy sufficiency appear to become ineffective? The "hedonia hypothesis of obesity" suggests that foods imbued with pleasurable signals override homeostatic mechanisms and even alter their set-points (Saper et al., 2002; Lutter and Nestler, 2009; Berthoud, 2011).

The results presented in Chapter 2 offered support for the "hedonia hypothesis"; it was found that just a single, short (1 h) exposure of mice to an energy-dense, highly palatable food (PF) leads to overconsumption of PF, even when the animals are pre-treated with leptin, a peptide hormone that potently signals satiety and adequate energy reserves (Section 1.2.3). The task of the present experiments was to identify the central molecules, sites of action and mechanisms that permit hedonic processes to counter the activity of highly conserved endogenous processes that normally serve to maintain appropriate body mass through regulated eating to meet energy demands in a contextually-relevant manner.

The pharmacological, neuroanatomical and behavioural approaches used here identified the lateral hypothalamus (LH), an area known to be activated by hunger, as a site wherein endocrine and behavioural signals converge and compete to drive overeating of PF (Kelley et al., 2005; Berthoud and Münzberg, 2011). Specifically, the results show that PF leads to activation of orexin neurons. These neurons are normally subject to inhibition by leptin (Laque et al., 2013; Goforth et al., 2014); however, we show that leptin fails to suppress orexin activity during exposure to palatable food despite the fact that leptin signalling *per se* is not disrupted by PF. In light of these observations, it is proposed that overeating of PF results from the overshadowing of leptin inhibition of

orexin neurons by hedonia-driven signals that impinge upon and disinhibit orexin neurons.

3.2. Materials and Methods

Animals

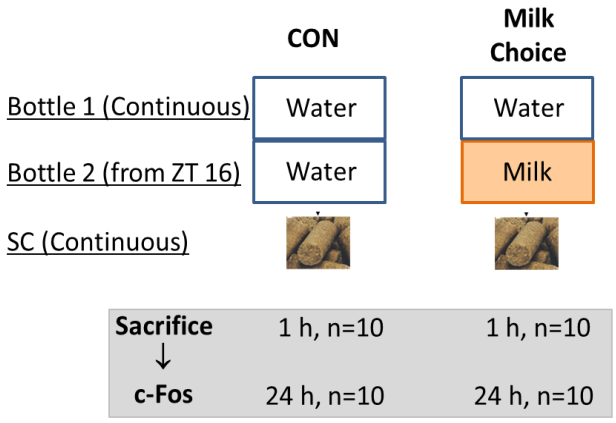
Experiments were performed on male C57BL/6N mice purchased at the age of 2 months (Charles River Laboratories, Sulzfeld, Germany); animals were used after at least 2 weeks of habituation to the animal facility of the Max Planck Institute of Psychiatry (Munich, Germany). Mice were single-housed under constant temperature (21 ± 2 °C), humidity (50 ± 10%) and a daily 12 h light/12 h dark cycle. Food and water were available *ad libitum* throughout, unless specified otherwise. Procedures on animals were approved by the local ethical commission (State Government of Upper Bavaria) and complied with EU Directive 2010/63/*EU*.

Test foods

During acclimation and unless specified otherwise, animals were maintained on standard chow (SC, 1320, 2.84 kcal/g, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany). To estimate brain activation during hedonic experience, mice were given access to SC and 60 ml of dairy cooking crème (Molkerei Weihenstephan, Germany; diluted in water to provide 1.64 kcal/g, comprised of 5% fat). To evaluate hedonic overriding of leptin, SC intake (single choice) was compared to food consumption when animals could choose between SC and palatable high-fat/high sucrose food (PF; D12451, ResearchDiets Inc., New Brunswick, NJ, USA, 4.73 kcal/g, 45% fat).

Mapping of brain activation after hedonic experience

Twenty-four adult male mice (aged 3 months) were habituated to presentation of two 75 ml drinking bottles for 5 days before testing. During test sessions the second drinking bottle was replaced (ZT 16) with a fresh bottle of water or milk (Scheme 3.1).



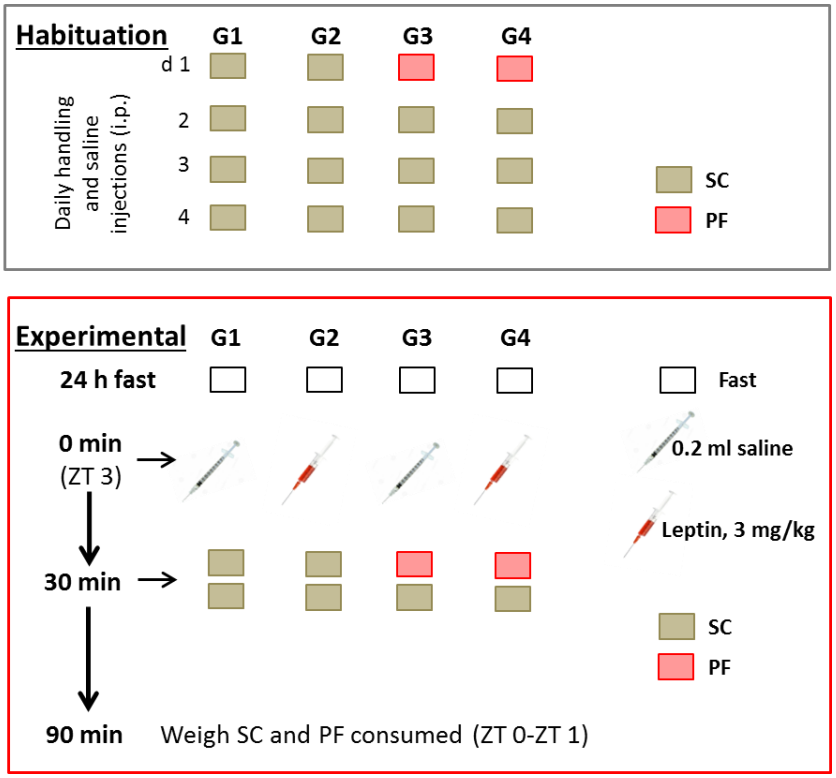
Scheme 3.1. Experimental design to map brain activation in response to hedonic stimuli. A control group of mice was sacrificed after 1 or 24 h of presentation of bottles containing water only (n = 6 per time-point). In three groups of mice, one of the drinking bottles was replaced with a bottle of milk (see Test foods, above) at ZT 16, and groups (n = 6 per group) were sacrificed after 1 or 24 h. All animals had ad libitum access to SC throughout the experiment.

At the indicated times, mice were sacrificed (cervical dislocation). Blood was collected from subgroups (n = 5-10 per group) from each of the treatment groups and their brains were excised

and flash-frozen in isopentane/dry ice and stored at (- 80° C) until processed for detection of C-fos mRNA by *in* situ hybridization histochemistry (ISHH) or immunohistochemistry (IHC), as described below.

Neural correlates of hedonic overriding of leptin signals

A total of 88 single-housed male mice (C57BL/6J), aged 3 months old, were used in this experiment, following the design depicted in Scheme 3.2. Individual body weights and food intake were monitored on 4 consecutive days before the actual experiment; during this preparatory phase, animals were also habituated to handling and injection procedures (0.2 ml saline i.p.). Animals were assigned to one of 4 treatment groups, ensuring that body weights did not significantly differ between groups; a total of 18 mice formed each group (G1-G4) (Scheme 3.2). Following a 24 h fast, animals were injected (i.p.) with either saline (G1 and G3) or leptin (3mg/kg; # LEP-6, Protein Laboratories Rehovot Ltd., Rehovot, Israel) at ZT 3; 30 min later, mice were given access (1 h) to either SC only (G1 and G2) or to a choice of SC and PF (G3 and G4). The latter groups had previously received PF for 24 h, five days before the experiment so as to familiarize them to this novel diet. Individual food intake (FI) was recorded during the last experimental hour.



Scheme 3.2. Experimental design to study the neural correlates of palatable food (PF) overriding the effects of leptin. Mice (n = 88) were divided into 4 groups (G1-G4). Five days before the experiment, G3 and G4 mice were exposed to PF for 24 h to allow animals to avoid the confound of "novelty" during the actual testing. Testing involved mice being fasted for 24 h, followed by i.p. injections of saline or leptin (3mg/kg) 30 min before presentation of the food choices, as shown. Food intake was measured 1 h after presentation of the choices.

Animals were sacrificed immediately at the end of the 1 h test feeding period by cervical dislocation; brains were excised and trunk blood was collected (n = 14/group) for determination of leptin, insulin, ghrelin, TNFα and IL-6 using a multiplex assay (see below). Fresh-frozen brains from 7 animals of each group were cryo-sectioned (10 µm from selected brain regions; Paxinos and Franklin, 2001) and stored at -20 ˚C until processed for ISHH detection of the RNAs encoding c-fos, orexin, melanin-concentrating hormone(MCH), neuropeptide Y (NPY) (see below), or for IHC detection of c-Fos protein. Seven mice from each group provided hypothalami for subsequent analysis of pSTAT3 by immunoblotting. In addition, tissue punches of the orbitofrontal cortex (OFC), nucleus accumbens (NAc) and ventral pallidum (VP) for neurotransmitter analysis by high performance liquid chromatography (HPLC) and orexin by radioimmunoassay (n = 7 per group) (see below); tissue punches were prepared after cryosectioning of brains (200 µm), using a NIH Style Neuro Punch instrument (Fine Science Tools, Heidelberg, Germany) using known coordinates (Paxinos and Franklin, 2001), and stored at -20 ˚C until neurotransmitter measurements. Lastly, 6 mice from each group were deeply anesthetised with pentobarbital-sodium (Narcoren, Merial GmbH, Hallbergmoos, Germany) before receiving intra-cardiac perfusions of 0.9% saline (50 ml), followed by 50 ml of freshly-prepared 4% *p*-formaldehyde in PBS; these brains were subsequently post-fixed in 4% *p*-formaldehyde for 24 h, cryo-preserved in sucrose (30% in PBS) and stored at -20 ˚C until cryo-sectioning. Sections (25 µm) of the lateral hypothalamus (Bregma -1.06 until -2.30) were cut on a freezing microtome, divided into six sequential pools and stored in antifreeze solution (30% ethylene glycol; 25% glycerol; 0.05 M phosphate buffer; pH 7.4) at -20°C until immunohistochemical detection of c-fos and pSTAT3; orexin-A/orexin-B, c-Fos/orexin-B, c-Fos/POMC, pSTAT3/galanin (all double-labelling), and c-Fos/orexin-A/galanin and c-fos/galanin/TH (all triple labelling) (see below and in Section 4.2).

mRNA detection by in situ hybridization histochemistry (ISHH)

Sagittal sections (10 µM; Lateral 3.25, 2.52, 1.56, 0.96, 0.48, 0.24 mm) and coronal sections were used to detect c-Fos mRNA (10 µM; Bregma 2.34, 1.70, 0.74, -0.70, -1.43, -3.16) or to detect orexin and MCH mRNA (10 µm; Bregma -1.46, -1.70). Sections from fresh-frozen brains were thaw-mounted on Super-Frost Plus glass slides (Thermo Fischer, Braunschweig, Germany), fixed in cold 4% formaldehyde in phosphate-buffered saline (PBS, pH 7.4), treated with acetic anhydride (0.25% in 0.1 M triethanolamine/NaCl, pH 8.0), dehydrated, delipidated and air-dried.

All sections were simultaneously hybridized with an oligonucleotide probe complementary to the probe of interest (see Table 3.1 for sequences; oligonucleotides custom-made by Sigma-Aldrich Chemie GmbH, Steinheim, Germany) that had been 3' end-labeled with ^{35}S d-ATP (Perkin Elmer, Waltham, MA, USA) using terminal deoxynucleotidyl transferase (Invitrogen, Karlsruhe, Germany), in a humidified environment (overnight, 37 ˚C); to control for specificity, alternate sections were hybridized with a respective radioactively labelled sense sequence. After washing to a final stringency with 1 X SSC and a 1:1 pre-warmed mixture of formamide and 4 x SSC at 40° C in a water bath, rinsing and dehydrating, slides were exposed to X-ray film (Carestream Biomax MR Film, Kodak, Rochester NY, USA) for 24 h (OX and MCH), 3 days (NPY) and 10 days (c-fos) before developing. After capturing the images, either quantitative measurement of optic density using Image J software (http://imagej.nih.gov/ij/download/zips/-j149.zip) or qualitative inspection of signal intesity was conducted. A ^{14}C standard with a range of 0-35 nCi/mg

(American Radiolabeled Chemicals Inc., St. Louis, MO, USA) was used to estimate radioactive emission.

TABLE 3.1. Antisense oligoprobes for ISHH

Target mRNA	Oligoprobe sequence
c-Fos	5'-GTT GAC AGG AGA GCC CAT GCT GGA GAA GGA GTC GGC TGG GGA ATG
Prepro-orexin	5'AGCAGCGTCACGGCGGCCCAGGGAACCTTTGTAG
Pro-MCH	5'CAACATGGTCGGTAGACTCTTCCCAGCATACACCTGAGCATGTCAA
NPY	5'GTCCTCTGCTGGCGCGTCCTCGCCCGGATTGTCCGGCTTGGAGGGGTA

Immunohistochemistry (IHC)

General protocol: Unless specifically mentioned, all reagents were diluted in 0.01 M PBS (pH 7.4); this buffer was also used for washing between each step (3 x 5 min at room temperature). Both, thaw-mounted (c-Fos) and free-floating (all other antigens) sections that had been previously washed in PBS were pre-treated with 0.5% hydrogen peroxide (15 min) to quench endogenous peroxidases, permeabilized with 0.2% Triton X-100 (15 min) and blocked in 2% normal horse serum (20 min) to prevent non-specific antibody binding. This was followed by incubation in primary and appropriate direct-conjugated fluorescent antibodies or biotinylated secondary antibodies (1:1000; 2 h at room temperature; Jackson ImmunoResearch Laboratories, West Grove, PA, USA); the latter signals were amplified using avidin–biotin complex (ABC; 1:1000, 2 h at room temperature; Vector Laboratories, Burlingame, CA, USA). Intermittent washes were made with 0.01 M Tris (pH 7.4). When required, a fluorescent signal amplification step, consisting of incubation in biotin-tyramide (1: 1000, in 0.05 M Tris, containing 0.006% H_2O_2, pH 7.6; 20 min) (Hopman et al., 1998) and secondary streptavidin fluorescent antibody (Alexa 488-streptavidin; 1:1000, 12 h; Molecular Probes, Thermo Fisher), was introduced after treatment of sections with ABC. Sections prepared using immunofluorescence were air-dried and coverslipped under Mowiol (Sigma Aldrich, Deisenhofen, Germany). Non-fluorescent detection of immunohostochemical signal was achieved with diaminobenzidine (DAB); in some cases, using nickel-intensified DAB (0.05% DAB, 0.15% Ni-ammonium-sulfate, 0.006% H_2O_2 in 0.1 M Tris buffer; pH 7.6). Following signal development, sections were mounted on glass slides, dehydrated, cleared in xylene and coverslipped with DPX (Fluka Chemie AG, Buchs, Switzerland).

Light and fluorescent microscopic images were captured using an AxioCam MRc 5 digital camera mounted on a Zeiss AxioImager M1 microscope, outfitted with AxioVision 4.6 software (Carl Zeiss, Göttingen, Germany). Confocal images were captured with an inverted Nikon Eclipse Ti-E microscope, equipped with an A1R confocal system (Nikon, Vienna, Austria). Digital images were processed with Adobe Photoshop CS software (Adobe Systems, San José, CA) and equally adjusted for brightness and contrast.

c-Fos: Coronal sections (10 µM; Bregma 2.34, 1.70, 0.74, -0.70, -1.43, -3.16) from fresh frozen brains were mounted on glass slides and fixed in cold 4% *p*-formaldehyde (10 min) in phosphate-buffered saline (PBS, pH 7.4). After peroxidase and non-specific binding blocking steps (see above), slides were incubated with rabbit anti-c-Fos antibody (1:5000, 18h, 4° C; PC 38, Millipore, Billerica, MA, USA), followed by anti-rabbit biotinylated secondary antibody and ABC. Signal was revealed using Ni-intensified DAB (black precipitate).

Orexin-A/orexin-B: Co-localization of orexin-A and orexin-B was tested using rabbit anti-orexin-A (1:5000, 18 h, Phoenix Pharmaceuticals, Burlingame, CA, USA) and goat anti-orexin-B (1:2000, 18h, 4° C; sc-8071, Santa Cruz Biotechnology, Dallas, TX USA) and anti-rabbit-fluorescein isothiocyanate (FITC) and anti-goat-Cy3 secondary antibodies, respectively.

C-fos/orexin-B: Free-floating sections from the lateral hypothalamus (every 6th section from Bregma -1.06 to -2.30) were incubated with goat anti-orexin-B (1:3000, 48 h, 4° C; sc-8071, Santa Cruz Biotechnology Inc.) and rabbit anti-c-Fos (1:6000, 48 h 4° C; PC 38, Millipore). First, c-Fos was visualized using ABC and Ni-DAB; the latter signal was further intensified using silver-gold (strong black signal), as described by Liposits (Liposits et al., 1984). Orexin-B immunoreactivity was then revealed following several washes, incubation in blocking serum, anti-goat biotinylated antibody, ABC and DAB (brown-orange signal).

c-Fos/POMC: Double labelling of c-Fos and POMC in lateral hypothalamic sections (every 6th section from Bregma -1.06 to -2.30) was achieved using goat anti-c-Fos (1:10000, 48 h, 4° C; sc-52, Santa Cruz Biotechnology) and rabbit anti-POMC (1:5000, 4° C; H-029-30 Phoenix Pharmaceuticals). C-Fos signal was detected using anti-goat biotinylated antibody, ABC and biotin-tyramide amplification and strepatavidin 488 (green fluorescence) and anti-rabbit Cy3 secondary antibodies were used to detect the POMC signal (red fluorescene).

pSTAT3: Primary rabbit anti-pSTAT3 (1:500, 48h, 4° C; #9131, Cell Signalling Technology, Boston, MA, USA) antiserum was used. pSTAT3 signal was biotin-tyramide amplified and detected with streptavidin 488.

Capillary electrophoretic detection of STAT3 immunoreactivity

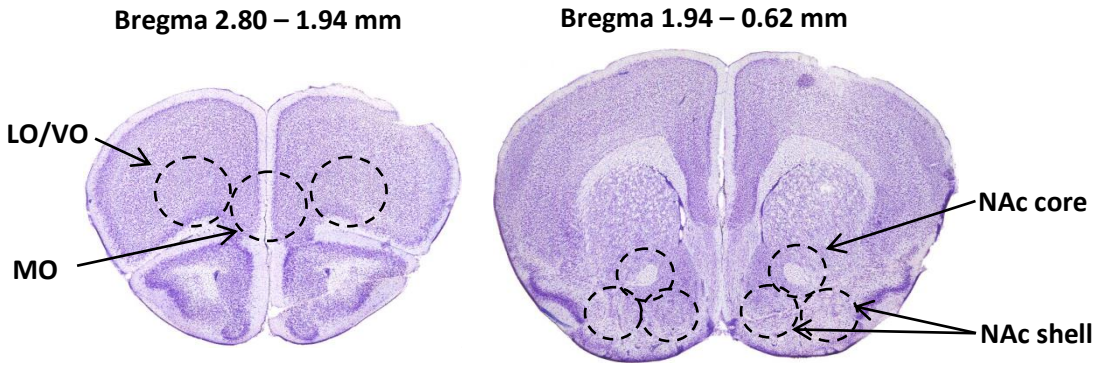
Dissected hypothalami were homogenized (100 mM Tris-HCl, pH 8; 1 mM EDTA, 250 mM NaCl, 25 mM MgCl, 10% glycerol, 1% Nonidet, and Roche cOmplete *Protease Inhibitor* Cocktail and phosphatase inhibitor cocktails 2 and 3, all from **from Sigma-Aldrich)**. The protein content of each sample was determined by Lowry assay (Lowry et al., 1951) and optical densities were read on a Synergy 2 multiplate instrument (BioTek Instruments, Winooski, VT, USA), using Biotek's proprietary Gen5 DataAnalysis software. Samples were prepared for semi-automatic capillary electrophoresis using a *Wes Mouse* Masterkit (PS-MK17, ProteinSimple, San Jose, CA, USA) and an additional anti-rabbit secondary antibody (042-206, ProteinSimple); a Wes™ (ProteinSimple) apparatus was used for electrophoretic separation and detection. The proteins pSTAT3 and total STAT3 were detected in hypothalamic lysates (2 µg protein/µl per capillary) by incubation with anti-pSTAT3 (1:25, #9145, Cell Signaling Technologies), anti-STAT3 (1:100, #9139, Cell Signaling Technologies) and actin (1:3000 for pSTAT3 and 1:1000 for STAT3, #4967, Cell Signaling Techonologies) for 2 h at room temperature. Both, total STAT3 and pSTAT3 signals were normalized against actin signals and pSTAT3 signals were, in turn, normalized against the respective total STAT3 signals.

Corticosterone radioimmunoassay (CORT RIA)

Trunk blood was collected, allowed to coagulate (4h, RT) and serum extracted after centrifugation (2100 *g,* 15 min, 4° C). A corticosterone RIA kit (RIA-1364, DRG, Marburg, Germany) was used to detect corticosterone concentrations in serum samples (1: 200 dilution).

Orexin radioimmunoassay (OX RIA)

Tissue levels of orexin A were estimated in bilateral micropunches of selected brain areas (Scheme 3.3) using an orexin-A RIA kit (#RK-003-30, Phoenix Pharmaceuticals, Burlingame, CA, USA). Peptides were extracted by sonication of tissue samples (0.1 M perchloric acid) and centrifugation (20,800 *g*, 15 min, 4° C). Supernatants were lyophilised (4°C), re-suspended in water and then processed according to the RIA kit manufacturers' protocol. The lower and upper detection limits of the assay were 10 and 1280 pg/ml, respectively. Sample concentrations were normalized to individual sample protein concentrations (Lowry assay of supernatants re-suspended in 3N NaOH).



Scheme 3.3. Location of punches. Punches from medial orbitofrontal (MO) and ventral/lateral orbitofrontal from subsequent 200 µm sections (Bregma 2.80- 1.94mm) and punches of nucleus accumbens (NAc) core (Bregma 1.70 – 0.62 mm) and shell (Bregma 1.94 – 0.62 mm) were collected for orexin RIA.

Multiplex assay of serum leptin, insulin, tumour necrosis factor- α and interleukin-6

Serum leptin (sensitivity: 40 pg/ml), insulin (30 pg/ml), ghrelin (3 pg/ml), tumour necrosis factor-α (TNF-α; 16 pg/ml)) and interleukin-6 (IL-6; 15 pg/ml) were simultaneously measured in serum, using a Luminex®-based technology Milliplex MAP kit (#MMHMAG-44K-07.Mouse, Merck Chemicals, Am Kronberger, Schwalbach, Germany) and a Bio-Plex (Bio-Rad Laboratories Gmbh, Munich, Bayern, Germany) instrument for signal detection. Values below assay sensitivity were excluded from the analysis.

Statistics

Data (given in text as mean ± SD; shown in figures as mean SD or ± SEM) were analysed for statistical differences using Prism 6 software (GraphPad, San Diego, CA) after checking for normality. Student's *t*-test (normally distributed data) or the Mann-Whitney test (non-Gaussian) were used to compare data from two groups. To analyse more than two experimental groups, two-factor analysis of variance (2 way-ANOVA) was used, as appropriate. 2 way-ANOVAs were followed by Tukey's multiple comparison (*post hoc*) tests. The level of significance was set at $P \leq 0.05$.

3.3. Results

3.3.1. Identification of brain areas that may mediate hedonic eating

Milk consumption in a satiated state

The aim of this experiment was to use the expression pattern of c-Fos, an immediate early gene, to map areas of the brain activated by a liquid hedonic food experience. Experimental animals could choose between palatable milk and SC, whereas control animals were only presented with water and SC. As shown in Fig. 3.1, milk was conspicuously preferred and consumed even though it was presented when animals were satiated (experimental group 24 h consumption of milk: 30 ± 7.6 grams or 6.6 ± 1.9 kcal/g BW *vs.* control group 24 h consumption of SC: 4 ± 0.46 grams or 0.39 ± 0.043 kcal/g BW).

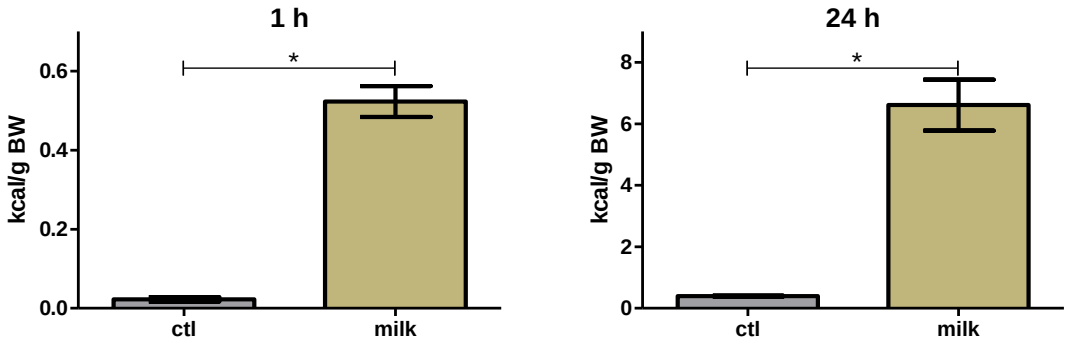


Figure 3.1. Mice overconsume milk (5%). SC intake in a no-choice condition compared to milk consumption in a satiated state (ZT 16) 1h (*left panel*) and 24 h (*right panel*) of consumption. Absolute energy consumed per g BW is shown as mean ± SEM (n=10 per group); * indicates $P < 0.05$.

Neural activation profile after eating palatable liquid meal (milk)

Qualitative analysis of c-Fos mRNA (ISHH) and immunoreactive (IHC) signal revealed neuronal activation in several brain areas, examples of which are shown in Fig. 3.2 (*left* and *right*, respectively); Table 3.2 presents the RNA hybridisation results according to broad (relevant) functional categories. Briefly, following consumption of the hedonic food for 1 h, c-Fos expression was upregulated in the piriform and insular cortices (the primary olfactory and taste areas, respectively), motor cortex and association areas (orbitofrontal cortex, lateral septum) as well as the central and basolateral amygdala that are involved in (emotional) learning and valuation of foods. In addition, c-Fos levels were increased in hypothalamic nuclei involved in mediating anorexigenic behaviour (VMH, DMH). Notably, neuronal activation after presentation of the palatable liquid food (milk) for 1 h was also high in the posterior lateral hypothalamus (perifornical area), an area known to be orexigenic that is to be activated during states of hunger.

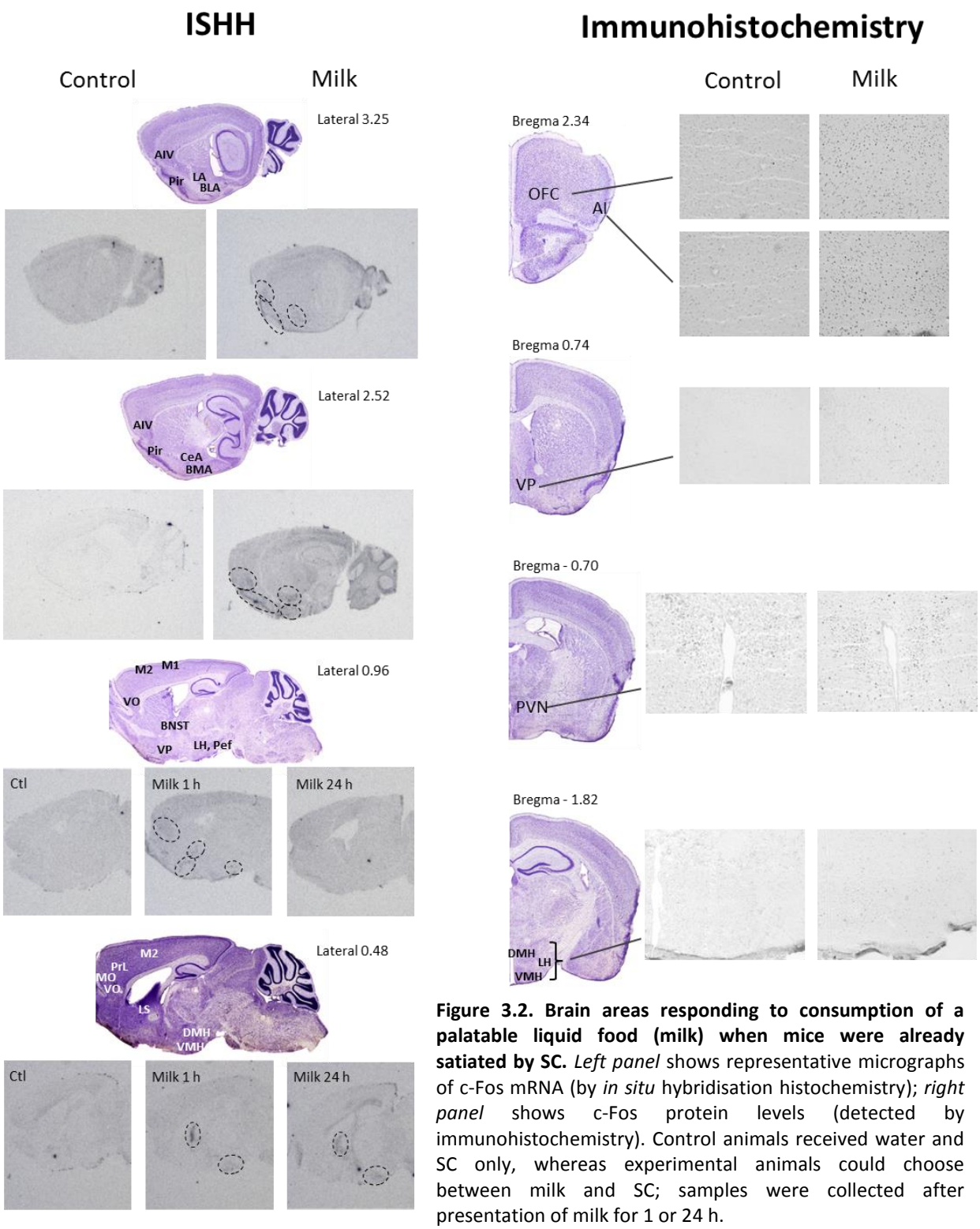


Figure 3.2. Brain areas responding to consumption of a palatable liquid food (milk) when mice were already satiated by SC. *Left panel* shows representative micrographs of c-Fos mRNA (by *in situ* hybridisation histochemistry); *right panel* shows c-Fos protein levels (detected by immunohistochemistry). Control animals received water and SC only, whereas experimental animals could choose between milk and SC; samples were collected after presentation of milk for 1 or 24 h.

TABLE 3.2. Relative changes in c-Fos mRNA expression after consumption of a hedonic solution (milk) vs. SC by control animals. Data based on sagittal sections, examples shown in Fig. 3.2 (n = 3 per group).

Function*	Region of interest	c-Fos mRNA expression (relative change)
Sensory	Agranular insular cortex, *AID, AIV*	++
	Piriform cortex, *Pir*	+++
Motor	Dorsal striatum, *CPu*	-
	Motor cortex, *M1, M2*	+
	Substantia Nigra, *SNR, SNC*	-
Association	Orbitofrontal cortex, *VO, LO, MO*	+
	Prefrontal cortex, *IL, PL*	+
	Anterior cingulate cortex, *Cg1, Cg2*	-
	Habenula, *LHbM, MHb*	-
Emotion and memory (limbic areas)	Bed nucleus of stria terminalis, *BNST*	++
	Amygdala, *BLA, BMA, LA, CeA*	++
	Lateral septum, *LSI*	+++
Hedonic, reward	Nucleus accumbens, NacSh, NacC	-
	Ventral pallidum, *VP*	++
	Ventral tegmental area, *VTA, PBP*	-
Endocrine	Hypothalamus, *ARC, VMH, DMH*	++
	Lateral hypothalamus, *LH, PeF*	++
Brainstem	Locus Coerulus, *LC*	-
	Substantia Nigra, *SN*	-
	Parabranchial nucleus, *PBN*	-
	Nucleus of Solitary tract *NTS*	-
	Pontine nuclei, *Pn*	++

*Broad functional categories that do not necessarily consider overlapping functions.

Upregulation of prepro-orexin RNA expression by a hedonic stimulus

Neurons in the LH express orexin; orexin-expressing neurons are known to be activated during fasting and inhibited after by satiety signals such as leptin (Yamanaka et al., 2003). Since the LH was activated by the hedonic food (milk, increased c-Fos expression) (Fig. 3.2), examination of the role of orexin neurons in hedonic feeding was considered important. Indeed, as depicted in Fig. 3.3, milk consumption for 1 h triggered a qualitative increase in orexin expression in the LH. Although extended exposure to milk (24 h) led to a downregulation of prepro-orexin RNA levels, introduction of a subset of animals to another hedonic food (palatable high-fat/high carbohydrate food, PF) after the 24 h exposure to milk, led to another increase in orexin expression (Fig. 3.3). Thus, processing of hedonic information (palatability, novelty and arousal) activates orexin neurons and increases transcription from the *orexin* gene.

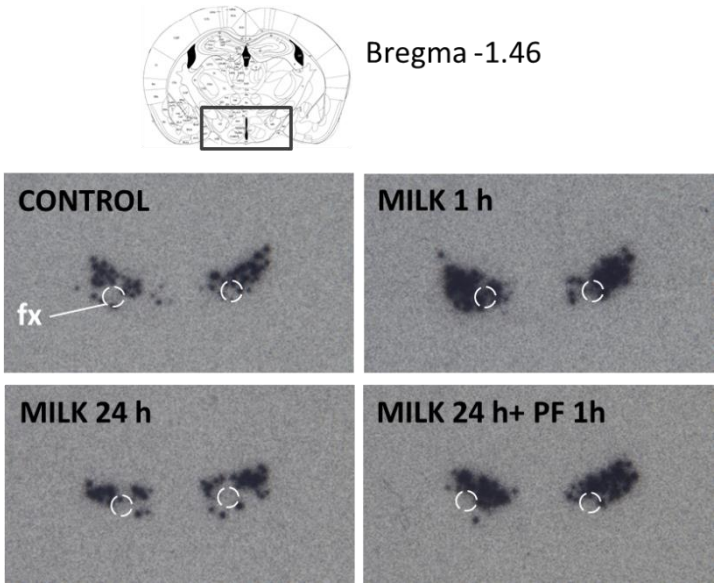


Figure 3.3. Prepro-orexin RNA is increased in the lateral hypothalamus (LH) after exposure to hedonic foods. SC intake in a no-choice condition compared to milk consumption in a satiated state (ZT 16) for 1 and 24 h of milk consumption and after 24 h of milk exposure followed by another 1h of exposure to novel palatable food (PF) (qualitative analysis, n= 3 per group).

Corticosterone (CORT) levels are increased in response to a hedonic stimulus

Like stress, appetitive stimuli usually elevate the organism's state of arousal (Berridge et al., 2010). Lateral hypothalamic orexin neurons are reciprocally connected to corticotropin-releasing hormone (CRH) neurons in the PVN (Winsky-Sommerer et al., 2004; Berridge et al., 2010); the latter are central to activation of the pituitary-adrenal axis (and therefore, gluccorticoid secretion) during stress and are integrated into the rest of neuroaxis that regulates the non-endocrine response to stress, including arousal (Arborelius et al., 1999; Kvetnansky et al., 2009). Consistent with a previous report that orexin of LH origin can stimulate the secretion of the glucocorticoid corticosterone (Bonnavion et al., 2015), consumption of milk for 1 h was associated with an increase in serum corticosterone levels (Fig. 3.4). In parallel to the above-reported results on orexin expression (Fig. 3.3), corticosterone secretion was at baseline after 24 h of feeding on milk, but was again elevated when a different hedonic stimulus (palatable high fat-high carbohydrate food, PF) was provided for 1 h thereafter (Fig. 3.4).

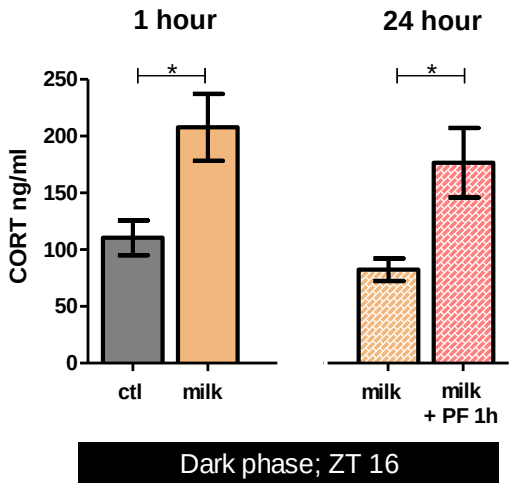


Figure 3.4. Serum corticosterone (CORT) profiles in control mice and mice receiving hedonic stimuli for the times indicated. CORT was measured from serum at ZT 16 in mice exposed to SC (n=10) which served as a control group and in animals after 1h (n=10) and 24h (n=6) of milk consumption, and in mice exposed to milk for 24 h and then to offered access novel palatable food (PF) for 1 h (n=4). * indicates $P < 0.05$.

3.3.2. Leptin does not suppress palatable food (PF) intake

Confirming the results presented in Chapter 2, an intraperitoneal injection of leptin (3 mg/kg, 30 min before presentation of SC/SC to controls and SC/PF to experimental animals for 1 h) to 24 h-fasted mice did not significantly affect food consumption in any of the groups ($F_{1, 64} = 2.183$, $P = 0.1445$), but PF was robustly eaten by both saline- and leptin-treated mice ($F_{1, 64} = 121.8$, $P < 0.0001$) (Fig. 3.5, left panel). Since a much smaller amount of SC was consumed by the control group than the amount of PF eaten by the experimental group, data was analysed by z-scores (leptin + PF normalized to saline + PF; leptin + SC normalized to saline + SC), to discern effects of leptin treatment (Fig. 3.5, right panel). The analysis of z-scores revealed a significant main effect of drug ($F_{1, 81} = 22.87$, $P < 0.0001$) and a tendency towards interaction between food type and drug ($F_{1, 81} = 2.967$, $P = 0.0888$). Post hoc tests confirmed that leptin suppressed SC intake more potently than PF intake.

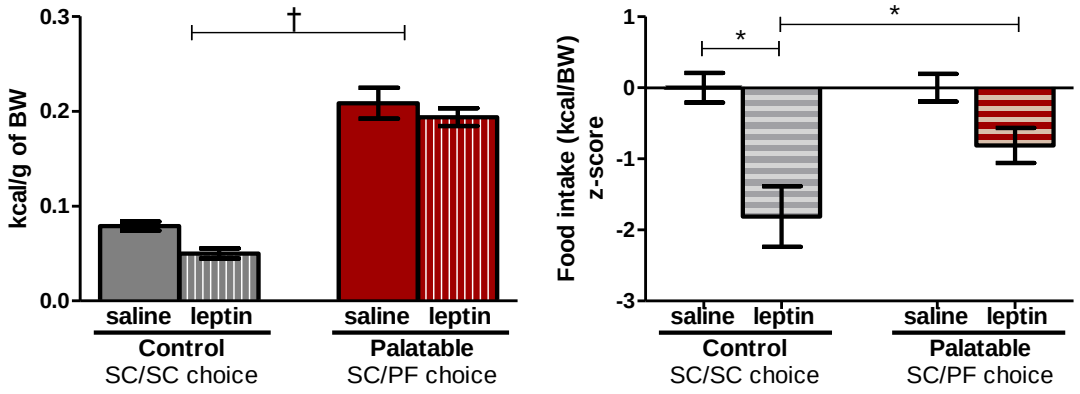


Figure 3.5. Leptin suppresses SC, but not PF, intake during the first hour of food exposure. Leptin (3 mg/kg, i.p.) or saline were injected to fasted mice at ZT 3; 30 h after these injections, food was presented and consumption measured after 1 h. Absolute energy consumed per g BW is shown as mean + SEM (left panel) and energy consumed per g BW by each group is shown as z- scores (right panel). * indicates $P < 0.05$. † indicates significant differences between SC and PF groups, $P < 0.05$.

In order to confirm the efficacy of the injected leptin, serum leptin levels were measured as well as those of other peripheral feeding peptides at the end of the experiment (1.5 h after leptin injection). As shown in Fig. 3.6A, blood leptin concentrations were significantly elevated after drug administration ($F_{1, 29} = 76.8$, $P < 0.0001$, confirmed by post-hoc tests). Exogenous leptin produced dichotomous effects on serum insulin levels: while it caused an approximately 2-fold, but non-significant decrease in animals receiving SC (Fig. 3.6B), leptin injection did not alter insulin levels post-PF consumption; main drug effect: $F_{1, 29} = 3.419$, $P = 0.07$; effect of food type: $F_{1, 29} = 0.17$, $P = 0.68$; *drug* X *food type* interaction: $F_{1, 29} = 1.56$, $P = 0.22$. Serum ghrelin levels were generally low after 1 h of eating (expected for a hunger signalling peptide) (Fig. 3.6C); however, there was a

main effect of leptin treatment ($F_{1, 27}$ = 4.244, P = 0.0491), consistent with the amount of food consumed by the different groups (Fig 3.5, *left panel*).

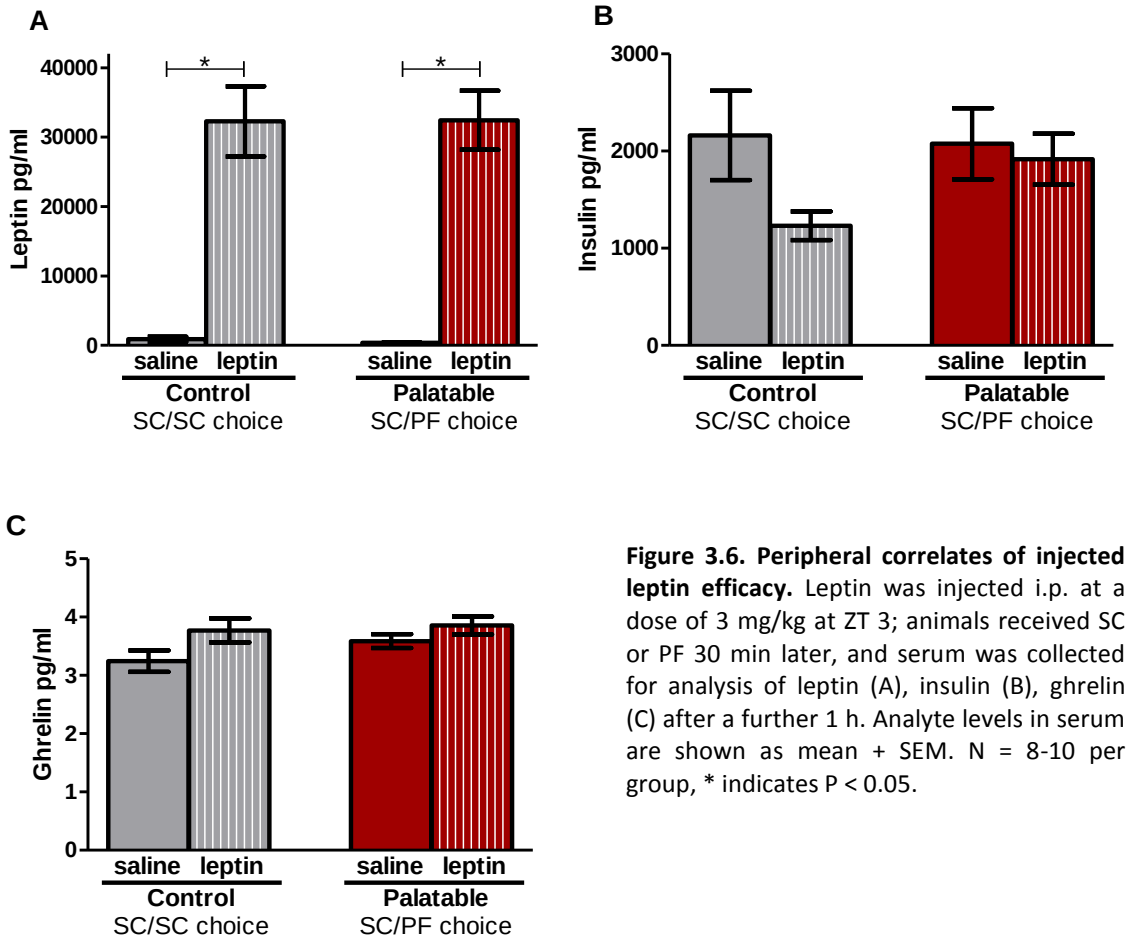


Figure 3.6. Peripheral correlates of injected leptin efficacy. Leptin was injected i.p. at a dose of 3 mg/kg at ZT 3; animals received SC or PF 30 min later, and serum was collected for analysis of leptin (A), insulin (B), ghrelin (C) after a further 1 h. Analyte levels in serum are shown as mean + SEM. N = 8-10 per group, * indicates P < 0.05.

Orexin neurons in lateral hypothalamus escape inhibition by leptin

Quantitative ISHH analysis showed that leptin fails to suppress prepro-orexin mRNA expression in the LH when 24 h-fasted mice are presented with PF in a choice paradigm or SC in a control condition; notably however, PF, but not SC, consumption was followed by increased prepro-orexin expression (Fig 3.7A; $F_{1, 20}$ = 16.92, P = 0.0005). Expression levels of another prominent LH orexigenic peptide, melanin-concentrating hormone (MCH), were not influenced by neither food type nor pharmacological treatment (Fig 3.7B). These findings suggest that while orexin expression is sensitive to the hedonic properties of food, MCH expression primarily responds to hunger.

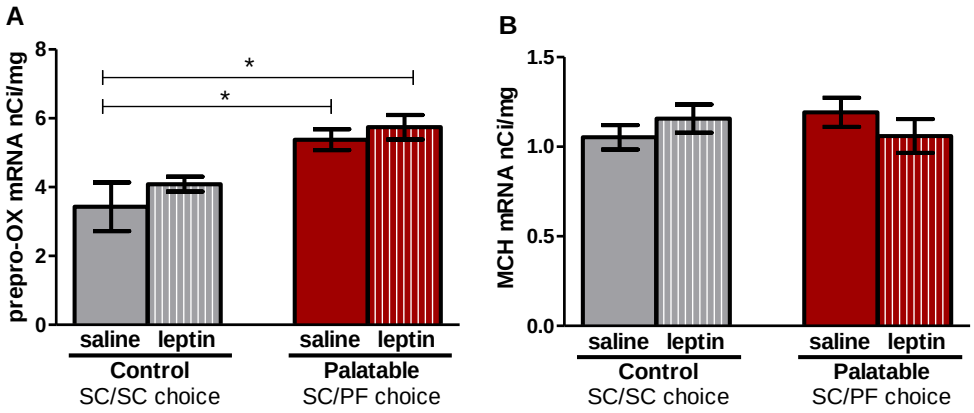


Figure 3.7. Palatable food increases (A) prepro-orexin (OX) but not (B) melanin-concentrating hormone (MCH) expression in the lateral hypothalamus. Leptin (3 mg/kg) was injected 30 min before food presentation for 1 h. N= 6-8 per group. * indicates $P < 0.05$ (Tukey's post hoc test).

Orexin A and orexin B are cleaved from prepro-orexin; although the effects of orexin A on food intake are longer-lasting than those of orexin B, the two peptides co-localize in the same neurons in the LH and have similar potencies in the regulation of feeding (Sakurai et al., 1998). Co-localization of both peptides in the cell bodies of LH orexin neurons was here confirmed in the mouse brain (Fig. 3.8).

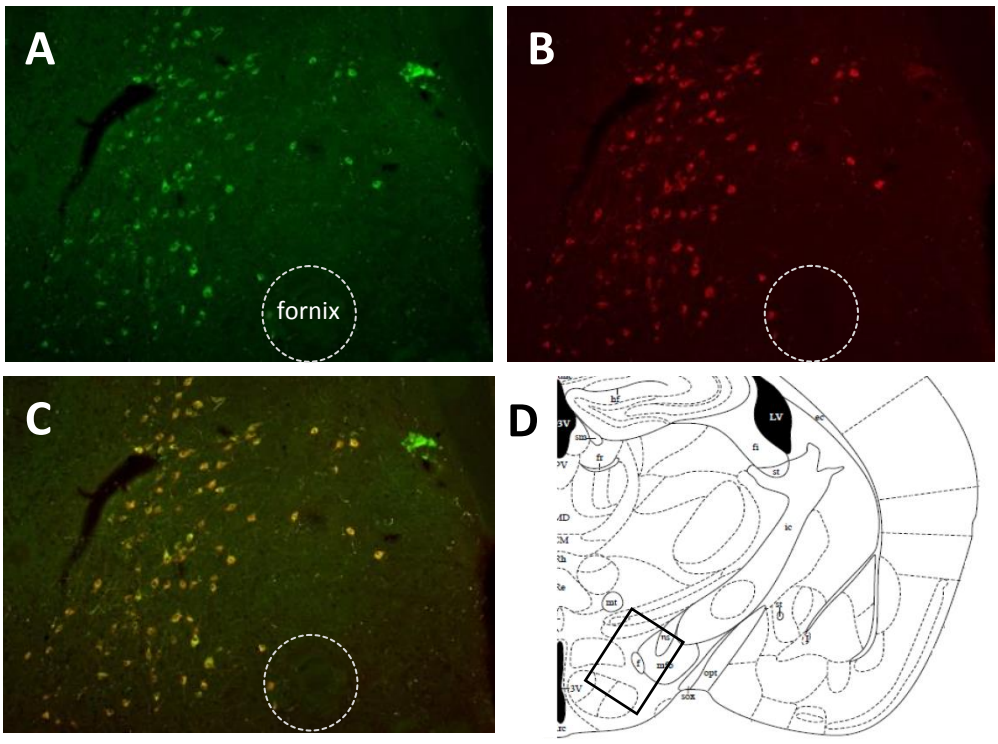


Figure 3.8. Co-localization of orexin-A and orexin-B in the lateral hypothalamus (LH) of the mouse. Representative photomicrographs of LH sections with staining against orexin-A (green) (A) and orexin-B (red) (B) and the overlay (yellow-orange) (C). Bregma -1.46 mm.

Previous studies found that leptin decreases orexin expression and orexin neuron activity (Lopez et al., 2000; Goforth et al., 2014). In the current study, leptin reduced c-Fos immunoreactive orexin cells in the LH of animals that consumed SC but failed to alter the increase in activation (c-Fos immunosignal) among LH orexin neurons of mice that consumed PF (Fig. 3.9) (drug effect: $F_{1, 21} = 27.38$, $P < 0.0001$; food type effect: $F_{1, 21} = 13.92$, $P = 0.0012$; *Drug* X *Food type* interaction: $F_{1, 21} = 12.16$, $P = 0.022$, with post hoc tests confirming differential effects of leptin on suppression of orexin neuron activation in a food type-dependent manner).

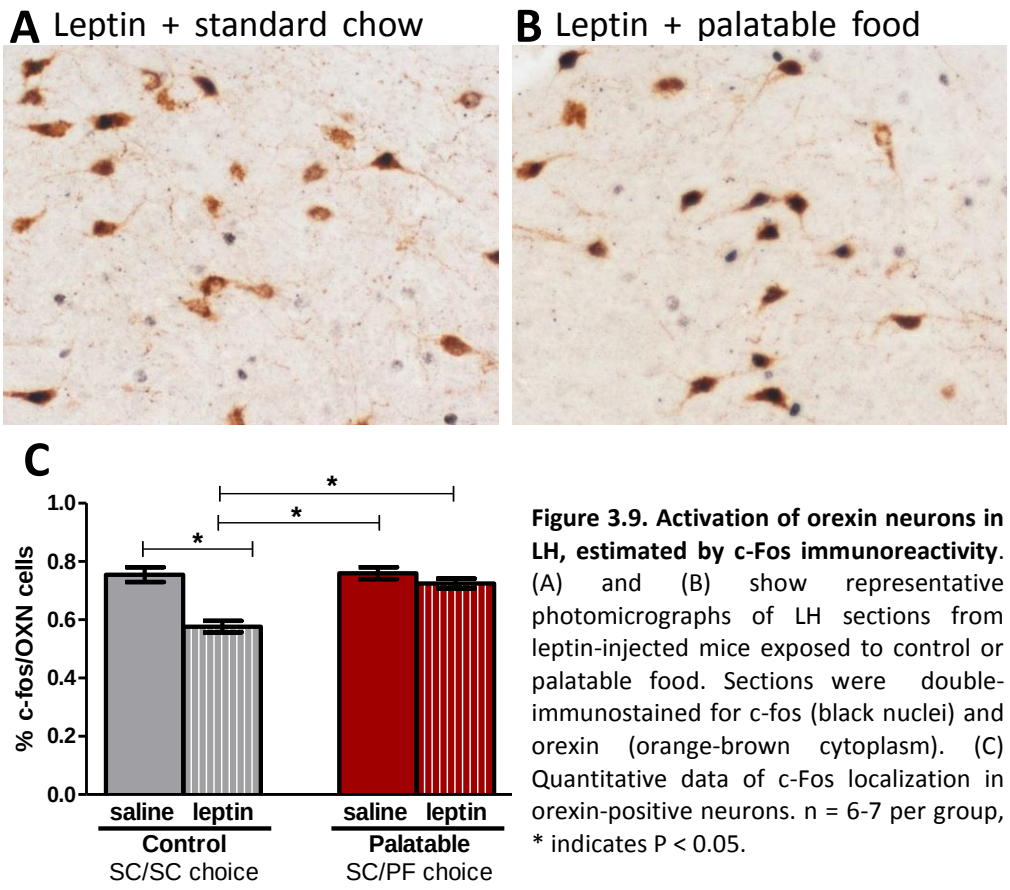


Figure 3.9. Activation of orexin neurons in LH, estimated by c-Fos immunoreactivity. (A) and (B) show representative photomicrographs of LH sections from leptin-injected mice exposed to control or palatable food. Sections were double-immunostained for c-fos (black nuclei) and orexin (orange-brown cytoplasm). (C) Quantitative data of c-Fos localization in orexin-positive neurons. n = 6-7 per group, * indicates $P < 0.05$.

Orexin peptide content is not changed in projection areas

Among other areas, LH orexin neurons project to forebrain regions that modulate hedonic feeding by associating sensory, visceral and reward-related information (Peyron et al., 1998; Marcus et al., 2001). As depicted in Fig. 3.10, orexin levels measured by radioimmunoassay on tissue punches, did not differ between the shell and core of the nucleus accumbens (NAc) and medial and lateral orbitofrontal cortex (OFC) of any of the treatment groups, i.e. neither food type choice (SC/SC *vs.* SC/PF) nor drug treatment (saline *vs.* leptin) significantly altered orexin levels at orexin neuron terminals.

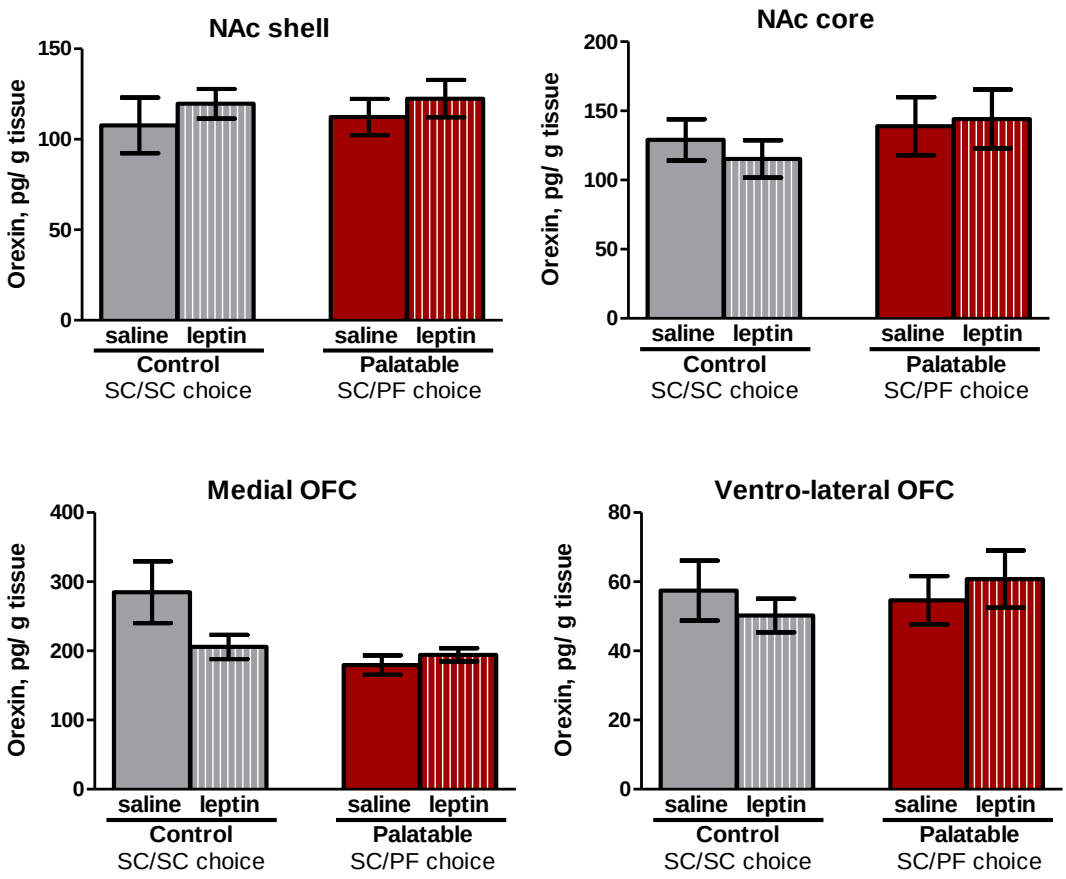


Figure 3.10. Orexin peptide content in forebrain projection areas. Upper row diplays orexin levels in NAc shell (Bregma 1.94 - 0.62 mm) and NAc core punches; lower row shows orexin levels in punches from the medial and ventro-lateral orbitofrontal cortex (OFC) (Bregma 2.80 - 1.94mm). Measurements were made in tissues obtained after mice that had been injected with saline or leptin for 30 min before being given access to either SC/SC or SC/PF choices for 1 h (n = 7 per group).

Leptin signalling is intact in the hypothalamus

To test whether the failure of leptin injections to suppress PF consumption was due to an interruption of leptin signalling mechanisms, we examined the efficacy of leptin signal transduction by measuring levels of phospho-STAT3 in the hypothalamus of animals that had been presented with SC/SC or SC/PF choices for 1 h following i.p. saline or leptin injected 30 min before food was introduced. It was found that pSTAT3 (relative to total STAT3 levels) was similarly induced in all treatment groups (Fig. 3.11): 2-way ANOVA revealed a strong main effect of leptin treatment ($F_{1, 20} = 22.08$, $P = 0.0001$), without any effect of food type or *Drug X Food type* interactions ($F_{1, 20} = 0.082$, $P = 0.77$). These quantitative data were confirmed by immunohistochemical detection of pSTAT3 signal in various hypothalamic leptin-target areas (ARC, VMH, DMH, LH) (Fig. 3.11B-I).

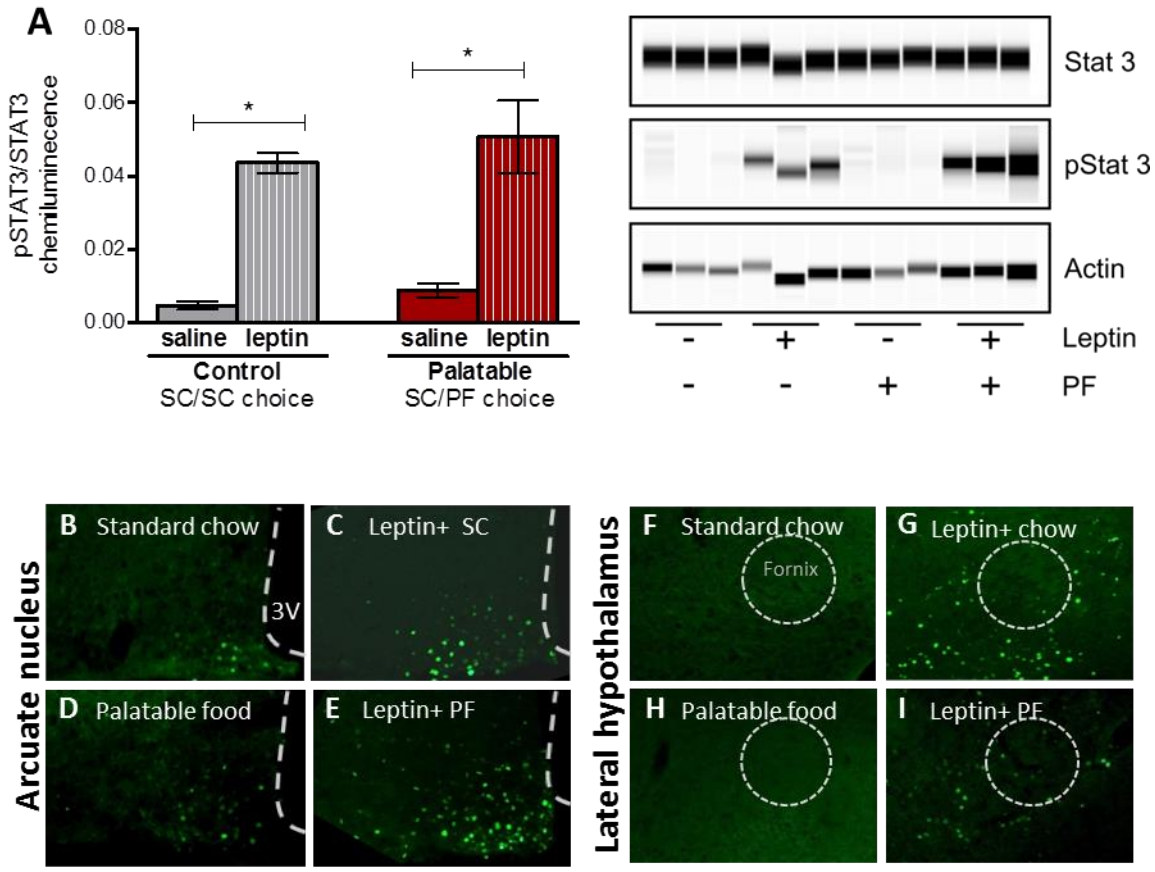


Figure 3.11. **Leptin induces phosphorylation of STAT3 in hypothalamus. (A)** Quantitative determination of pSTAT3 and total STAT3 levels by capillary electrophoresis in hypothalamic lysates *(left panel*) and lane view presentation of representative samples (*right panel*). **(B-E)** Qualitative (*right panel*) determination pSTAT3 immunoreactivity (green) in cryo-sections containing the arcuate nucleus (Arc; Bregma -1.46) from mice injected with leptin before being given access to either SC or PF for 1 h thereafter. **(F-I)** Immunoreactivity (green) in cryo-sections containing the lateral hypothalamus (LH; Bregma -1.82) from mice injected with leptin 30 min before being given access to either SC or PF for 1 h thereafter.

Further verification of the efficacy of exogenous leptin was provided by the demonstration that i.p. leptin resulted in an activation of proopiomelanocortin (POMC) neurons in the arcuate nucleus (ARC); POMC neurons are primary mediators of the anorexigenic effects of leptin (Sohn et al., 2013). As depicted in Fig. 3.12A-E, both, PF and leptin (in combination with SC and PF) resulted in increased c-Fos immunoreactivity in the POMC-immunoreactive neurons in the ARC (main effects of leptin and food type: $F_{1,\ 20}$ = 19.86, P = 0.0002 and $F_{1,\ 20}$ = 12.10, P = 0.002, respectively; no interaction between factors: $F_{1,\ 20}$ = 2.658, P = 0.12).

Lastly, since orexigenic neuropeptide Y (NPY) and anorexigenic POMC neurons reciprocally inhibit each other, NPY mRNA expression was measured after leptin injection and PF (NPY detection by immunohistochemistry requires colchicine pre-treatment which could influence the behavioural measures). As shown in Fig. 3.12F, both, leptin and PF down-regulated NPY mRNA expression, as would be expected based

on the c-Fos/POMC expression pattern (Fig. 3.12A-E; no significant main effects; *Leptin* X *Food type* interaction: $F_{1, 25} = 7.541$, $P = 0.01$).

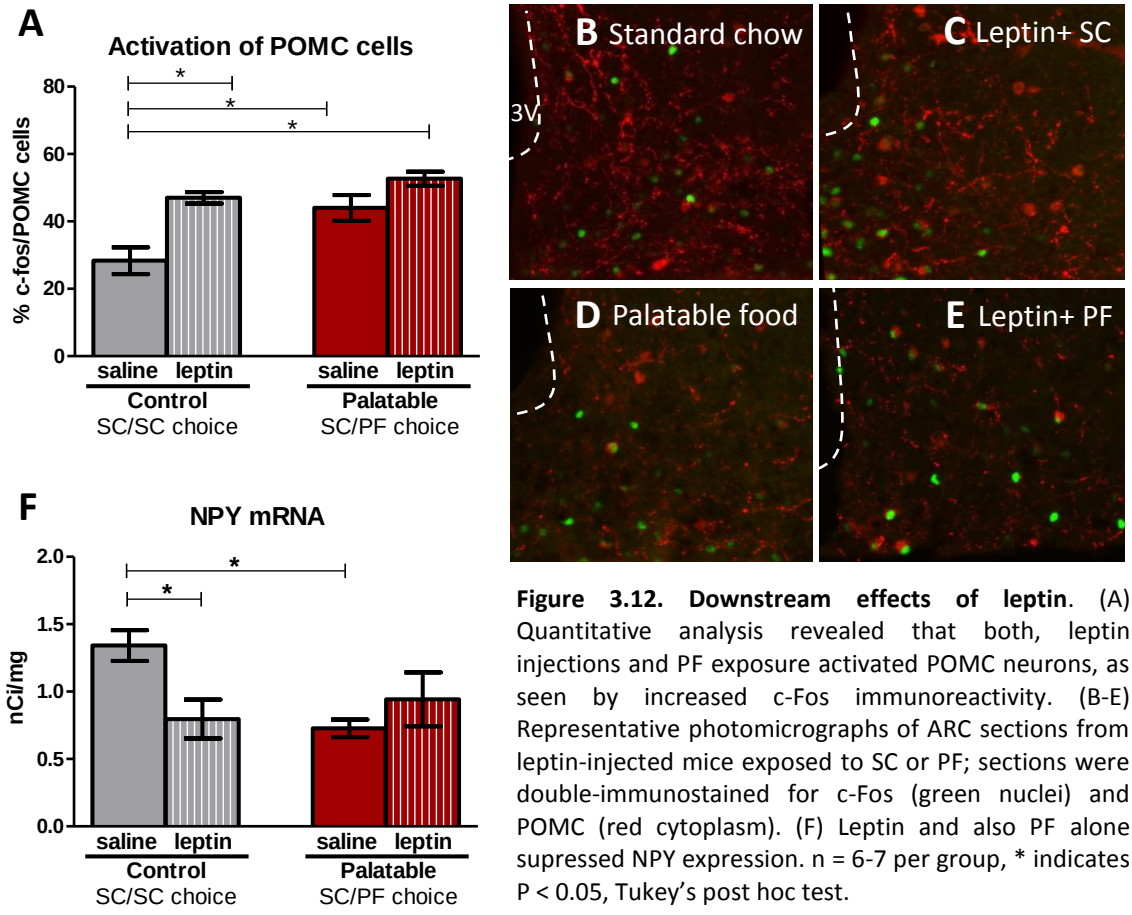


Figure 3.12. Downstream effects of leptin. (A) Quantitative analysis revealed that both, leptin injections and PF exposure activated POMC neurons, as seen by increased c-Fos immunoreactivity. (B-E) Representative photomicrographs of ARC sections from leptin-injected mice exposed to SC or PF; sections were double-immunostained for c-Fos (green nuclei) and POMC (red cytoplasm). (F) Leptin and also PF alone supressed NPY expression. n = 6-7 per group, * indicates $P < 0.05$, Tukey's post hoc test.

3.4. Discussion

The central question addressed in this set of experiments concerned the identity of the molecules (and their sites of action) whose appetite-suppressing actions are apparently countermanded by hedonic stimuli. Here, and in the previous chapter, a single exposure to an unfamiliar palatable food (PF) was shown to be sufficient to induce overeating in mice. One key finding of the present work was that, while the anorexigenic peptide leptin suppressed consumption of standard chow (SC), leptin failed to restrain PF intake in animals that were previously fasted for 24 h. Under normal physiological conditions, adipocyte secretion of leptin signals satiety and curtails eating by acting on a variety of brain centres, including the hypothalamus which serves as the central integrator of information regarding hunger, satiety and energy status (Elias et al., 1998; Sohn et al., 2013). More specifically, the lateral hypothalamus (LH) plays a critical role in the regulation of feeding behaviour by additionally integrating information about the

sensory and motivational value of appetitive stimuli as well the affective state of the individual (Kelley et al., 2005; Berthoud and Münzberg, 2011).

Leptin deficiency and leptin insensitivity are associated with overeating and obesity (Myers et al., 2010; Morton et al., 2014). However, apart from the data reported in Chapter 2, most work on the effects of leptin has been performed in subjects that were chronically exposed to an energy-dense food and leptin resistant. Here we demonstrate that the failure of leptin to limit PF intake does not reflect impairments in leptin signalling, as judged by ability of the hormone to induce phosphorylation of STAT3 (pSTAT3, a well-characterized marker of leptin receptor activation) (Banks et al., 2000; Fruhbeck, 2006) in various hypothalamic nuclei, including the LH and arcuate nucleus (ARC) when exposed to PF after a fast (Fig 3.11). The activity of both of these leptin receptor-expressing nuclei (Hakansson et al., 1998) (Fig. 3.2) was rapidly increased upon exposure of satiated mice (with presumably high leptin levels) to a hedonic food, as indicated by increases in their expression of the immediate early gene c-Fos (Table 3.2). In addition to increasing hypothalamic neural activity, hedonic food also upregulated c-Fos expression in a number of other functional brain units, including those involved in sensory perception and processing, motor functions, peripheral-brain communication, and association, learning/memory, and reward functions (Fig. 3.2, Table 3.2) (Valdivia et al., 2014).

Pro-opiomelanocortin (POMC)-expressing neurons in the ARC comprise an important part of the leptin-activated anorexigenic circuitry. For example, their activation is normally induced by leptin signalling and results in the inhibition of orexigenic NPY neurons (Velloso and Schwartz, 2011; van Swieten et al., 2014). In the choice paradigm used in these studies, both leptin and PF activated POMC cells and decreased NPY mRNA expression levels in the ARC (Fig. 3.12). It is highly likely that in addition to leptin, other peripheral anorexigenic signals triggered by PF (e.g. glucose, insulin, fatty acids and peptide YY (Batterham et al., 2002; Williams et al., 2011; Sohn et al., 2013; Magnan et al., 2015) would be operative in activating POMC neurons. Thus, the mismatch between the anorexigenic effects of leptin and PF on the ARC (expected) and lack of leptin effects on behaviour (unexpected) suggest a role for additional up- or downstream modulatory mechanisms that permit hedonic feeding in the face of a strong appetite-reducing homeostatic signal (leptin). Accordingly, we next focussed on the LH, and area endowed with orexin neurons whose activity increases in response to hunger (Yamanaka et al., 2003).

Besides the fact that the LH represents a hub from which signals descend to trigger the motoric aspects of eating (Berthoud and Münzberg, 2011), the decision to follow events in the LH was guided primarily by the observation that the LH of pre-satiated mice responded to a hedonic food with increased c-Fos expression (Fig. 3.2). Moreover, presentation of a hedonic food upregulated orexin mRNA expression in the LH of

satiated mice. In addition, presentation of a second type of PF to animals already satiated on the first (and orexin transcription had returned to baseline), again led to an increase in orexin gene expression (Fig. 3.3). These observations match those of a previous study which described orexin neuron activation in mice that ate a high-fat food despite being satiated on standard food (Valdivia et al., 2014). It should also be mentioned that, in the present study, the effects of PF exposure on LH neurons was found to be confined to orexin-expressing cells: the mRNA expression levels of another orexigenic peptide, melanin-concentrating hormone (MCH), were not altered by PF intake (Fig. 3.7). Given that leptin is known to silence orexin neuron activity and secretion (Yamanaka et al., 2003; Goforth et al., 2014), it is important to note that exogenous leptin proved ineffective against the orexin neuron-activating effects of PF consumption (Fig. 3.9), thus possibly explaining our behavioural observations (Fig. 3.5). Together, these results suggest that, in addition to their better-known role in signalling hunger, LH orexin neurons also stimulate eating of palatable stimuli, independently of satiety state.

The orexin content of various forebrain areas such as the orbitofrontal cortex (OFC, medial and ventro-lateral subdivisions) and nucleus accumbens (NAc, shell and core) which are implicated in the integration of sensory inputs and generation of hedonic drive (Rolls and Baylis, 1994; de Araujo et al., 2003; Gottfried et al., 2003; Kringelbach et al., 2003; Small et al., 2003; Norgren et al., 2006; Small, 2012; Castro et al., 2015) were unchanged by any of the food type exposures and treatment combinations tested. The LH and hedonia-processing centres are connected bidirectionally (Berthoud and Münzberg, 2011); inputs from the hedonic centres are therefore plausible conduits of hedonic overdrive and the overriding of homeostatic mechanisms that converge in the LH (cf. Saper et al., 2002; Lutter and Nestler, 2009; Berthoud, 2011; Berridge and Kringelbach, 2013). Indeed, neurons in the medial nucleus accumbens (NAc) shell, that integrate inputs from sensory- and motivation-related areas, project to the LH (Mogenson et al., 1983; Thompson and Swanson, 2010) where they inhibit orexin neurons. On the other hand, inhibition of NAc activity disinhibits orexin neurons and induces feeding (Stratford and Kelley, 1999; Saper et al., 2002; Zheng et al., 2003; Baldo et al., 2004; Krause et al., 2010). That PF presentation inhibited the NAc, leading to NAc-mediated disinhibition of LH orexin neurons cannot be dismissed on the basis of the present analysis: as PF did not increase c-Fos immunoreactivity in the NAc, it is possible that NAc was in fact inhibited and that led to orexigenic effects (at present, there are no markers that allow histochemical mapping of neuronal inhibition; immediate early gene markers only reflect activation).

Strong activation (c-Fos immunoreactivity) of the ventral pallidum (VP), a so-called "hedonic hotspot" (Ho and Berridge, 2014) was observed when mice were given access to a hedonic food (Fig. 3.2, Table 3.2). The VP is innervated by orexigenic neurons originating in the LH which appear to be responsible for the orofacial expression of

"liking" of pleasure-giving foods (Ho and Berridge, 2013; Castro et al., 2015). The VP is connected to the NAc by GABAergic afferents and efferents (Smith and Berridge, 2007). The NAc itself receives direct and indirect inputs, albeit neurochemically still poorly characterized, from sensory areas (e.g. insula, piriform cortex) as well as the orbitofrontal cortex (OFC); the latter structure plays an important role in integration of sensory stimuli, value attribution, expectation and decision making, and is therefore viewed as a critical link between the reception and consumption of rewarding stimuli (e.g. food) and feelings of pleasure (hedonia) (Gottfried et al., 2003; Kringelbach et al., 2003; Small et al., 2003; Small, 2012; Rudebeck et al., 2013). Based on these chemo-cytoarchitectural arrangements and the present results, it is proposed that, following consumption of a PF, the NAc receives inputs from higher sensory-valuation centres and, in turn, disinhibits orexin neurons in the LH. The circuit is closed by orexinergic projections to the VP and probably, the VTA, thus enhancing "liking" and motivation to eat ("wanting") (Zheng et al., 2007). We hypothesize that the LH may serve as a point of convergence of homeostatic and hedonic signals and play a central part in driving the excessive ingestion of PF.

A key question that remains open concerns the mechanism(s) through which hedonic processes nullify the appetite-suppressing actions of leptin during PF exposure; these effects of leptin normally result from its ability to induce POMC-derived anorexigenic α-melanoctye-stimulating hormone (α-MSH) while blocking the orexigenic actions of ARC-produced NPY and LH-produced orexin. Since leptin signalling was not interrupted in the hypothalamus (increased pSTAT3 levels), the possibility that effects of leptin on a subpopulation of LH neurons that express galanin/neurotensin/GABA neurons are impaired seems plausible. The latter neurons were previously shown to innervate (Louis et al., 2010; Laque et al., 2013) and inhibit orexin expression and release (Leinninger et al., 2009; Goforth et al., 2014) from neighbouring cells; interestingly, electrophysiological studies on hypothalamic slices demonstrated that leptin-dependent hyperpolarization of orexinergic neurons can be enhanced by galanin, but not by neurotensin or GABA (Goforth et al., 2014). Experiments reported in the following chapter (Chapter 4) aimed at examining the role of galanin-expressing neurons in the LH in the abrogation of leptin efficacy in the face of PF-generated hedonic signals.

3.5. Supplementary observations

- Proinflammatory cytokines
- Mesocorticolimbic dopamine system activity

Proinflammatory cytokines

Two pro-inflammatory cytokines, tumour necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6), are subject to modulation by exposure to energy dense foods[3]. In the absence of a leading hypothesis of what factors might lead to hedonic-driven overeating, preliminary investigations examined the serum levels of these two cytokines, taking advantage of their inclusion in the multiplex assay kit used (see Chapter 3, Materials & Methods) (Figure S 3.1).

Leptin treatment increased serum TNF-α levels (main effect $F_{1, 29} = 17.10$, $P = 0.0003$) after both SC and PF intake, and post-hoc tests revealed the increase was statistically significant only after eating SC. Levels of IL-6 were also elevated in response to leptin (main effect $F_{1, 32} = 9.552$, $P = 0.0041$, post-hoc tests confirmed the increase after PF) with no main effect of food type and no interaction between the factors. These results make it unlikely that TNF-α and IL-6 contribute to the mechanisms that lead to the overriding of leptin signals. Nevertheless, since obesity is accompanied by elevated leptin levels and (frequently) by systemic inflammation, these inflammatory processes have been suggested to participate in the development of insulin and leptin resistance and weight[4] gain; thus, the present observations might be worthy of future study, especially because elevated leptin levels may also contribute to the development of

[3] De Git KC & Adan R (2015) Leptin resistance in diet-induced obesity: the role of hypothalamic inflammation. ***Obesity rev*** *16*, 207-224.; Weisberg SP et al., (2003) Obesity is associated with macrophage accumulation in adipose tissue. ***J Clin Invest*** 112, 1796-1808.; Xu H et al., (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. ***J Clin Invest*** 112, 1821-1830.

[4] Buettner C et al (2008) Leptin controls adipose tissue lipogenesis via central, STAT3-independent mechanisms. ***Nat Med*** 14, 667-675; Loffreda S et al (1998) Leptin regulates proinflammatory immune responses. ***FASEB J*** 12, 57-65; Mantzoros CS et al (1997) Leptin concentrations in relation to body mass index and the tumor necrosis factor-alpha system in humans. ***J Clin Endocr Metab*** 82, 3408-3413; Olofsson LE et al (2013) Modulation of AgRP-neuronal function by SOCS3 as an initiating event in diet-induced hypothalamic leptin resistance. ***Proc Natl Acad Sci USA*** 110, E697-706; Shi X et al (2013) Nuclear factor kappaB (NF-kappaB) suppresses food intake and energy expenditure in mice by directly activating the Pomc promoter. ***Diabetologia*** *56*, 925-936.; Senn JJ et al (2003) Suppressor of cytokine signaling-3 (SOCS-3), a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes. ***J Biol Chem*** 278, 13740-13746; Weisberg SP et al (2003) Obesity is associated with macrophage accumulation in adipose tissue. ***J Clin Invest*** 112, 1796-1808; Xu H et al (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. ***J Clin Invest*** 112, 1821-1830; You T et al (2005) Abdominal adipose tissue cytokine gene expression: relationship to obesity and metabolic risk factors. ***Am J Physiol*** 288, E741-7.

hypothalamic inflammation and subsequent obesity[5] by initially inducing inflammation in the periphery.

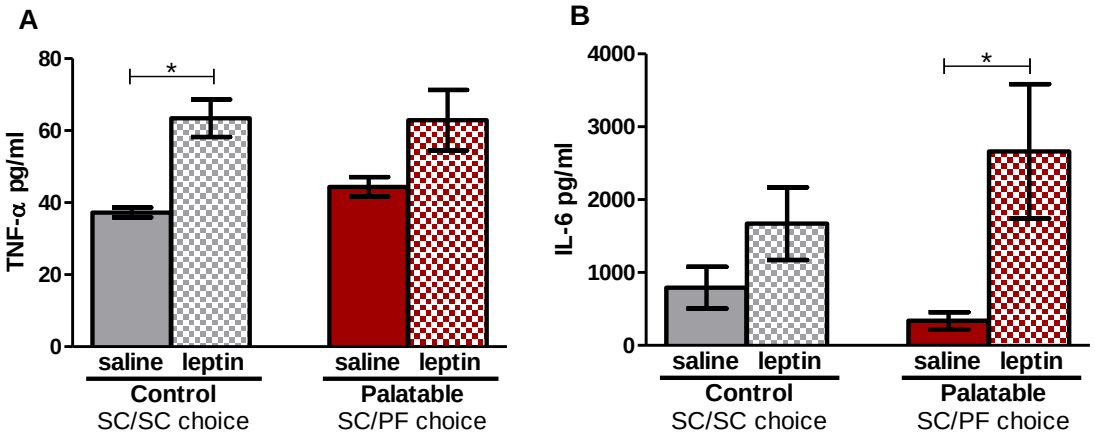


Figure S 3.1. Peripheral correlates of injected leptin efficacy. Leptin was injected i.p. at a dose of 3 mg/kg at ZT 3; animals received SC or PF 30 min later, and serum was collected for analysis of TNF-α (A), IL-6 (B) after a further 1 h. Analyte levels in serum are shown as mean + SEM. N = 8-10 per group, * indicates $P < 0.05$.

[5] Thaler et al. (2012) Obesity is associated with hypothalamic injury in rodents and humans. ***J Clin Invest*** 122:153–162.

Mesocorticolimbic dopamine system responses

Elevated levels of dopamine (DA) are required for motivation, learning and motor action[6]. The theories suggesting that overeating is similar to drug addiction consider dysfunction in dopaminergic transmission to be causal to obesity[7]. Other data which are relevant to our central question show that leptin decreases the motivation to eat by inhibiting DAergic cell bodies in ventral tegmental area (VTA), thus reducing mesocorticolimbic DA tone[8]. The pilot studies reported here were done on the premise that the loss of leptin efficacy during hedonic feeding might be accompanied by alterations in DAergic activity in the VTA and its projection areas. Dopamine and its metabolites were assayed (reverse-phase high performance liquid chromatography [HPLC] with electrochemical detection)[9] in punches from the orbitofrontal cortex and nucleus accumbens (NAc) obtained from animals treated as shown in Scheme 3.2.

The VTA projects to the frontal cortex and nucleus accumbens (NAc)[10]. The NAc and orbitofrontal cortex (OFC, within the frontal cortex) are implicated in the integration of

[6] Kosobud AE et al. (1994) Behavioral associations of neuronal activity in the ventral tegmental area of the rat. ***J Neurosci****:* 14:7117–7129; Wise RA (2005) Forebrain substrates of reward and motivation. ***J Comp Neurol*** 493:115–121; Shimura T et al. (2002) Ventral tegmental lesions reduce overconsumption of normally preferred taste fluid in rats. ***Behav Brain Res*** 134:123–130; Touzani K et al. (2008) Activation of dopamine D1-like receptors in nucleus accumbens is critical for the acquisition, but not the expression, of nutrient-conditioned flavor preferences in rats. ***Eur J Neurosci*** 27:1525–1533.

[7] Volkow ND et al. (2008) Overlapping neuronal circuits in addiction and obesity: evidence of systems pathology. ***Phil Trans of Royal Soc London.*** *Series B, Biological sciences* 363:3191–3200; Gipson CD et al. (2013) Relapse induced by cues predicting cocaine depends on rapid, transient synaptic potentiation. ***Neuron*** 77:867–872.

[8] Figlewicz DP et al. (2003) Expression of receptors for insulin and leptin in the ventral tegmental area/substantia nigra (VTA/SN) of the rat. ***Brain Res*** 964:107–115; Hommel JD et al. (2006) Leptin receptor signaling in midbrain dopamine neurons regulates feeding. ***Neuron*** 51:801–810; Morton GJ et al.(2009) The action of leptin in the ventral tegmental area to decrease food intake is dependent on Jak-2 signaling. ***Am J Phys Endo Metab*** 297:E202-10.

[9] <u>Catecholamine measurements:</u> *Tissue levels of dopamine (DA) and DA metabolites were estimated in bilateral micropunches of selected brain areas (see Scheme 3.3). Tissue lysates were those used to assay orexin-A (Section 3.2). High performance liquid chromatography (HPLC) was used to estimate DA, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytryptamine (3-MT) and homovanillic acid (HVA), 5 levels in tissue lysates. The reverse-phase HPLC protocol with electrochemical detection (UltiMate3000 / CoulochemIII, ThermoFischer, USA) was developed by Elmira Anderzhanova (Max Planck Institute of Psychiatry, Munich). All reagents used for the mobile phase were of analytical grade (Carl Roth GmbH or MERCK KGaA, Germany). A citrate-phosphate mobile phase containing 8.5 % of acetonitrile (pH 3.00) was used. Monoamines were separated on an analytical column (C18, 150 mm×3 mm, 3 um, YMC Triart, YMC Europe GmbH, Germany) at a flow rate of 0.5 ml/min. The potentials of the working-electrodes were set at -75 mV +220 mV, the guard cell potential was set at +350 mV. Monoamine concentrations were calibrated against peak areas obtained for respective external standards. The detection limits for all compounds were between 0.032 and 0.050 nM Anderzhanova et al. (2013). Absolute neurotransmitter levels were normalized to the protein concentration in individual punches (Lowry assay).*

[10] Wise RA (2005) Forebrain substrates of reward and motivation. ***J Comp Neurol*** 493:115–121.

sensory inputs and generation of hedonic drive which are modulated by dopamine[11]. The activity of a neurotransmitter system may be indirectly assessed by measuring turnover of the transmitter[12]. Dopamine metabolism results in monoamine oxidase (MAO)-catalysed 3,4-dihydroxyphenylacetic acid (DOPAC) and via catechol-*OI*-methyltransferase (COMT), in the intermediate metabolite 3-methoxytryptamine (3-MT); both metabolites are subsequently metabolised by COMT into homovanillic acid (HVA). Turnover rates of DA were estimated from ratios of the DA metabolites to total DA (DOPAC:DA, 3-MT:DA, HVA:DA, (DOPAC + 3-MT + HVA):DA). In the current experiment, the only parameter that showed significant changes in response to PF or leptin treatment was DA turnover estimated by 3-MT/DA (Fig. S 3.2). Since COMT converts DA to 3-MT at extraneuronal sites, brain levels of 3-MT reflect DA released at synaptic terminals, providing an accurate measure of DA release when estimated in brain lysates[13].

Activation of the medial OFC correlates with exposure to high-caloric stimuli, especially in obese people[14]; additionally, greater medial OFC volume is associated with adaptive taste reinforcement-learning (presumably in a DA-dependent fashion) in lean but not obese subjects[15], although some reports also show reduced medial OFC activity in obese subjects vs. lean experiencing the sensory components of food[16]. Here, PF increased DAergic tone (3-MT/DA) in medial OFC ($F_{1, 24}$ = 11.08, P = 0.0028), whereas leptin did not

[11] Rolls ET, Baylis LL (1994) Gustatory, olfactory, and visual convergence within the primate orbitofrontal cortex. ***J Neurosci*** 14:5437–5452; de Araujo, Ivan E, Rolls ET, Kringelbach ML, McGlone F, Phillips N (2003) Taste-olfactory convergence, and the representation of the pleasantness of flavour, in the human brain. ***Eur J Neurosci*** 18:2059–2068; Gottfried JA et al. (2003) Encoding predictive reward value in human amygdala and orbitofrontal cortex. ***Science*** 301:1104–1107; Kringelbach ML et al. (2003) Activation of the human orbitofrontal cortex to a liquid food stimulus is correlated with its subjective pleasantness. ***Cereb cortex*** 13:1064–1071; Small DM et al. (2003) Feeding-induced dopamine release in dorsal striatum correlates with meal pleasantness ratings in healthy human volunteers. ***NeuroImage*** 19:1709–1715; Norgren R et al. (2006) Gustatory reward and the nucleus accumbens. ***Phys Behav*** 89:531–535; Small DM (2012) Flavor is in the brain. ***Phys Behav*** 107:540–552; Castro DC et al. (2015) Lateral hypothalamus, nucleus accumbens, and ventral pallidum roles in eating and hunger: interactions between homeostatic and reward circuitry. ***Front Syst Neurosci*** 9:90.

[12] Antkiewicz-Michaluk L et al. (2001) Different action on dopamine catabolic pathways of two endogenous 1,2,3,4-tetrahydroisoquinolines with similar antidopaminergic properties. ***J Neurochem*** 78:100–108. Cooper JR et al. (2003) Chapter 9. Dopamine. Dopamine metabolism. Biochemical Basis of Neuropharmacology.: Oxford University Press.

[13] Wood PL, Altar CA (1988) Dopamine release in vivo from nigrostriatal, mesolimbic, and mesocortical neurons: utility of 3-methoxytyramine measurements***. Pharm Rev*** 40:163–187; Roth JA (1992) Membrane-bound catechol-O-methyltransferase: a re-evaluation of its role in the O-methylation of the catecholamine neurotransmitters. ***Rev Phys Biochem Pharm*** 120:1–29; Cooper JR et al. (2003) Chapter 9. Dopamine. Dopamine metabolism. Biochemical Basis of Neuropharmacology: Oxford University Press

[14] Stoeckel LE, et al. (2008) Widespread reward-system activation in obese women in response to pictures of high-calorie foods. ***NeuroImage*** 41:636–647

[15] Shott ME et al. (2015) Orbitofrontal cortex volume and brain reward response in obesity. ***Int J Obesity (2005)*** 39:214–221.

[16]DelParigi et al. (2005) Sensory experience of food and obesity: a positron emission tomography study of the brain regions affected by tasting a liquid meal after a prolonged fast. ***NeuroImage*** 24:436–443; Karhunen LJ et al.(2000) Regional cerebral blood flow during exposure to food in obese binge eating women. ***Psych Res*** 99:29–42;

influence this parameter ($F_{1, 24}$ = 0.1748, P = 0.68) (Fig S 3.2A). Together with the previous (albeit contrasting reports), we hypothesize that elevated DA release in the medial OFC may be involved in generating hedonia-driven appetite, a subject worthwhile of future studies on the modulation of hedonia by leptin; alternatively, it is

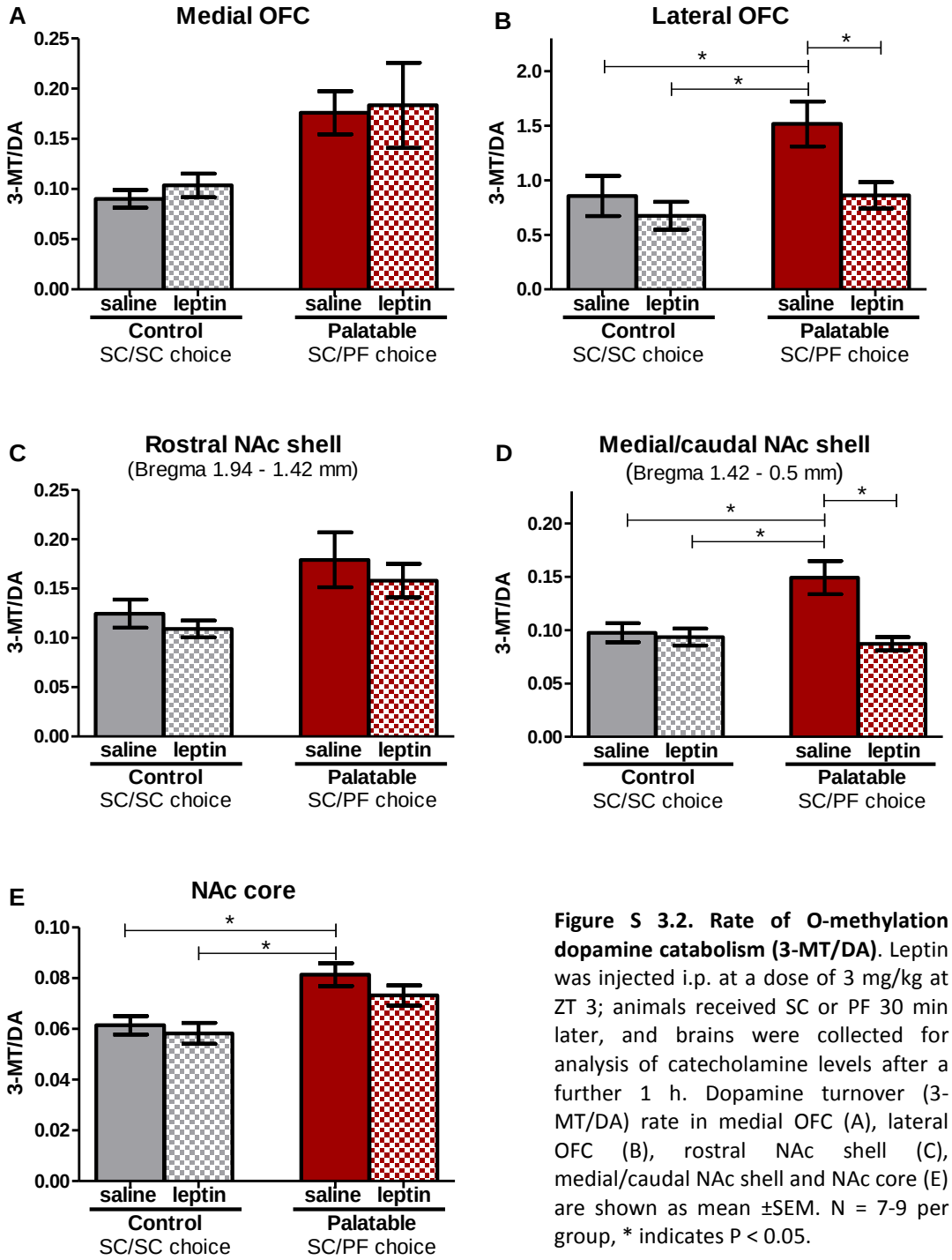


Figure S 3.2. Rate of O-methylation dopamine catabolism (3-MT/DA). Leptin was injected i.p. at a dose of 3 mg/kg at ZT 3; animals received SC or PF 30 min later, and brains were collected for analysis of catecholamine levels after a further 1 h. Dopamine turnover (3-MT/DA) rate in medial OFC (A), lateral OFC (B), rostral NAc shell (C), medial/caudal NAc shell and NAc core (E) are shown as mean ±SEM. N = 7-9 per group, * indicates P < 0.05.

possible that DA in the medial OFC mediates appropriate sensory learning processes[17] and thus contributes to the acquisition of sustained food preferences.

Dopamine turnover (3-MT:DA) in the ventro-lateral OFC was increased upon exposure to PF, but was decreased after leptin administration to levels seen in animals exposed to control food (SC) (Fig S 3.2B) (main effect of food type: $F_{1, 23} = 6.987$, $P = 0.015$; drug effect: $F_{1, 23} = 6.765$, $P = 0.016$; *drug* X *food type* interaction: $F_{1, 23} = 2.168$, $P = 0.15$). Not much is known about the contributions of the lateral OFC to feeding; however, human studies suggest that increased activation of this subregion reflects its greater sensitivity to food reward[18] and that changes in dopamine signalling efficacy in the lateral OFC correlate with greater body weight gain[19]. If an elevated DAergic tone drives feeding, then decreased DAergic activity in the lateral OFC seen here would predict decreased PF consumption.

The rostral NAc shell is especially relevant for hedonic feeding; as expected, we found elevated DA turnover in this region after PF consumption; however, leptin did not have any effect on the consumption of SC or PF (Fig S 3.2C) (food type: $F_{1, 25} = 7.234$, $P = 0.0126$; drug effect: $F_{1, 25} = 0.8978$, $P = 0.35$). Previously, injections of the μ-opioid receptor agonist DAMGO (D-ala^2-N-Me-Phe^4-Glycol^5-enkephalin) into the rat rostrodorsal part of the NAc shell were reported to enhance the expression of hedonic "liking" responses to the taste of an appetitive sucrose solution[20]. In contrast, we observed that leptin decreases PF-triggered DA turnover in the medial/caudal NAc shell (drug effect: $F_{1, 24} = 10.32$, $P = 0.0037$; food type: $F_{1, 24} = 4.862$, $P = 0.0373$). Projections from the rostrodorsal NAc shell are similar to those of the rest of the NAc, except that the rostral NAc shell innervates the lateral preoptic area and the lateral hypothalamus (LH); the latter are dense and overlap extensively with those of the lateral septum[21]. Importantly, we found that the lateral septum and LH are activated by hedonic experience (Fig. 3.2, Table 3.2) and that orexin neurons in the LH are the most likely location where leptin fails to act when PF is available (Fig. 3.5). The rostral NAc shell

[17] Stoeckel LE, et al. (2008) Widespread reward-system activation in obese women in response to pictures of high-calorie foods. ***NeuroImage*** 41:636–647; Shott ME et al. (2015) Orbitofrontal cortex volume and brain reward response in obesity. ***Int J Obesity*** *(2005)* 39:214–221.

[18] Stice E et al. (2009) Relation of obesity to consummatory and anticipatory food reward. Phys Behav 97:551–560; Stoeckel LE, et al. (2008) Widespread reward-system activation in obese women in response to pictures of high-calorie foods. ***NeuroImage*** 41:636–647.; Rothemund Y et al. (2007) Differential activation of the dorsal striatum by high-calorie visual food stimuli in obese individuals. ***NeuroImage*** 37:410–421.

[19] Stice E et al. (2010) Reward circuitry responsivity to food predicts future increases in body mass: moderating effects of DRD2 and DRD4. ***NeuroImage*** 50:1618–1625.

[20] Pecina S, Berridge KC (2005) Hedonic hot spot in nucleus accumbens shell: where do mu-opioids cause increased hedonic impact of sweetness? ***J Neurosci*** 25:11777–11786.

[21] Zahm DS, et al. (2013) On lateral septum-like characteristics of outputs from the accumbal hedonic "hotspot" of Peciña and Berridge with commentary on the transitional nature of basal forebrain "boundaries". ***J Comp Neurol*** 521:50–68.

could thus be one of the key regions through which hedonic feeding is driven or amplified.

Our experiments also revealed increased DA turnover in the NAc core after PF, an effect that was not modulated by leptin upon exposure to either SC or PF (Fig S 3.2E) (food type: $F_{1, 24} = 18.35$, $P = 0.0003$; drug effect: $F_{1, 24} = 1.956$, $P = 0.1748$). By acting in this structure, DA mediates the acquisition and/or performance of classical conditioning behaviour; in addition, the Nac core is implicated in compulsive overeating[22]. The increased levels of DA in the NAc core observed in our experiment suggest that PF preference may be initiated in the NAc core.

There are varying views about the role of DA in reward, with some studies suggesting that increased DA release in the NAc is not necessary for rodents to learn to recognise palatable food or to mediate predictive associations[23]. This view is supported by the results of a recent study in which optogenetic stimulation of DA neurons in the VTA facilitated instrumental responses in a food seeking operant task, but did not increase the amount of food consumed[24]. It is also important to note, that DA (and other neurotransmitters) does not produce behaviours (e.g. motor response or reward), but rather acts as a neuromodulator that adjusts the response to variable input signals of the state of the organism[25].

[22] Brown RM et al. (2015) Addiction-like synaptic impairments in diet-induced obesity. ***Biol Psy***.; Di Ciano P et al. (2001) Differential involvement of NMDA, AMPA/kainate, and dopamine receptors in the nucleus accumbens core in the acquisition and performance of pavlovian approach behavior. ***J Neurosci*** 21:9471–9477.

[23] Berridge KC, Robinson TE (1998) What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? ***Brain Res*** 28:309–369; Aberman JE, Salamone JD (1999) Nucleus accumbens dopamine depletions make rats more sensitive to high ratio requirements but do not impair primary food reinforcement**.** ***Neurosci*** 92:545–552; Cannon CM, Palmiter RD (2003) Reward without dopamine. ***J Neurosci*** 23:10827–10831.

[24] Adamantidis AR, Tsai H, Boutrel B, Zhang F, Stuber GD, Budygin EA, Touriño C, Bonci A, Deisseroth K, Lecea L de (2011) Optogenetic interrogation of dopaminergic modulation of the multiple phases of reward-seeking behavior. ***J Neurosci*** 31:10829–10835.

[25] Cooper JR et al. (2003) Chapter 9. Dopamine. Dopamine metabolism. Biochemical Basis of Neuropharmacology.: Oxford University Press.

CHAPTER 4

POTENTIAL OF GALANIN TO RESTORE ANOREXIGENIC EFFECTS OF LEPTIN DURING EXPOSURE TO A HEDONIC FOOD

Abstract

Our previous work indicated that disinhibition of orexin neurons in the lateral hypothalamus (LH) may underlie overconsumption of an unfamiliar palatable high-fat high-carbohydrate food (palatable food, PF), due to an overriding of the appetite-suppressing effects of leptin. In this study, we tested the possibility of an intermediary role for galanin neurons in this phenomenon. Leptin signalling to orexin neurons in the LH is indirect, and depends on leptin receptor-mediated effects on neighbouring galanin (GAL) cells; recent studies have shown that galanin, a peptide usually associated with increased food ingestion, inhibits orexin neurons and supresses feeding behaviour. Here, we confirmed that galaninergic neurons indeed terminate in the vicinity of orexin neurons and that microinjections of a galanin receptor 2 (Gal2R) agonist (M1145) into the LH at least partially restores the ability of leptin to reduce PF intake. These initial results support the view that the LH is a site at which leptin signals encounter conflicting (e.g. hedonic) inputs. These novel insights highlight the need for more research into the nature and mode of action of extra-hypothalamic inputs that signal reward to the LH as well as the intricate intrahypothalamic circuitry and mechanisms that sense satiety and coordinate go/no-go decisions about feeding.

Highlights

- Hedonic food signals converge on the lateral hypothalamus (LH) and compete with leptin-mediated signals of satiety.
- Leptin appear to inhibit orexinergic neurons in the LH via galanin receptor 2 mediation.

4.1 Introduction

We previously postulated (Chapter 3) that palatable food (PF) interferes with the ability of leptin to exert its anorexigenic effects by interrupting a leptinergic pathway(s) that inhibit orexin neurons in the lateral hypothalamus (LH); the latter are known to increase arousal and food intake (Yamanaka et al., 2003). Our previous study showed that orexin neurons are activated (increased c-Fos expression) after exposure to PF despite the presence of leptin (Fig. 3.9). Although orexin neurons themselves do not bear leptin receptors (LepRb) (Louis et al., 2010), recent evidence indicates that they are indirectly regulated by leptin (Goforth et al., 2014). Indeed, orexin neurons in the LH are densely innervated by neighbouring LepRb-positive neurons (Louis et al., 2010; Leinninger et al., 2011) which co-express the neuropeptides galanin (Gal) and neurotensin (Nts) as well as the inhibitory neurotransmitter GABA (Laque et al., 2013). Importantly, a subsequent elegant study using DREADD (designer receptor exclusively activated by designer drugs), $LepRb^{Nts}KO$ mice and electrophysiological analyses of hypothalamic slices revealed that orexin neurons are not subject to GABA-dependent inhibition but rather, are subject to hyperpolarization via K_{ATP} channels and presynaptic inhibition of excitatory neurotransmission (Goforth et al., 2014). The authors of that work argued that, since Nts excites orexin neurons, Nts is unlikely to mediate the effects of leptin on orexin and instead demonstrated that Gal receptor signalling is required for leptin to exert its effects on orexinergic neuron activity (Goforth et al., 2014).

The orexigenic properties of Gal are well described (Crawley et al., 1990; Kyrkouli et al., 1990; Schick et al., 1993; Saar et al., 2011), especially with respect to preferential consumption of high-fat foods over high-carbohydrate options (Tempel et al., 1988; Kyrkouli et al., 1990; Leibowitz, 2005; Adams et al., 2008). It should be noted, however, that while many of the early reports on the orexinergic effects of Gal were based on intra-cerebroventricular administration of the peptide, a few studies addressed the site-specificity of its effects. Gal microinjections into the rat PVN and medial preoptic (mPOA) nucleus (Tempel et al., 1988; Patterson et al., 2006), but not into the arcuate (ARC), dorsomedial (DM), rostral preoptic (rPOA) nuclei or the LH (Patterson et al., 2006), elicited feeding behaviour. It should also be mentioned that most of the research on the appetite-modulating effects of Gal has been conducted in rats, raising questions regarding the validity of generalizations across species; as an example, galanin-like peptide (GALP) exerts opposite effects in mice and rats (Shioda et al., 2011). Indeed, Patterson et al., (Patterson et al., 2006) failed to detect any effects of intra-LH injections of Gal on feeding in rats, in contrast to the above-mentioned studies which demonstratie that, in the mouse LH, leptin activates Gal neurons which, in turn, inhibit orexin neuron activity and suppress feeding (Laque et al., 2013; Goforth et al., 2014). Notwithstanding the potential importance of species differences and site-specific effects, it appears that Gal dosage may also determine the directionality of effects (induction *vs.* suppression of feeding); for example, one study demonstrated that

intraventricular Gal may exert biphasic effects on feeding, with low and high doses of the peptide acting to, respectively, inhibit and stimulate feeding (Parrado et al., 2007).

Following up on the observations that Gal can inhibit orexin neurons in a leptin-dependent fashion (Laque et al., 2013; Goforth et al., 2014), and given our own observations that presentation of a hedonic food does not interfere with leptin signalling in its hypothalamic targets (upregulation of pSTAT3 in the LH and ARC, as reported in Chapter 3), we here asked whether inadequate inhibitory galaninergic inputs to orexin neurons might account for the inability of leptin to suppress PF ingestion. The rationale for this study included the suggestion that the LH integrates hedonic and metabolic information (Berthoud and Münzberg, 2011) (Chapter 3). Two of the three known galanin receptors (Gal1R and Gal2R) are expressed abundantly in the hypothalamus (Bjorklund et al., 2002; Hökfelt, 2010) where they may act individually or as heteromers (Fuxe et al., 2012). Here, we examined whether the intra-LH administration of either a Gal1R (M617; Lundström et al., 2005; Blackshear et al., 2007) or a Gal2R (M1145; Runesson et al., 2009; Saar et al., 2011) agonist (each at two doses) could restore leptin-induced suppression of feeding when fasted mice were presented with a PF.

4.2 Materials and Methods

Animals

Experiments were performed on male C57BL/6 mice purchased at the age of 2 months (Charles River Laboratories, Sulzfeld, Germany); animals were used after at least 2 weeks of acclimation to the animal facility of the Max Planck Institute of Psychiatry (Munich, Germany). Mice were single-housed under a daily 12 h light-12 h dark cycle. Food and water were available *ad libitum* throughout, unless specified otherwise. Procedures on animals were approved by the local ethical commission (State Government of Upper Bavaria) and complied with EU Directive 2010/63/*EU*.

Test foods

During acclimation and unless specified otherwise, animals were maintained on standard chow (SC, 1320, 2.84 kcal/g, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany). To evaluate hedonic feeding, food consumption was measured over a period of 1 h when animals had access to SC and palatable high-fat/high sucrose food (PF; D12451, ResearchDiets Inc., New Brunswick, NJ, USA, 4.73 kcal/g, 45% fat) simultaneously.

Drugs

Galanin receptor 1 (M617) and 2 (M1145) agonists were obtained from Tocris (Bristol, UK). The compounds were dissolved in vehicle (sterile saline).

Intra-LH microinjections

A total of 39 single-housed male C57BL/6J mice were used in the study. Mice were anaesthetized with isofluorane, placed in a stereotaxic frame (Stoelting Co., Wood Dale, USA) and subcutaneously injected with an analgesic (0.5 mg/kg, Metacam, Braun Melsungen AG, Melsungen, Germany) and atropine sulphate (0.5 mg/kg, Atropinsulfat, Braun, Melsungen AG). Bilateral intra-LH cannulation involved placement of guide cannulae with stylets (Plastics One, Roanoke, VA); the coordinates used to target the LH were A/P: −1.5, M/L ± 1 and D/V −5.0 from Bregma, based on the atlas of Paxinos and Franklin (Paxinos and Franklin, Keith B. J, 2001). The stylets were later replaced with removable dummy injectors (Plastics One) and the guide cannulae fixed to the skull with dental acrylic resin (Paladur, Heraeus Kulzer, Hanau, Germany). Animals were allowed to recover (home cages) for 2 weeks before experimental manipulations. Bilateral infusion cannulae (projecting 4.2 mm below the end of the guide cannulae) were used for the microinjections. Either 0.5 µl of sterile 0.9% NaCl or galanin agonists (M617 or M1145) were delivered over 3 minutes, using a Hamilton microinfusion pump. Infusion cannulae were held in place for 3 minutes after microinjection, then removed and replace with dummy injectors. Following the behavioural tests (see below), injector cannulae were again inserted and 0.5 µl methylene blue was injected into the injection sites. Brains were snap-frozen, cut on a cryostat and stained with cresyl violet to validate the placement of cannulae and correct site of the microinjections (ongoing).

Behavioural testing

Testing commenced 2 weeks after placement of intra-LH guide cannulae; during this recovery period, animals were habituated to handling and received i.p. injections of saline (0.2 ml) on 5 occasions; animals were familiarized with the PF by exposing them to the diet for 24 h, 5 d before behavioural testing. Animal body weights were monitored and animals were divided into groups (control, Gal1R agonist, Gal2R agonist), ensuring that body weights were similar between the groups. Behavioural testing was preceded by a 24 h fast, after which all groups received leptin (3 mg/kg i.p; # LEP-6, Protein Laboratories Rehovot Ltd, Rehovot, Israel) at ZT 3. After an interval of 20 min, animals received intra-LH microinjections (0.5 µl) of either saline, Gal1R or Gal2R agonists (0.1 nM); 10 min later, all mice were provided with a choice of SC and PF, the ingestion of which was measured at 20 min, 40 min and 1, 3, 6, 24 h after food presentation. The experimental procedure was repeated after a 6 d rest period; animals then received intra-LH microinjections of saline, Gal1R or Gal2R agonists (1 nM) and their consumption SC or PF monitored over 1 h, as before.

Immunohistochemistry (IHC)

Galanin immunoreactivity was visualized by applying the general protocol described in Section 3.3. to free-floating paraformaldehyde (PFA, 4%)-fixed brain sections (including the hypothalamus). The brains originated from mice used in the previous chapter (Scheme 3.2).

pSTAT3/galanin. Sections were incubated with rabbit anti-pSTAT3 (1:500, 48 h, 4° C; #9131, Cell Signalling Technology, Boston, MA, USA) and sheep anti-galanin (1:50 000, 48 h, kindly provided by Dr. István Merchenthaler, University of Maryland, College Park, MD). Antigens were detected with Alexa 488-streptavidin after biotin-tyramide amplification (pSTAT3) or direct conjugated anti-sheep CY3 antibody (galanin).

Galanin/C-Fos/orexin-A. Sections were sequentially incubated (4° C) with sheep anti-galanin (1:50,000, 48 h), rabbit anti-c-Fos (1:5,000, 48 h; PC38, Millipore) and rabbit anti-orexin-A (1:5,000, 18 h; Phoenix Pharmaceuticals, Burlingame, CA, USA). The c-Fos signal was amplified with biotin-tyramide and visualized using Alexa 488-streptavidin. Since the antisera against anti-c-Fos and orexin were both raised in rabbits, orexin detection was performed by directly conjugating the anti-orexin to Texas Red®-X (Z-25370, Zenon® Tricolor Rabbit IgG Labeling Kit #2, Thermo-Fischer Scientific). Galanin signal was detected with an anti-sheep AMCA secondary antibody (blue).

Image capture. Microscopic images were captured using an AxioCam MRc 5 digital camera mounted on a Zeiss AxioImager M1 microscope, outfitted with AxioVision 4.6 software (Carl Zeiss, Göttingen, Germany). Confocal images were captured with an inverted Nikon Eclipse Ti-E microscope, equipped with an A1R confocal system (Nikon, Vienna, Austria). Digital images were processed with Adobe Photoshop CS software (Adobe Systems, San José, CA); all images were equally adjusted for brightness and contrast.

Statistics

Data (given in text as mean $\pm$ SD; shown in figures as $\pm$ SEM) were analysed for statistical differences using Prism 6 software (GraphPad, San Diego, CA) after checking for normality. Student's *t*-test (normally distributed data) or the Mann-Whitney test (non-Gaussian) were used to compare data from two groups. To analyse more than two experimental groups, analysis of variance (ANOVA) or the non-parametric Kruskal-Wallis test were used, as appropriate. ANOVAs were followed by Tukey's and Dunns' tests; Kruskal-Wallis tests were followed by Dunns' multiple comparison (*post hoc*) test. The level of significance was set at $P \leq 0.05$.

4.3 Results

Galaninergic innervation of lateral hypothalamic orexin neurons

Galanin-immunopositive cells cannot be visualized without arresting axonal transport with intracerebroventricular injections of colchicine (Melander et al., 1986; Laque et al., 2013), a drug that also interferes with behaviour. Accordingly, we here used an alterantive strategy (Louis et al., 2010; Leinninger et al., 2011) to detect orexin neurons in the LH receiving galaninergic inputs. Qualitative analysis (Fig. 4.1 and Fig. 4.2) revealed galanin-positive processes in close proximity to pSTAT3-positive (assumed to be LepRb-expressing galanin neurons) and orexin-positive neurons in the LH, close to the fornix. Both treatment combinations (leptin+SC and leptin+PF) led to concomitant increases in pSTAT3, galanin and orexin immunoreactivity.

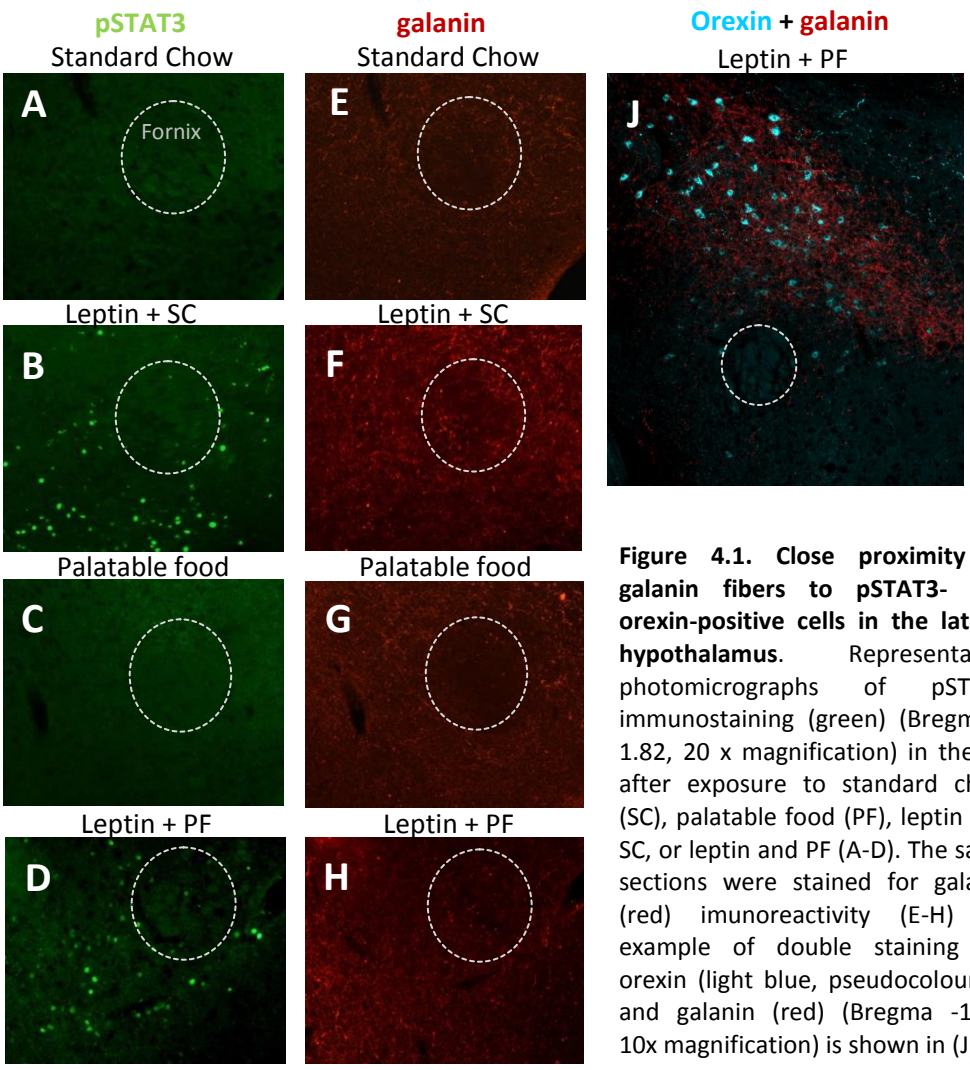


Figure 4.1. Close proximity of galanin fibers to pSTAT3- and orexin-positive cells in the lateral hypothalamus. Representative photomicrographs of pSTAT3 immunostaining (green) (Bregma -1.82, 20 x magnification) in the LH after exposure to standard chow (SC), palatable food (PF), leptin and SC, or leptin and PF (A-D). The same sections were stained for galanin (red) imunoreactivity (E-H) An example of double staining for orexin (light blue, pseudocoloured) and galanin (red) (Bregma -1.46, 10x magnification) is shown in (J).

Galanin partially restores leptin efficacy during hedonia-driven overeating

Neither 0.1 nor 1 nM microinjections of M617, a galanin receptor 1 (Gal1R) agonist into the LH, together with a peripheral injection of leptin, affected PF consumption at any of the time points measured (data not shown). On the other hand, a 0.1 nM dose of intra-LH M1145, a galanin receptor 2 (Gal2R) agonist, produced a marginally significant increase in the ability of leptin to suppress PF ingestion over 1 h ($P = 0.065$, $T = 1.925$) (Fig. 4.3A). Administration of a higher dose (1 nM) of the Gal2R agonist suppressed PF intake further, albeit with some delay (6-24 h) ($P = 0.046$, $T = 2.099$) (Fig. 4.3D). It is worth mentioning here that, the intra-LH microinjection was not responsible for the

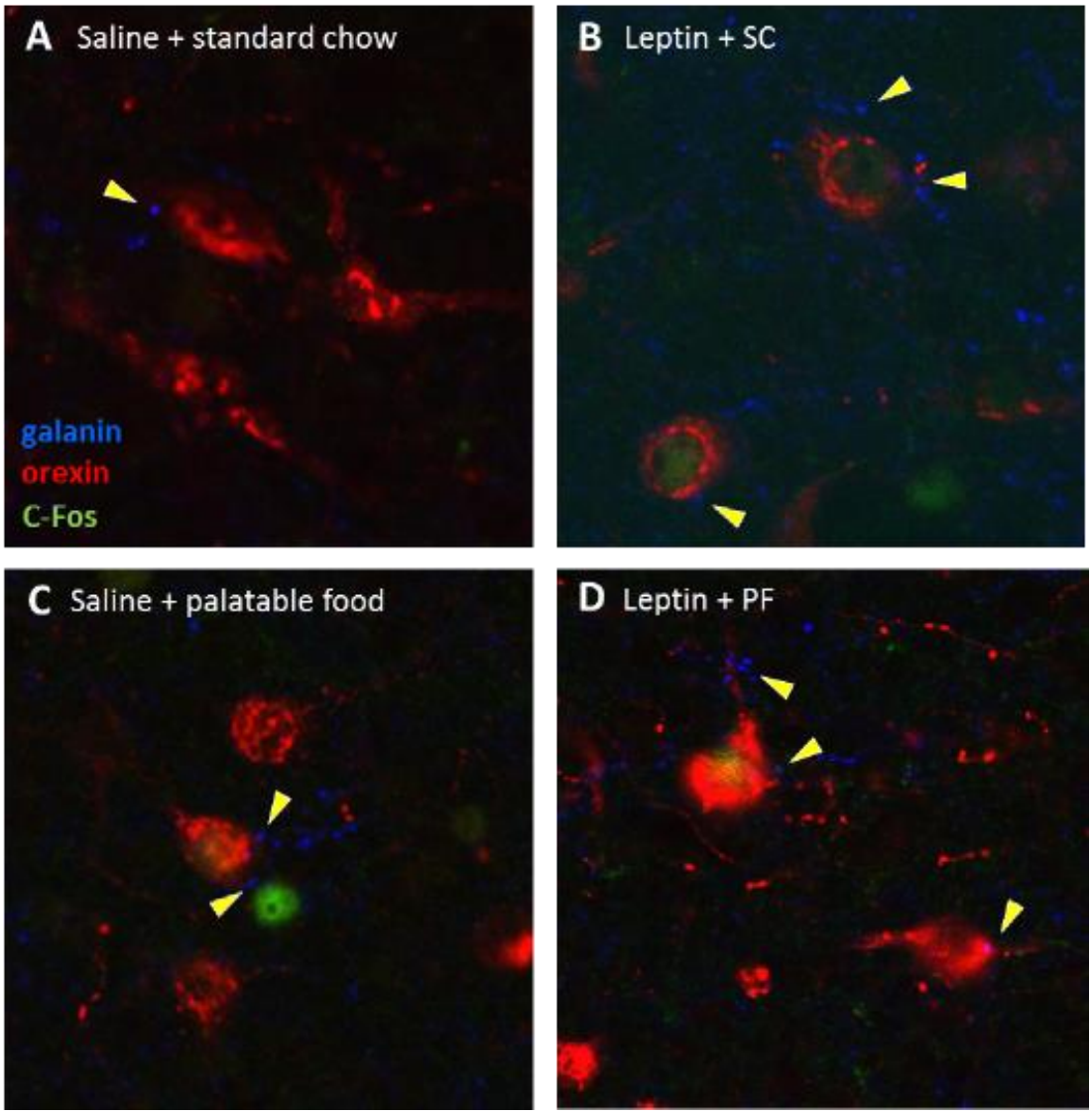
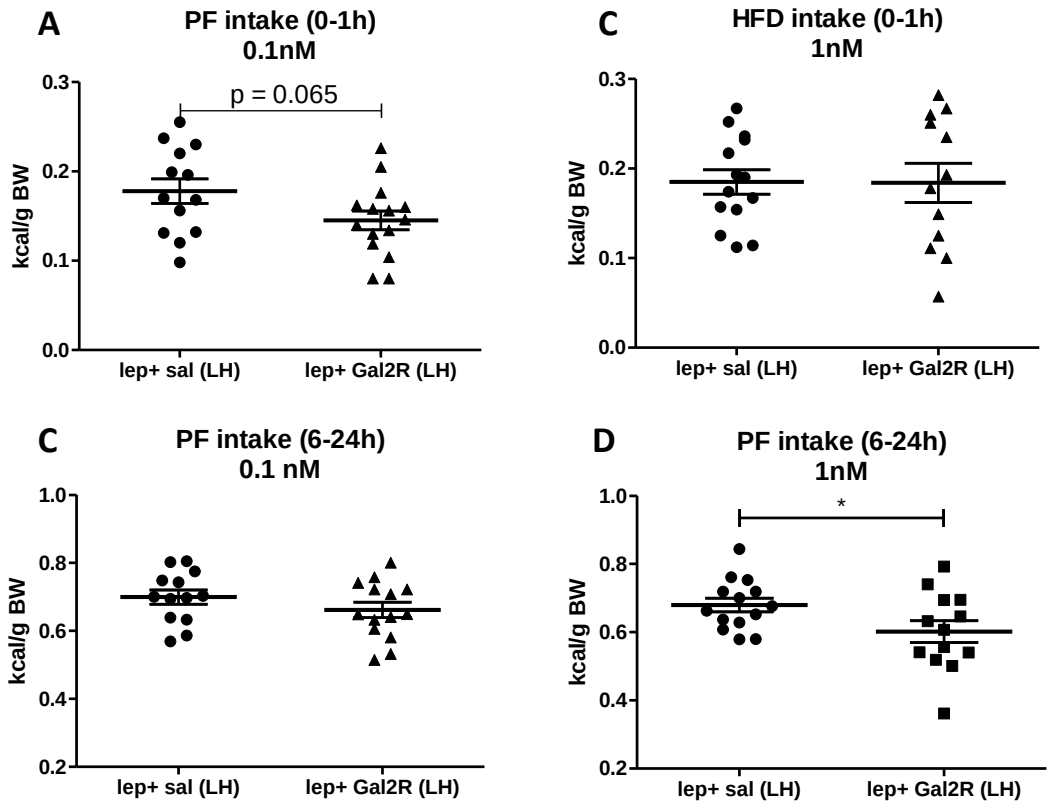


Figure 4.2. Galaninergic terminals in the vicinity of orexin cells in the lateral hypothalamus (LH), revealed by confocal microscopy. (A-D) Images of galanin-immunoreactive fibers (blue) that neighbour orexin A- (red) and c-Fos (green)- immunoreactive neurons in the LH. Likely sites of contact are indicated by arrowheads.

poor responses to the Gal receptor agonists (see Fig. 4.3E) in which the results of the present experiment are compared with those obtained in Fig. 3.5. In line with our previous observations, i.p. leptin failed to decrease eating of a hedonic food (Fig. 4.3E). Indeed, as shown in Fig. 4.3E the intra-LH Gal2R agonist injection potentiated the anorexigenic effects of leptin on PF intake (1-way ANOVA: $F_{4,84} = 2.824$, $P = 0.03$).



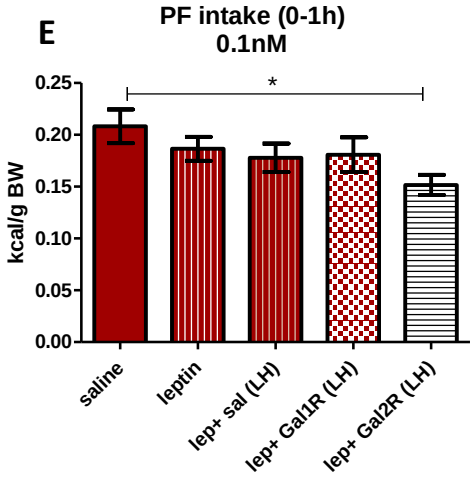


Figure 4.3. Galanin receptor 2 (Gal2R) agonist restores efficacy of peripheral leptin on PF intake. (A-E) Palatable food (PF) consumption by mice that received an injection of leptin (3 mg/kg, i.p.) and an intra-LH injection of a Gal2R agonist (M1145; 0.1 nM or 1 nM) after a 24-h fast. Saline (vehicle for both drugs) was injected in control animals. Leptin or saline i.p. injections were administered at $t_{-30\ min}$; Gal2R agonist (M1145) or saline were bilaterally injected intra-LH at $t_{-10\ min}$; (A-B) Cumulative PF intake (expressed as kcal/g BW) after 0.1 nM or 1 nM microinjections was measured over $t_{0\text{-}60\ min}$; (B-C) cumulative PF intake was monitored over $t_{6\text{-}24\ h}$. (E) PF intake over 1 h following a 24-h fast. The first two columns show results from a previous experiment (Fig. 3.5) (n = 23 per group); columns 3-5 represent data obtained in animals that also received saline or GalR1 (M617; 0.1 nM) or GalR2 (M1145; 0.1 nM) intra-LH microinjections 10 min before food presentation (n = 11-15 per group) in the present experiment.

4.4 Discussion

The finding that leptin cannot suppress palatable food (PF, high-fat high-carbohydrate food) ingestion by mice (Chapter 3) suggested a break in the chain of events that culminate in the suppression of orexin neuron activity. Having obtained evidence regarding the integrity of leptin signalling pathways and the anorexigenic activity of the

mediobasal hypothalamus, the present work focused on the lateral hypothalamus (LH), an area that integrates metabolic and sensory information from the periphery and other brain areas (Berthoud and Münzberg, 2011). Specifically, the present work attempted to examine the neural substrates and mechanisms that may be disrupted when mice encounter a PF.

Important clues that guided the present investigations came from recent studies that identified leptin receptor (LepRb)-expressing neurons in the LH that were found to play a critical role in the regulation of the activity of orexin neurons (Leinninger et al., 2009; Leinninger et al., 2011; Goforth et al., 2014). Chemophenotyping of these LepRb-bearing neurons, that densely project to proximate orexin neurons, showed them to co-express galanin (Gal), neurotensin and GABA (Louis et al., 2010; Laque et al., 2013). Here, we confirmed that fibres from Gal-immunopositive neurons, that are reported to respond to exogenous leptin with an increase in pSTAT3 levels (Laque et al., 2013), are apposed to orexin-expressing cell bodies in the LH. The elegant work by Goforth and colleagues demonstrated that Gal, but not neither neurotensin nor GABA, mediates the inhibitory actions of leptin on orexin neurons (Goforth et al., 2014). Accordingly, we tested the hypothesis that the failure of leptin to suppress PF ingestion results from inadequate Gal-mediated inhibition of orexin neuron activity. The validity of this hypothesis was supported by our observations that site-specific microinjections of a synthetic galanin receptor 2 (GAL2R; M1145) agonist into the LH partially restores leptin-induced suppression of PF consumption. Similar injections of a GAL1R agonist (M617) did not have any effect.

While the results of our preliminary experiments require further testing and inclusion of further control groups, they hint at a potentially interesting mechanism of hedonically-driven PF overeating. Our findings contrast with the view that Gal1R, rather than Gal2R, modulate orexinergic tone and regulate feeding (Gorbatyuk and Hokfelt, 1998; Zorrilla et al., 2007; Saar et al., 2011), a view supported by the observation that developmental deletion of LepRb from galanin neurons is accompanied by a reduction of Gal1R, but not Gal2R (Laque et al., 2015). While only GAL1R expression has, to date, been detected on orexin neurons (Laque et al., 2015), GAL2R expression on orexin cells has to yet be validated. Gal2R appear to be coupled to an inhibitory G protein (Bjorklund et al., 2002) that could trigger hyperpolarization of orexin cells by direct hyperpolarization of OX neurons or by presynaptic modulation of inputs to orexin neurons. This reasoning is consistent with results obtained from hypothalamic slice preparations where leptin was found to decrease the frequency (but not amplitude) of sEPSCs in orexin neurons (Goforth et al., 2014). Such modulations could potentially block excitatory inputs originating in areas involved in hedonic processing and appetitive motivation such as the nucleus accumbens (NAc), ventral pallidum (VP) and prefrontal cortex (Groenewegen et al., 1993; Zheng et al., 2003; Smith and Berridge, 2007; Mena et al., 2013; Castro et al., 2015). In this scenario, the control of PF consumption would depend on a balance

between hedonic and GALergic inputs; overeating would occur if hedonic stimulation sufficiently exceeds a certain threshold so as to override the inhibitory actions of GAL on orexin neurons.

It is important to reiterate that intra-LH administration of the GAL2R agonist (M1145) restored, albeit weakly, leptin-induced suppression of PF consumption; indeed, while co-administration of leptin and M1145 produced a stronger effect than when leptin was administered alone, the potentiating effects of M1145 only became evident when PF consumption was compared to PF consumption under baseline conditions. Indeed, small effect sizes were also observed by other authors who applied Gal (and Gal analogues) *in vivo* (Smith et al., 1996; Smith et al., 1997) and *in vitro* (Goforth et al., 2014). The doses chosen in the present work were based on previous studies (Tempel et al., 1988; Schick et al., 1993; Smith et al., 1996; Smith et al., 1997; Parrado et al., 2007; Anderson et al., 2013), and M617 and M1145 are currently the most selective, high affinity ligands for GAL1R and GAL2R, respectively (Saar et al., 2011). In light of these facts, our results suggest that Gal may not be the only modulator of orexin-triggered feeding. Besides their reception of leptin and other appetite-suppressing signals of peripheral origin (e.g. insulin, cholecystokinin), LH neurons receive a multiplicity of inputs from brain areas involved in emotion, memory and sensing bodily energy requirements (Saper et al., 1979; Kelley et al., 2005; Berthoud and Münzberg, 2011).

In addition to their hedonic (sensory) properties, hedonic foods, such as the one used in this study, are usually of high energetic and nutritional value, a factor that could contribute to homeostatic imbalance. In particular, glucose and fatty acids (FAs) are readily sensed in the hypothalamus where they act to decrease appetite (Oomura et al., 1969; Oomura et al., 1975; Woods et al., 1984; Jo et al., 2009; Le Foll et al., 2014; Magnan et al., 2015) (see Section 1.2.2). In addition, FAs can also activate orexigenic pathways (DiPatrizio and Piomelli, 2015; Steinbusch et al., 2015). Interestingly, as compared to other hypothalamic nuclei (ARC, VMN, DMH, PVN), the LH displays increased neural activation (upregulated c-Fos levels) after FA intake (Cruciani-Guglielmacci et al., 2004); thus, it is plausible that FAs participate in the modulation of GAL neuron responses to PF. Our analysis revealed a delayed onset of action of intra-LH-delivered M1145 (GAL2R agonist), suggesting that it may be important to consider another factor, namely, fat metabolism (cf. Le Foll et al., 2014) in the mechanisms that allow PF consumption during states of satiety. Experiments are currently being designed to address these additional aspects.

Together, our findings confer with the suggestion that a diversity of signals contribute to the probabilistic, but highly coordinated, intake of standard chow (SC) (Woods and Begg, 2015); understanding the control of the ingestion of hedonically-loaded PF represents a challenge of immense dimensions, yet one of critical importance if the etiopathogenic mechanisms of obesity are to be resolved.

CHAPTER 5

GENERAL DISCUSSION

Introductory remarks

On the one hand, energy consumption in excess of physiological needs reflects the high palatability of energy-dense foods, and on the other, an abundance of high-fat, high-carbohydrate foods; the latter is particularly relevant to modern humans who also are exposed to irresponsible advertising campaigns. About 40% of the human population is overweight (OECD, 2014; WHO, 2015). Many overweight and obese people exhibit hypertension, cardiac disease and high levels of blood sugar and triglycerides, and have increased risk for other diseases such as cancer (WHO, 2015). In addition, obesity can interfere with normal neuronal function, with adverse consequences for cognitive and emotional behaviour as well as food choice and the physiological regulation of feeding (Luppino et al., 2010; Cazettes et al., 2011; Cohen et al., 2011; Gontier et al., 2015). The most successful treatment of obesity and its comorbidities is bariatric surgery, but the intervention impairs absorption of many essential nutrients and is neither cheap nor feasible for application in the large number of obese people in Western societies (Vincent and le Roux, 2008; Flynn et al., 2015). It would therefore seem more pragmatic to shift the focus from studying obese subjects to understanding the processes that lead to obesity that would facilitate the identification of preventative measures. There is a surprising paucity of data regarding the mechanisms involved in the initiation of overeating and therefore, obesity; these mechanisms are likely complex, involving integration of physiological and behavioural signals and responses.

Numerous studies, using a wide spectrum of approaches, have attempted to tackle the question of what causes obesity. While some studies suggest genetic predisposition or epigenetic inheritance (Sandhu et al., 2002; Perusse et al., 2005; Kilpeläinen et al., 2011; Llewellyn et al., 2013; van Dijk et al., 2015) as key mechanisms, others point to changes in the gut microbiome (Ley et al., 2005; Cho et al., 2012) or environmental toxins (Grun and Blumberg, 2006; Manikkam et al., 2013) as potential causal factors. Nevertheless, most authors agree that excessive energy intake (with respect to energy expenditure) is, unarguably, the most prominent factor that drives weight gain (Cox et al., 1999; Mela, 2006; Blundell et al., 2015).

Hypothesis

The main question addressed in this thesis concerned the mechanisms that lead to initial overeating of palatable, energy-dense foods. These initial processes are important as they may shape the formation of food preferences and therefore, obesity. Here, the **working hypothesis was that, hedonic processes elicited by a food's palatability, energy, texture and/or novelty are likely to interfere with physiological satiety signals; specifically, we were interested in whether and how acute exposure to a palatable food (PF) influences the transduction of signals that normally serve to curtail excessive**

eating. Briefly, we considered that the sensory properties of food (which contribute to their palatability) dominate over initial body weight set-points or homeostatic signals in the regulation of eating (Wirtshafter and Davis, 1977). From an evolutionary perspective, it would appear that hedonic (pleasurable) processes facilitate adaptation and survival and are thus, evolutionarily advantageous (Berridge and Kringelbach, 2013). One may ask how or why highly-evolved and robust physiological processes become hijacked by extraneous stimuli that induce obesity and therefore reduce health quality. At least as far as humans are concerned, the answer to this question may largely lie in the fact that, in evolutionary terms, modern humans have experienced a relatively rapid growth in the abundance of energy-dense foods to which their energy-sensing oral and post-oral mechanisms have not had a chance to adapt to in an optimal manner (see box 1.1 and Section 1.3.2).

To test this, we developed a new behavioural paradigm and used neuroanatomical, molecular, biochemical and pharmacological approaches to examine responses and adaptations in various relevant brain areas following acute exposures of mice to food types varying in sensory and energetic composition.

Design of behavioural paradigm to test hypothesis

Given the above background, the first goal of this thesis was to establish a behavioural paradigm that would allow investigation of the interactions between hedonic and homeostatic signals in mice. Rodent studies enable the use of standardized experimental conditions as well as the study of the cellular and molecular basis of the behaviours; examination of these aspects is limited by the non-invasive methods that, for ethical and practical reasons, must be used in humans. The “food choice paradigm” introduced involved exposing mice to a palatable high-calorie (high-fat/high-carbohydrate, PF) and less-palatable (balanced, standard, SC) food options under different physiological conditions and after pharmacological mimicry of different states of satiety (leptin injected to 24 h-fasted mice and ghrelin administered to SC-satiated mice, when endogenous levels of the respective hormones would be low). Our experiments showed that mice eat palatable calorie-rich foods in excess of their normal daily needs when hungry and injected with the satiety hormone leptin or after satiation with SC; moreover, a further increase in PF ingestion was seen when SC-satiated mice were injected with ghrelin, a hunger-signalling hormone. These observations provided the necessary validity to the choice paradigm as a useful one for studying hedonic eating.

It is important to note that the simple food choice paradigms (see schemes 2.2, 2.3, 3.1, 3.2) were used to provide initial insights into the regulation of eating, a primitive but complex behaviour. Many factors that are critically involved in the regulation of feeding, e.g. previous experience (learning and memory), sensory perception, decision-making, habits, cognition, exercise, environmental modulation and social environment, could not

be addressed by the present experiments. Nevertheless, several findings reported in this thesis can be integrated into existing knowledge and developed into future research questions. For example it would be interesting to know whether the acute effects of leptin modify the formation of memory regarding a particular type of food and whether leptin modulates habit formation. How hedonic feeding and satiety responses are influenced by exercise or exposure to enriched environments would be other potential subjects of enquiry.

Practical considerations regarding behavioural paradigm

A major challenge in research on the regulation of feeding is that the behaviour is not based on simple homeostatic reflexes (Woods and Begg, 2015), but rather engages a variety of brain circuits (involved in processing hunger state, emotions, circadian influences etc) that are activated simultaneously or in rapid sequence. The hypophagic response to just a single hormone, in seemingly identical experimental settings, appears to be probabilistic and difficult to replicate, as exemplified by response to leptin, a major anorexigenic signal (see Woods and Begg, 2015). Indeed, since the differences in intake of palatable vs. less-palatable food in response to leptin were not large, albeit significant in the majority of experiments reported in this thesis, care was taken to ascertain robustness of results by repeating all behavioural experiments on at least two occasions.

Generally, our experimental design, using the choice paradigm, involved providing a food choice comprised of high-fat/high-carbohydrate and standard chow (SC), so as to mimic a situation in which hedonic overeating would likely occur (initially, the choice paradigm also included low-fat/high-carbohydrate food, but since this food option was not consumed, it was eventually not included as a choice). Further, data analysis of palatable food intake involved comparison with control food consumption in a “no-choice” situation, i.e. when mice only had access to a SC. This was necessitated by the fact that mice exclusively consumed the high-fat/high-carbohydrate food in the choice paradigm, almost completely neglecting the SC (thus, from Chapter 3 onwards, the term palatable food [PF] was used to describe high-fat/high-carbohydrate food). Given the physical (and presumably sensory) properties of PF (high calorific, fat and carbohydrate content), PF was considered to represent a food with high hedonic value.

Although both, the oro-sensory and post-oral effects of food are known to be important for the formation of food preferences (de Araujo, 2011), these aspects were not studied separately in the present work. However, it is important to mention that both, oral and post-oral signals activate the mesolimbic dopamine system which is critical for the motivation to eat and acquisition of food preferences (Kosobud et al., 1994; Hajnal et al., 2004; Liang et al., 2006; de Araujo et al., 2008; Ferreira et al., 2012; Tellez et al., 2013a; Han et al., 2016) (see section 1.3.2).

Hedonic properties of food subjugate homeostatic controls of feeding

An important result from the present experiments was the demonstration that exogenous leptin loses its efficacy at suppressing food consumption in the presence of PF. Conversely, presentation of PF was found to potentiate the orexigenic effects of ghrelin. While similar results were previously described for ghrelin (Egecioglu et al., 2010; Merkestein et al., 2012; Perelló and Zigman, 2012), the loss of leptin efficiency when PF is presented has not been reported hitherto. Rather, leptin has been reported to reduce reward salience and preference for PF (Figlewicz et al., 2001; Figlewicz et al., 2004; Hommel et al., 2006; Domingos et al., 2011). Previous authors ascribed overeating to deficient leptin availability and/or impaired leptin signalling, a conclusion drawn on the basis of observations in subjects that were already obese (Myers et al., 2010; Morton et al., 2014). In marked contrast, our results showing that PF overeating occurs in lean animals are interesting insofar that leptin efficiently activated a major downstream target, the transcription factor *signal transducer and activator of transcription 3* (STAT3) in leptin-responsive hypothalamic neurons (also see later). Here, it should be noted that the failure of leptin to suppress PF intake proved to be highly reproducible between independent experiments (see Fig. 2.6, 2.9, 3.5).

Interestingly, the effects of PF (overeating) prevailed only transiently (1 h) over the normal effects of leptin; it was observed that PF consumption was reduced by acute leptin when animals were exposed to PF for an extended period (for 24 h). We also monitored the effect of endogenous satiety signals (no leptin injections) on caloric intake (PF choice vs. SC control groups) over 1 week. In fact, the calorific intake from PF choice and SC control groups gradually became similar, reaching identical amounts at the end of the week. These findings suggest that hedonic overriding of physiological satiety signals gradually wanes with time, perhaps reflecting changes in the perceived hedonic value and/or novelty value of PF. Thus, the physiological mechanisms regulating satiety appear to be restored to normal as animals become familiar with the novel/hedonic experience provided by initial exposure to PF. A previous study from our lab (Harb et al., 2014) reported similar findings, albeit in aged mice.

Neural substrates that potentially mediate the effects of PF

We considered that PF can potantially interfere with the physiological control of feeding behaviour either i) by activating brain areas that induce hedonic feeding (and subsequently, dopamine-dependent processes that modulate motivation and reward-related behaviour), and/or ii) by blunting the response of feeding regulatory circuits to satiety signals. Specifically, we considered the possibility that the effects of PF involve reduced expression of satiety signals and/or their receptors, increased expression of

hunger signals and/or their receptors, and/or modulation of post-receptor signalling or of downstream-target neurons of feeding peptides (Erlanson-Albertsson, 2005).

Our experiments showed that acute PF exposure does not alter the hypothalamic proximal response to leptin (upregulated pSTAT3) (Banks et al., 2000; Fruhbeck, 2006). In addition, they show that PF activates leptin receptor (LepRb)-expressing anorexigenic proopiomelanocortin (POMC) neurons (Elias et al., 1998a; Cowley et al., 2001; Sohn et al., 2013), as judged by c-Fos immunoreactivity. Further, blood ghrelin levels were not affected by either PF or leptin administration, and neurons expressing orexigenic neuropeptide Y/Agouti-related peptide (NPY/AgRP), that are normally stimulated by ghrelin and inhibited by POMC neurons (Velloso and Schwartz, 2011; van Swieten et al., 2014), displayed reduced levels of NPY mRNA expression following leptin administration and acute PF exposure to 24 h-fasted mice. These results suggest that PF suppresses the initial signals of appetite, probably via increased activation of POMC neurons. Importantly, our observations indicate that overeating of PF occurs despite anorexigenic signals from first order neurons (responding to peripheral satiety-regulatory peptides such as leptin).

In addition to the hypothalamus, leptin receptors are expressed in the ventral tegmental area (VTA), an important site for neuroadaptations to rewarding stimuli. Various studies suggest that leptin suppresses feeding by inhibiting the activity of dopamine (DA) neurons in the VTA (Figlewicz et al., 2003; Figlewicz et al., 2004; Hommel et al., 2006; Morton et al., 2014; van der Plasse et al., 2015). The VTA could thus be another site at which leptin effects on feeding are overridden when hedonic foods are available. While there are reports that high-fat food impairs pSTAT3 induction in the VTA (Matheny et al., 2011), other authors found that the VTA and NAc remain responsive to central leptin even when POMC neurons in the Arc display leptin resistance (van den Heuvel et al., 2014). In an attempt to examine this possibility, we measured pSTAT3 levels in the VTA in animals that had been injected with leptin before exposure to the food choice paradigm, but failed to observe any leptin-induced increase in pSTAT3 immunoreactivity in this structure. In light of other reports of leptin-triggered STAT3 phosphorylation in the VTA (Fulton et al., 2006; Hommel et al., 2006; Morton et al., 2014), it would appear that dose and route of administration (ICV or i.p.) may determine the magnitude of the pSTAT3 response. It is also possible that other signalling pathways, phosphatidylinositol-3 kinase (PI3k) and extracellular signal-regulated kinase-1 and -2 (ERK1/2), mediate the effects of leptin in DA neurons (Trinko et al., 2011; Thompson and Borgland, 2013). These pathways are, however, constitutively activated and respond to several different signals (including insulin) (Konner and Bruning, 2012) and thus, only poorly reflect leptin signalling efficacy. Here, we sought an alternative approach to this question by indirectly measuring activation of VTA neurons after leptin administration, namely by estimating DA turnover in candidate forebrain regions (Wise, 1996).

The DAergic tone (3-MT:DA, a proxy of DA release) in the NAc and OFC was increased after mice consumed PF, a finding consistent with previous reports that detected activation of the "motivation circuitry" after ingestion of rewarding (including energy-rich) food (Hernandez and Hoebel, 1988; Wilson et al., 1995; Small et al., 2003; Norgren et al., 2006). Interestingly, DA turnover in the ventro-lateral OFC and medial/caudal NAc shell was not influenced by exogenous leptin in animals eating SC, but was reduced in those eating PF. Unexpectedly, leptin potently suppressed DA turnover in the animals eating PF. It is plausible that effect of leptin on DA system activity emerges only during bouts of (hedonic) overeating and, that this effect appears with a delay (DA tone was measured after 1 h of ingestion); in this case, leptin sensing in the VTA would serve as an additional slow-acting homeostatic mechanism (to hypothalamic leptin signalling) that enables responses to stimuli of high incentive value. This explanation would match the food consumption profiles observed here, in which food consumption responds to satiety signals only later than 1 h of food exposure (the same time when leptin decreases DA turnover). In addition, it is important to note, that leptin affected DA tone in a site-specific manner in animals eating PF. Whereas PF itself increased DA tone in the medial OFC (area responsible for value attribution and greater responsiveness to high-caloric stimuli), NAc core (linked to compulsive behaviours) and rostral NAc shell (hedonic "liking" of foods) (Pecina and Berridge, 2005; Stoeckel et al., 2008; Adamantidis et al., 2011; Brown RM et al., 2015), elevated DA turnover was not altered in these areas by leptin administration. Accordingly, it is suggested that high DA system activity in the aforementioned areas represents the mechanism through which hedonic inputs first, further increase food intake and second, influence the development of future preferences for PF; the latter most likely involve memory and habit formation since DA is important for reward-learning in these areas (Adamantidis et al., 2011; Brown et al., 2015).

The mismatch between the primary satiety response (intact, as judged by pSTAT3 signalling, in the ARC and other hypothalamic nuclei, and possibly the VTA) and the loss of leptin effects on PF eating, suggests the involvement of additional up- or downstream modulatory mechanisms that allow hedonic feeding. Brain areas that are activated (increased c-Fos immunoreactivity) by ingestion of a hedonic food during a state of satiety include those that respond to sensory, nutrient and hedonic inputs (e.g insula, piriform cortex, ventral pallidum, ARC, VMH, DMH; see Table 3.2) and the posterior lateral hypothalamus (LH). Our studies focused on neurons in the LH since they are normally activated by hunger, an effect that reverses upon satiety due to increases in the secretion of leptin (Yamanaka et al., 2003).

The LH comprises two populations of appetite-inducing neurons: orexin (OX, also called hypocretin) and melanin-concentrating hormone (MCH) neurons. Our results show that in satiated, as well as in previously-fasted mice, acute (1 h) exposure to a PF selectively upregulates OX expression, without inducing any changes in the expression of MCH.

Orexin thus seems to have a dual function, to increase appetite in hungry animals (cf. Yamanaka et al., 2003) and to exert positive feedback on feeding behaviour in the presence of hedonic foods. We also observed that hedonic food presentation to satiated mice is accompanied by an increase of blood corticosterone (CORT) levels, with CORT titres increasing in parallel with OX expression levels; notably, the rise in CORT in this paradigm was not as high as that seen during typically stressful conditions. Other authors have shown that OX neurons stimulate the secretion of CORT (Bonnavion et al., 2015) and that, glucocorticoids, in turn, increase arousal and motivation (Arborelius et al., 1999; Kvetnansky et al., 2009). Accordingly, it is posited that the PF-induced increase in CORT levels, as a result of OX neuron activation, serves to increase arousal and generate locomotor activity with the ultimate purpose of seeking energy-dense foods.

Orexin neuron activity and expression are normally subject to inhibition by leptin (Lopez et al., 2000; Yamanaka et al., 2003; Laque et al., 2013; Goforth et al., 2014). Consistently, we here found that leptin potently inhibits activation of OX neurons; however, leptin efficacy was lost during exposure to PF (Fig. 3.5. and 3.9). Another finding was that the orexin content of various forebrain projection areas such as the orbitofrontal cortex (OFC, medial and ventro-lateral subdivisions) and nucleus accumbens (NAc, shell and core) which are implicated in the integration of sensory inputs and generation of hedonic drive (Rolls and Baylis, 1994; de Araujo et al., 2003; Gottfried et al., 2003; Kringelbach et al., 2003; Small et al., 2003; Norgren et al., 2006; Small, 2012; Castro et al., 2015) was unchanged by any of the food type exposures and drug treatment combinations tested. One explanation for this could be that the "snap-shot" OX profiles obtained after 1 h of eating were obtained after a sub-optimal delay such that changes induced by leptin and/or PF had either already occurred or were still in the process of becoming manifested. It is also possible that OX is differentially secreted at various projection sites or that OX neurons terminating in the VTA, rather than in forebrain regions, are those that respond to leptin and PF. For example, Valdivia *et al.* (2014) found that most OX neurons that are responsive to HFD innervate the VTA and that the activation of the VTA, but not other mesolimbic areas, by high-fat food is blocked by peripheral injection of an OX antagonist. It is also worth noting that OX signalling in the VTA is required for opioid agonists injected into the NAc to induce "hedonic" appetite (Zheng and Patterson, 2007). Together, the present and previously-published data offer many lines of future investigation.

The fact that leptin signalling *per se* is not disrupted by PF suggests the participation of other, hedonia-driven, inputs to OXergic neurons that result in the overshadowing of the normal inhibitory effects of leptin. We suggest that hedonic sensing neurons in the rostral nucleus accumbens (NAc) shell project to the LH where they disinhibit OX cells (by inhibiting inhibitory galanin/neurotensin/GABA neurons that mediate leptin suppression of OX neurons). In turn, OX cells projecting to the ventral pallidum (VP) (and probably back to NAc shell) generate hedonic "liking" of PF, while prominent OXergic

projections to the VTA recruit mesolimbic DAergic and brain stem areas to facilitate feeding. Indeed, the herein observed pattern of neuronal activation (c-Fos expression) in mice that consumed PF is consistent with the proposed circuitry, as shown in Figure 5.1 and discussed in greater detail below.

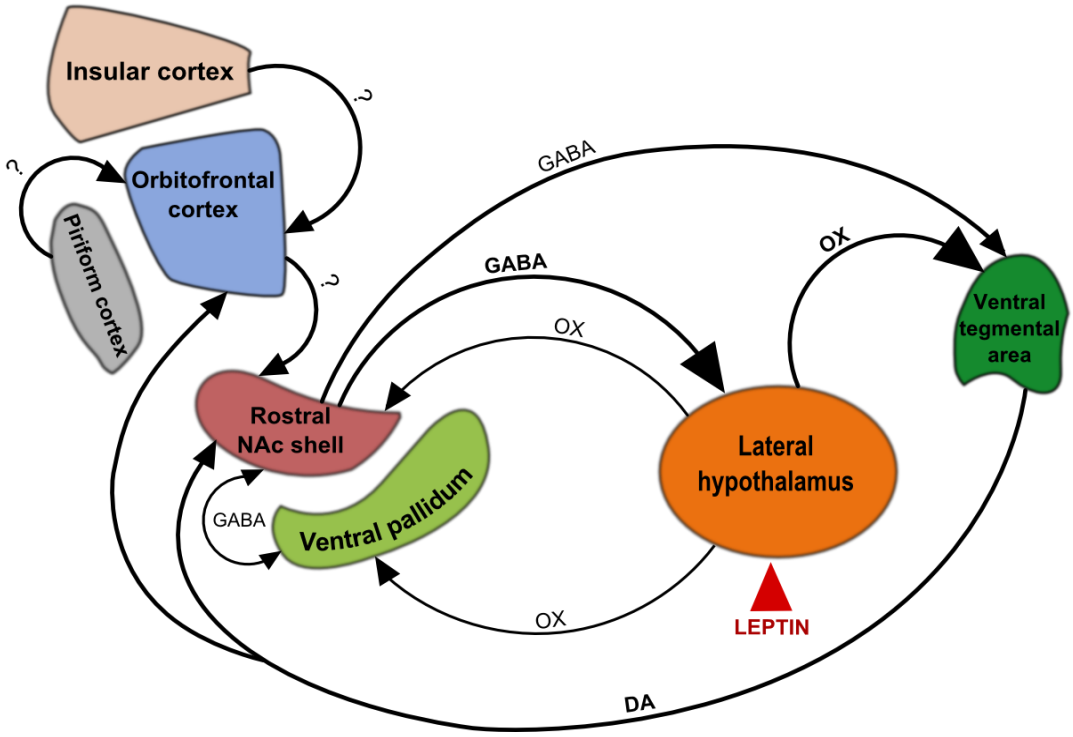


Figure 5.1. Proposed circuit involved in integration of physiological and hedonic signals.

Our results show that areas known to be involved in sensory processing (insular, piriform cortex) and with valuation and integration of sensory information (OFC) (Rolls and Baylis, 1994; Cohen et al., 2011; Steinbusch et al., 2015) are activated by hedonically-weighted food. Previous studies described projections from sensory- and motivation-related areas to the Nac shell (where the signals are integrated) and therefrom to the LH (Mogenson et al., 1983; Kelley et al., 2005; Thompson and Swanson, 2010; Berthoud and Münzberg, 2011). However, we failed to detect c-Fos immunoreactivity in the NAc shell after PF ingestion, an observation that fits with the hypothesis that inhibitory hyperpolarization of the NAc is the primary generator of appetitive motivation. According to that hypothesis, inhibition of NAc neurons leads to the release of downstream targets (e.g. VP, VTA and LH) from GABA-mediated suppression (Mogenson et al., 1983; Stratford and Kelley, 1999; Baldo et al., 2004; Stratford, 2005; Tindell et al., 2009; Smith et al., 2011; see Castro et al., 2015 for a comprehensive review). This view also fits with data showing that (neuronal activity-inhibiting) opioid agonists generate hedonic experiences (Zhang et al., 1998; Will et al., 2003; Kelley et al., 2005; Kelley et al., 2005b). On the other hand, there is contrary evidence regarding the importance of excitation of a subpopulation

(30-50%) of NAc neurons in the elicitation of responses to food reward and/or the anticipation of such rewards (Carelli et al., 2000; Hollander et al., 2002; Nicola et al., 2004; Roitman et al., 2005, 2010; Cameron et al., 2014; Castro et al., 2015). Thus, it seems plausible that the activity patterns of NAc shell neurons vary in different behavioural settings. It is also possible that feeding depends on differential, possibly mutual, excitation/inhibition of neuron subpopulations in the NAc, as suggested by Castro et al. (2015). Further investigation into the relationship between NAc shell neuronal activity and behavioural outcome is warranted.

The ventral pallidum (VP), a "hedonic hotspot" that amplifies liking reactions (Smith and Berridge, 2005), was also seen to be activated following PF exposure in the present work. The VP also serves as a relay through which signals from the NAc are projected to the LH. The VP is connected to the NAc by GABAergic afferents and efferents (Smith and Berridge, 2007) and reports that blockade of GABA-A receptors in the VP stimulates feeding (Stratford et al., 1999) are consistent with the concept that inhibitory outputs from the NAc must be suppressed to permit food intake. It should also be noted that the VP additionally receives OX projections from the LH that amplify hedonic liking (Ho and Berridge, 2013). It was observed in the present work that OX neurons (activated in response to hedonic food) did not respond to leptin; this finding suggests that these neurons receive sensory information from the NAc shell which is subsequently conveyed by OXergic projections to the VTA, VP and NAc shell (where orexin could modulate DA release) to amplify food intake, thus forming a positive feedback-loop (Zheng et al., 2007, Smith et al., 2011; Valdivia et al., 2014; Castro et al., 2016).

Our initial attempts to disentangle the specific mechanism through which leptin actions are abrogated by hedonic stimuli focused on the LH with the aim of identifying molecules that might mediate the effects of leptin on OX neurons. OX cells are known to respond to leptin with reduced activity, resulting in supressed feeding behaviour. It appears that OX neurons are indirectly subject to leptin inhibition since they do not express leptin receptors (Louis et al., 2010). In fact, recent studies have shown that leptin exerts its action on OX neurons via neighbouring galanin/neurotensin/GABA cells (Louis et al., 2010; Leinninger et al., 2011; Laque et al., 2013; Goforth et al., 2014). Moreover, galanin (Gal), but neither neurotensin nor GABA, signalling was shown to be necessary for leptin to exert its inhibitory effects on orexinergic neuron activity (Goforth et al., 2014). Interestingly, Gal is a peptide usually associated with increased food ingestion (Crawley et al., 1990; Kyrkouli et al., 1990; Schick et al., 1993; Saar et al., 2011) and greater preference for fatty foods over high-carbohydrate options (Tempel et al., 1988; Kyrkouli et al., 1990; Leibowitz, 2005; Adams et al., 2008). There is reason to believe, however, that the effect of Gal on food intake varies in a site-specific manner (Tempel et al., 1988; Patterson et al., 2006; Laque et al., 2013; Goforth et al., 2014) and that Gal may dose-dependently exert biphasic effects on feeding (Parrado et al., 2007).

Based on the above-mentioned prior knowledge, we first confirmed that galaninergic neurons indeed terminate in the vicinity of orexin cells. Next, site-specific pharmacological stimulation of galanin (Gal) receptors was used to examine whether inadequate inhibitory galaninergic inputs to orexin neurons might account for the inability of leptin to suppress PF ingestion. Our results showed that, a galanin receptor 2 (Gal2R) agonist (M1145), microinjected into the LH just before exposure to PF, partially restores the ability of leptin to reduce PF intake. Since the LH is a site wherein sensory, endocrine and behavioural signals converge and projections are sent to executive areas (Kelley et al., 2005; Berthoud and Münzberg, 2011), our observations support the view that hedonic food signals are sensed by the LH and compete with regulatory signals from satiety/hunger peptides to modulate motivation to obtain food and reward (Berthoud and Münzberg, 2011; Castro et al., 2015).

Further insights into how Gal-ergic modulation of OX cells is disrupted upon hedonic feeding will require a significant amount of additional work, including robust proof for the presence of Gal2R on orexin cells. Since Gal2R appear to be coupled to either inhibitory or stimulatory G proteins (Bjorklund et al., 2002), one can speculate that activated Gal2R trigger an inhibition of OX cells, either by direct hyperpolarization of OX neurons or by presynaptic modulation of inputs to OX neurons. The latter would be consistent with results obtained from hypothalamic slice preparations where leptin was found to decrease the frequency (but not amplitude) of sEPSCs in orexin neurons (Goforth et al., 2014). We hypothesize that Gal-ergic effects on OX neurons could potentially block excitatory inputs originating in areas involved in hedonic processing and appetitive motivation such as the nucleus accumbens (NAc), ventral pallidum (VP) and prefrontal cortex (Groenewegen et al., 1993; Zheng et al., 2003; Smith and Berridge, 2007; Mena et al., 2013; Castro et al., 2015). In this scenario, the control of PF consumption would depend on a balance between hedonic and GALergic inputs; overeating would occur if hedonic stimulation sufficiently exceeds a certain threshold so as to override the inhibitory actions of GAL on orexin neurons (proposed circuitry depicted on Fig 5.2).

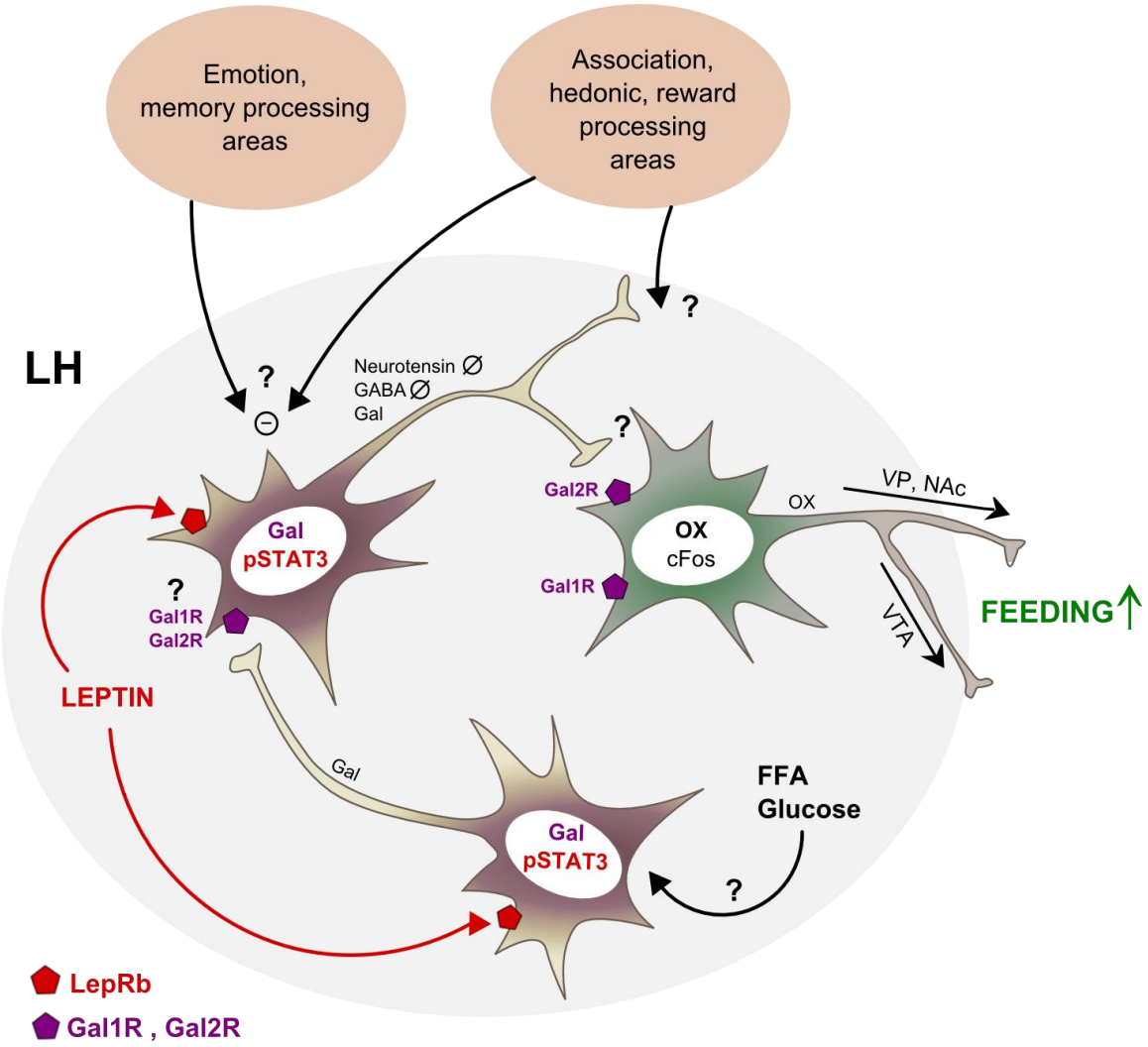


Figure 5.2. Integration of inputs from areas related to emotional and hedonic processing and leptin signals in the lateral hypothalamus (LH). Leptin signals that convey satiety are sensed in the LH by leptin receptors (LepRb) on neurons that express galanin (Gal), neurotensin and GABA. Gal is suggested to mediate the inhibitory effects of leptin on orexin (OX) neurons (Laque et al., 2013; Goforth et al., 2014). In the present work, OX neurons did not respond to leptin when exposed to palatable food, but intra-LH microinjections of a Gal receptor 2 (Gal2R) agonist potentiated the effects of leptin on food intake. This finding is in line with a previous demonstration that Gal hyperpolarizes OX neurons or at least modulates presynaptic inputs onto OX neurons (Goforth et al., 2014). There is a possibility that Gal receptors feedback on Gal neurons. The model proposes that, upon exposure to hedonic food, inhibitory projections from Gal cells to OX neurons receive inhibitory inputs from brain areas responsible for integrating hedonic, reward, memory and emotion processing. Non-esterified free fatty acids (FFA) and glucose are sensed in the LH and could also affect Gal neuron activation. Together, the model depicts how disinhibition of appetite-stimulating OX neurons can lead to increased food intake even when high levels of leptin prevail.

Our finding that intra-LH administration of the GAL2R agonist (M1145) restored, albeit weakly, leptin-induced suppression of PF consumption, suggests that Gal may not be the only modulator of orexin-triggered feeding. Besides their reception of leptin and other appetite-suppressing signals of peripheral origin (e.g. insulin, cholecystokinin), LH neurons receive several other inputs from brain areas involved in emotion, memory and

sensing bodily energy requirements (Saper et al., 1979; Kelley et al., 2005; Berthoud and Münzberg, 2011). In addition to their hedonic (sensory) properties, palatable foods are usually of high energetic and nutritional value, a factor that could contribute to homeostatic imbalance. In particular, glucose and fatty acids (FAs) are sensed in the hypothalamus where they act to decrease appetite (Oomura et al., 1969; Oomura et al., 1975; Woods et al., 1984; Jo et al., 2009; Le Foll et al., 2014; Magnan et al., 2015). Importantly, FAs could also activate orexigenic pathways (DiPatrizio and Piomelli, 2015; Steinbusch et al., 2015). It is thus plausible that FAs participate in the modulation of GAL neuron responses to PF. These additional aspects could be illuminated in future experiments.

Concluding remarks

The herein observed diminished ability of leptin to suppress the acute ingestion of novel palatable food provides a critical insight into the main objective of this thesis: to understand the initiation of hedonic overdrive that may lead to overeating. The results indicate that processes initiated during the first 24 h of overeating represent the turning on of behavioural and physiological adjustments that create an imbalance between energy intake and expenditure (reflected in body weight). Interestingly, when PF is freely available its overconsumption lasts for only a few days before calorific intake is adjusted back to the level seen in control animals. However, despite the gradual reduction in feeding, PF-fed mice continue to show increases in body weight. This possibly reflects a re-calibration of the defended body weight set-point in parallel with changes in the rate of energy metabolism (Levin, 2005; Müller et al., 2010). Further studies should be directed at proving whether the dys-synchrony between central and peripheral signals, triggered at the time of first exposure to a PF, indeed represents a critical event in the pathway to obesity.

Based on the observation that mice progressively decrease their intake of hedonic food over time, it is likely that “loss of novelty” contributes to the phenomenon, at least to some extent. Another potential contributory factor is habituation (to the sensory properties of food); indeed, we have observed that intermittent access to PF leads to bouts of overeating (binge eating) that do not wane in intensity over time (results not shown) (Rossetti et al., 2014, 2014; Martire et al., 2015). Investigation of the interplay between homeostatic and hedonic factors during exposure to intermittently-presented PF could thus be a worthwhile paradigm in future research since it integrates a variety of factors that are also relevant for overeating in humans and animals, e.g. memory, reward expectation, compulsivity.

The complexity of the integration of peripheral feeding signals and central hedonic processing necessitated focus on a limited number of homeostatic regulatory molecules (ghrelin, leptin, galanin). The complexity of the processes also played a role in our

choosing of simple test paradigms which necessarily precluded distinction of the roles of different sensory modalities (olfaction, taste, post-oral nutrient signalling) or of individual macronutrients (fat, carbohydrate, protein) in the hedonic experience. Nevertheless, the information generated here will serve as a useful basis for further stepwise progress in understanding the intricate interplay between these factors and the physiological processes that normally operate to curtail excessive overeating. In this respect, the choice paradigm developed here is envisaged to be important. Likewise, the support offered to the proposal that the LH is a brain site at which hedonic and homeostatic signals converge represents a significant contribution to the field. The data that leptin fails to suppress OX neuron activity during acute exposure to PF, due to interruption of Gal (Gal2R-mediated) inhibition of OX neurons, represents a novel demonstration that leads to new testable hypotheses.

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Pühendan oma doktoritöö emale, isale, Avele, minu vanavanematele ja Paabule, kelle toetus ja armastus on asendamatud.

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(In preparation, based on data presented in Chapters 2, 3 and 4)

Leidmaa E, Patchev AV, Gazea M, Pissioti A, Gassen N, Laszlo B, Kallo I, Liposits Z, Almeida OFX (in preparation). Hedonic food overrides the efficacy of leptin effects on orexin neurons by interfering with galaninergic system.

Leidmaa E, Gazea M, Anderzhanova E, Pissioti A, Stoffel R, Patchev AV, Almeida OFX (in preparation). Dysregulation in serotoninergic neurotransmission upon acute exposure to palatable food.

Publications

Gazea M, **Leidmaa E**, Anderzhanova E, Pissioti A, Flachskamm C, Patchev AV, Almeida OFX, Kimura M (in preparation) The central link between hypophagia and elevated wakefulness following HFD withdrawal in early life obese mice.

Gazea M, Patchev AV, **Leidmaa E**, Anderzhanova E, Flachskamm C, Almeida OFX, Kimura M (in preparation) Peripubertal diet-induced obesity programs sleep-wake behavior during aging in mice through alterations in serotonergic transmission.

Heinla I, **Leidmaa E**, Kongi K, Pennert A, Innos J, Nurk K, Tekko T, Singh K, Vanaveski T, Reimets R, Mandel M, Lang A, Lilleväli K, Kaasik A, Vasar E, Philips MA. (2015). Gene expression patterns and environmental enrichment-induced effects in the hippocampi of mice suggest importance of Lsamp in plasticity. *Front Neurosci*; 9:205.

Heinla I, **Leidmaa E**, Visnapuu T, Philips MA, Vasar E (2014). Enrichment and individual housing reinforce the differences in aggressiveness and amphetamine response in 129S6/SvEv and C57BL/6 strains. *Behav Brain Res*; 267:66-73.

Innos J, **Leidmaa E**, Philips MA, Sütt S, Alttoa A, Harro J, Kõks S, Vasar E (2013). Lsamp^{-}/$^{-}$ mice display lower sensitivity to amphetamine and have elevated 5-HT turnover. *Biochem Biophys Res Commun*; 430(1):413-8

Innos J, Philips MA, **Leidmaa E**, Heinla I, Raud S, Reemann P, Plaas M, Nurk K, Kurrikoff K, Matto V, Visnapuu T, Mardi P, Kõks S, Vasar E (2011). Lower anxiety and a decrease in agonistic behaviour in Lsamp-deficient mice. *Behav Brain Res*; 217(1):21-31

Affidavit

Eidesstattliche Versicherung/Affidavit

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation “Hedonic interruption of the physiological controls of eating: sites and mechanisms” selbstständig angefertigt habe, mich außer der angegebenen keener weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation “Hedonic interruption of the physiological controls of eating: sites and mechanisms” is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den 01. Februar 2016

Munich, 1st of Febuary 2016 Unterschrift, signature

List of author contributions

The experiments described in this thesis were designed by Este Leidmaa with the guidance from Professor Osborne Almeida and Dr. Alexandre Patchev. Professors Heidrun Potschka (LMU) and Christophe Magnan (Université Paris Diderot), members of the Thesis Advisory Committee, reviewed progress of the work on a regular basis.

Surgical procedures (stereotaxic-guided brain microinjections) and behavioural experiments and were organized and conducted by Este Leidmaa. Mary Gazea guided and assisted the surgical work; Anna Pissioti and Dr. Alexandre Patchev helped with collection of behavioural data.

Este Leidmaa prepared tissues and carried out all morphological, biochemical and molecular analysis.

Technical expertise and help was provided by the following individuals:

- Dr. Elmira Anderzhanova: neurotransmitter measurements by HPLC;
- Dr. Nils Gassen: advice and planning of analysis by capillary electrophoresis;
- Rainer Stoffel and Cornelia Flachskamm: assistance with radioimmunoassay for corticosterone and orexin;
- Dr. Imre Kallo and Professor Zsolt Liposits (with technical assistance from Laszlo Barna), Institute of Experimental Medicine (Hungarian Academy of Sciences): immunohistochemical characterization of neuropeptides in the lateral hypothalamus.

Este Leidmaa carried out statistical analyses of the data, prepared the figures and wrote and compiled the final version of the dissertation; Professor Osborne Almeida provided critique on draft versions of the dissertation.

I certify that the above statements are accurate.

Osborne Almeida
5 February, 2016

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