Roles of leptin receptor-expressing neurons in body weight regulation

Véronne de Vrind

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Functies van leptine receptor-expresserende neuronen in de regulatie van lichaamsgewicht

(met een samenvatting in het Nederlands)

Proefschrift

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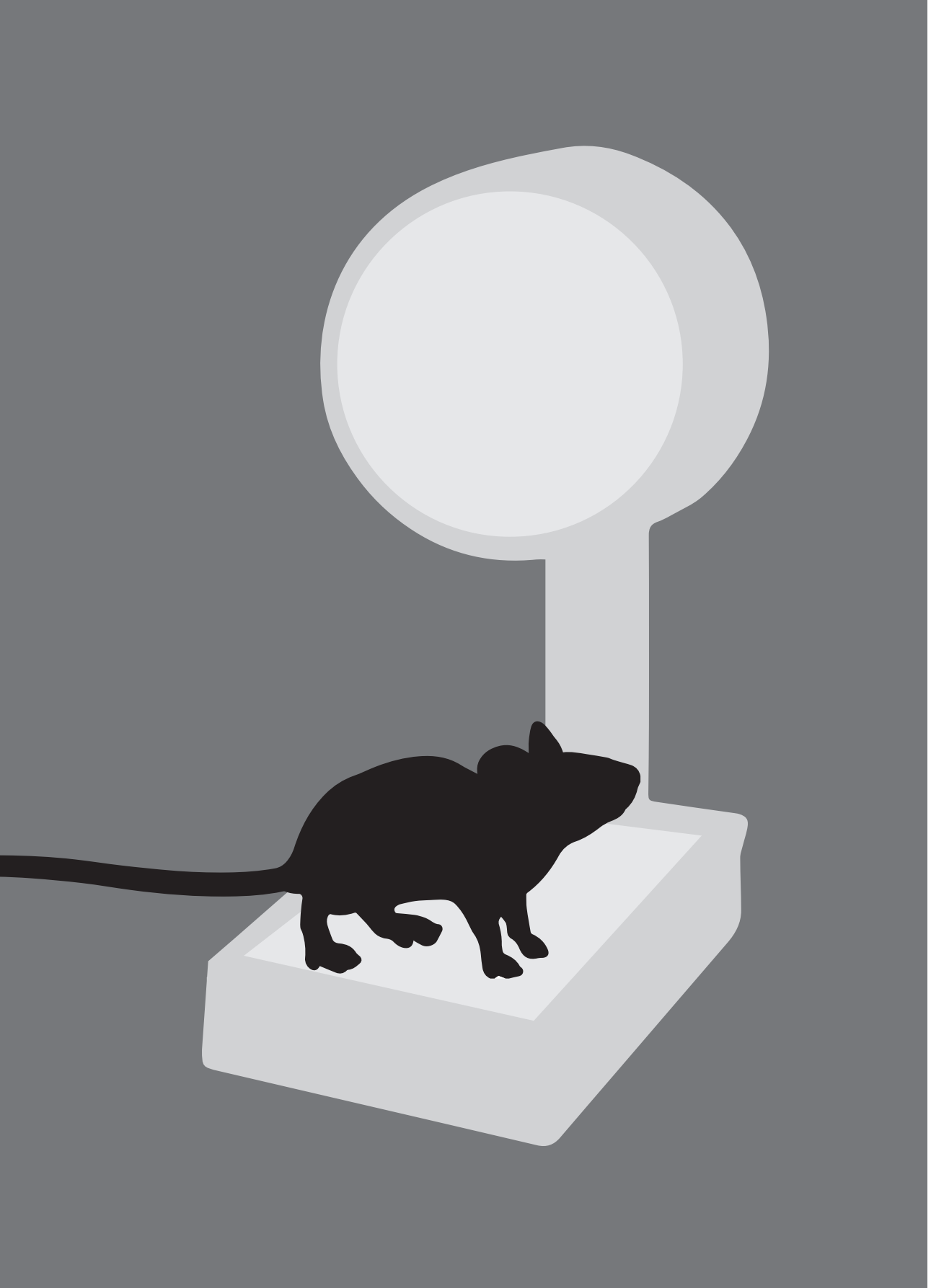
Table of Contents



Introduction

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Introduction

Obesity

Overweight and obesity are major health issues affecting a large number of people. The world health organization defines overweight and obesity as abnormal or excessive fat accumulation that presents a risk to health (1). Obesity is measured by body mass index (BMI), which is a person's weight in kilograms divided by the square of their length in meters. A person with a BMI > 25 is considered overweight and a BMI > 30 obese.

Overweight and obesity are becoming a problem of pandemic proportions. In 2017, a staggering amount of 49% of adults (18 years and above) in the Netherlands were overweight and 14% were obese (2). These numbers are not restricted to the Netherlands, as the worldwide statistics of 2016 are very similar (39% of adults overweight, 13% obese and 18% of children overweight or obese) (1). This epidemic has detrimental consequences. The increasing incidence of overweight predisposes millions of people to secondary diseases, such as type-2 diabetes, cardiovascular disease, cancer and osteoarthritis and reduces life quality and expectancy (3–6). As a result, obesity is the cause of 5% of deaths worldwide, and even 8% in high-income countries (7). Furthermore, the economical burden is alarming. Productivity loss is twice as high in obese individuals versus normal weight individuals and leads to economic costs due to more sick-leaves and disability pension (8, 9). The global economic burden of obesity is estimated to be 2.8% of global gross domestic product, which is barely less than the global impact from smoking or armed violence, war and terrorism, which are the two highest economic burdens worldwide (10).

The susceptibility to become obese is highly variable in humans (and rodents). While some individuals become obese, others remain lean when exposed to the same environment. The cause of this variable susceptibility is complex, because considerable evidence points towards influences by both genetics and the obesogenic environment we live in (in which we encounter an abundance of (palatable) food supply) (11). The high variability to become obese has led to multiple theories. For example, the thrifty genotype hypothesis and behavioural susceptibility theory suggest that natural selection has led to the maintenance of genes that enhance responsiveness to feeding opportunities and that enable individuals to efficiently process food to deposit fat during periods of food abundance (12). These genes essentially would have aided in the survival of individuals in an environment where food availability was uncertain, such as in the time of hunters and gatherers. However, in the current obesogenic environment where food is plentiful, genes that aid in weight gain can instead become detrimental. The thrifty hypothesis does not fully capture the complexity of predisposition to obesity, nor does any other model to date (13). With a continuous increase in the obesity epidemic, there is a need for further research into mechanisms regulating body weight in order to gain knowledge on why some people are more susceptible to obesity and perhaps provide new bases for therapeutic interventions.

Body weight regulation

Body weight is regulated by balancing energy intake and energy expenditure. Gaining weight is the result of an imbalance, which leads to the storage of excess energy in white adipose tissue (WAT). Energy intake is influenced by many factors, such as satiation/hunger, motivational drive and palatability of food. Energy expenditure is considered to be the result of basal metabolic rate, physical activity, diet-induced thermogenesis and thermogenesis that is required to maintain a stable body temperature. The latter consists of shivering and non-shivering thermogenesis. Non-shivering thermogenesis is heat produced by brown adipose tissue (BAT). BAT activity plays a large role in metabolism since it burns ingested fatty acids and glucose from the diet to produce heat. BAT size correlates negatively with BMI, which suggests that BAT activity plays an important role in human body weight regulation (14).

Since obesity is basically the result of greater energy intake than energy requirements, reducing weight should theoretically be easily achievable by suppressing intake and increasing energy expenditure. However, many people try to lose weight and on the long term this is effective in only ~20% of individuals (15). Even more, dieting is often followed by weight regain or even weight gain (16, 17). A challenging factor in losing weight is that the body counteracts weight loss by behavioural changes, such as increased food cravings or decreased energy expenditure (18–20).

How the body regulates body weight is an extensively studied subject, but is terribly complex. The brain, especially the hypothalamus, is known to play an essential role in energy balance. In this thesis, we seek to further understand how specific neurons in the brain contribute to energy homeostasis. To do so, we focus on one particularly interesting hormone, leptin, which is known as an adiposity signal that links peripheral energy storage in WAT to behaviours regulated by the brain. Leptin plays a crucial role in body weight regulation and is known for reducing food intake and increasing energy expenditure. In the following sections the discovery of leptin, how leptin signals adiposity levels to the brain and behaviours attributed to leptin will be described.

The discovery of leptin by leptin deficient *ob/ob* mice

In 1949, a recessive mutation was discovered that caused mice to become hyperphagic (increased appetite) and hypoactive resulting in obesity (four times the weight of normal littermates) (21). This mutation was designated by the symbol *ob*, from the

word obese, and mice homozygous for this mutation were called *ob/ob* mice (21). Parabiosis of *ob/ob* mice with wildtype mice, i.e. connecting the blood circulation of *ob/ob* mice to that of wildtype mice, resulted in a decrease in the severity of obesity of *ob/ob* mice while there was no effect on the wildtype mice (22). This revealed that *ob/ob* mice lack a factor that regulates body weight. More than 40 years after its discovery, in 1994, the *ob* gene was cloned for the first time (Zhang et al., 1994). In 1995, injecting the *ob* protein in *ob/ob* and wildtype mice was found to reduce body weight and food intake and increase energy expenditure (24). Due to a nonsense mutation at amino acid 105, the *ob* protein is not produced in *ob/ob* mice, showing that the lack of *ob* protein renders mice obese (23).

Since the *ob* protein made an animal thinner, the name leptin was proposed, which is derived from the Greek word leptós, meaning thin. Leptin is highly preserved across mammals, including humans, suggesting high functional importance. Leptin analogs also exist in amphibians, reptiles, fish and insects, but it remains to be determined whether leptin functions as an adiposity signal in non-mammals (25–27).

Leptin secretion

Leptin is mainly derived from WAT and circulates in levels proportional to the amount of WAT and the size of adipocytes (28, 29). As such, leptin levels generally correlate with BMI in both rodents and humans and convey information about the body's energy storage (28, 30–33). Obese individuals have high circulating leptin levels (hyperleptinemia). Since these individuals do not respond to the higher leptin levels by reducing food intake, individuals with diet-induced obesity (DIO) are thought to have become "leptin resistant".

Although there is a strong correlation between body weight and circulating leptin levels, studies report highly variable leptin levels at any level of body fat (32). This variability exists because more factors influence leptin secretion. Amongst others, gender (estrogens are associated with increased leptin levels), circadian rhythms and feeding (increased insulin and glucose after meals induce leptin secretion) affect leptin levels (28, 33–48).

Leptin receptor

To induce behavioural effects, leptin must cross the blood-brain-barrier and bind to leptin receptors (LepR) in the brain. The LepR is the product of the *db* gene (49). Soon after the discovery of the LepR, it became clear that at least six LepR isoforms exist (LepRa-f) (50, 51). The LepR belongs to the family of cytokine-1 receptors, which are transmembrane receptors that respond to cytokines. The LepR isoforms share a common extracellular ligand-binding domain and, aside from LepRe, the LepRs share common transmembrane domains, but have differing cytoplasmic domains. The LepRb has a longer cytoplasmic domain and is capable of signal transduction (52). Interestingly, in *db/db* LepRb deficient mice, an alternatively spliced transcript of LepRb is produced with a 106 base insertion, which leads to premature termination of the intracellular domain (53). Like *ob/ob* mice, *db/db* mice are obese. Since the long intracellular domain is necessary for signal transduction, the inability of leptin signalling is thought to cause obesity in *db/db* mice.

All LepR subtypes are capable of binding to leptin and endocytosis of leptin (54). The short forms of the LepR are highly expressed in microvessels, which make up the blood-brain-barrier, and are therefore thought to play a role in leptin transport from blood to the CNS (55). This is supported by the fact that the LepRb deficient *db/db* mice are still able to transfer leptin across the blood-brain-barrier (56). LepRb is expressed throughout the brain with prominent expression in the hypothalamus and is thought to mediate behavioural effects by leptin (57).

Central and peripheral leptin functions

Binding of leptin to LepR in the brain induces leptin's well-known reduction in food intake in mammals, but also for instance in fish and flies (25, 58). Besides feeding, three aspects of energy homeostasis that are influenced by leptin are of interest for this thesis, namely motivation for food reward, locomotion and thermogenesis. Of note, leptin plays a role in a far larger array of physiological responses ranging from reproduction and immunity to mood, cognition and neuroprotection (59, 60).

Motivation

In general, motivation refers to the willingness to work for something, such as a food reward. A validated method to test this is the progressive ratio task, in which animals must increasingly work to obtain a reward, which is generally a palatable sugar solution or sugar pellets (61). The work entails pressing a lever or performing nose pokes a certain amount of times. Once a ratio is completed, the animal receives a reward, after which the consecutive ratio requires a higher amount of presses or nose pokes for the same reward. The amount of presses/pokes made or rewards received at the end of the task are used as a measure of motivation.

In rodents, food restriction increases motivational behaviour and peripheral administration of leptin attenuates this (62–65). Similarly, in humans that lost weight by dieting, leptin reduces the activity of reward and hedonic feeding related brain areas in response to visual food cues (66, 67). Thus, leptin reduces motivation in both rodents and humans.

Locomotion

The effect of leptin on locomotor activity is less clear-cut and rather interesting: i) leptin increases locomotor activity in otherwise hypoactive obese *ob/ob* mice to levels seen in normal mice (68), ii) leptin has no effect on locomotion in normal fed mice (68, 69) and iii) leptin decreases locomotion in food restriction-induced hyperactive mice (70). These results suggest that leptin differently modulates locomotor activity depending on the amount of bodily energy reserves: leptin promotes locomotion when energy reserves are high, but decreases locomotion when energy reserves are low.

Thermogenesis

Thermogenesis is the process of heat production. Heat is generated by physical activity, diet-induced thermogenesis, shivering and non-shivering thermogenesis, which is heat produced by BAT. Peripheral administration of leptin has been shown to increase body temperature in mice (71, 72). More specifically, BAT temperature is increased with leptin administration or hyperleptinemia seen with obesity (73, 74). In both humans and rodents, the lack of leptin, such as in *ob/ob* mice or decreased leptin levels due to dieting, results in a lower body temperature compared to controls and this can be partially rescued by leptin treatment (75–77). A recent study in *ob/ob* mice showed that leptin increases body temperature by decreasing heat loss through the tail, implying that leptin is also capable of raising body temperature without directly activating thermogenesis (77). Thus, leptin raises body temperature directly via thermogenesis, but also via other indirect mechanisms such as reducing heat loss.

Variable leptin sensitivity

Although leptin is most-known for reducing food intake, it has become clear from rat studies that not all animals are sensitive to leptin's anorexic effects (78–80). While many rats reduce feeding after leptin injections when compared to vehicle injections, these studies showed that ~50% of rats increase feeding after leptin. These studies further showed that whether or not an animal is sensitive to leptin predicts the degree of weight gain on a high caloric diet: when given the same diet, animals that responded to leptin by decreasing food intake gained less weight compared to animals that increased food intake (78, 80).

Not only the feeding response to leptin but also the thermogenic response to leptin positively correlated with body weight gain on a high caloric diet (80). Rats that decreased feeding after exogenous leptin, also increased body temperature to a greater extent after leptin injections and ultimately gained less weight on a high caloric diet. These studies suggest that reduced sensitivity to the behavioural effects of leptin may predispose animals to become obese. Whether this variable leptin sensitivity is also generalizable to other rodent species or even humans remains to be determined. If this variability occurs in humans and predicts body weight gain, this may be an interesting diagnostic method to identify subpopulations that are at a greater risk of becoming obese.

Leptin in the arcuate nucleus

One of the best studied regions in leptin research is the arcuate nucleus (ARC), which contains one of the highest numbers of LepR (81). In the ARC, two main cell types express LepR: the anorexigenic (reducing food intake) cocaine- and amphetamine-regulated transcript (CART)/ pro-opiomelanocortin (POMC) and the orexigenic (increasing food intake) agouti related peptide (AgRP)/neuropeptide-Y (NPY) neurons. Leptin, in part, inhibits feeding by modulating the activity of both ARC neuronal populations by activation of different pathways (82). AgRP/NPY neurons are inhibited via the LepR-JAK2-MAPK pathway and POMC neurons are activated by the LepR-JAK2-PI3-k pathway, thus favouring the anorexigenic tone of the ARC and reducing food intake.

The ARC is thought to be an important region in the maintenance of obesity, because in rodents the ARC develops leptin resistance with diet-induced obesity and, thus, becomes less sensitive to leptin action (83–85). However, leptin action on ARC neuronal populations does not cover all aspects of central leptin signalling, as deletion of LepR from POMC/CART or AgRP/NPY in the ARC results in obesity that is less severe than that seen in *db/db* (86, 87). In line with this, restoration of leptin signalling in the ARC of *db/db* mice only modestly decreased body weight and food intake, but was unable to normalize these aspects (88). Since ARC LepR signalling cannot explain all behavioural effects of leptin, it is important that we study other LepR-expressing regions to increase our knowledge of the complex approach of leptin to regulate body weight.

This thesis focuses on LepR expressing regions that are associated with different aspects of body weight regulation, but that have gained less attention with respect to the functional implications of leptin. We chose four LepR expressing brain regions, because of the behaviours they are associated with: the midbrain -consisting of the ventral tegmental area (VTA) and substantia nigra (SN) - is known for its role in motivational behaviour; the lateral hypothalamus (LH) is known as the feeding center; and the dorsomedial hypothalamus (DMH) is a region involved in energy expenditure, mainly in the form of thermogenesis. The associated behaviours gave us a basis to further study the behavioural functions of LepR neurons in these regions

with respect to body weight regulation. A small review of the four regions will be given in the next section.

Leptin in the midbrain

The midbrain is a dopaminergic (DA) region divided into two main regions: the VTA and SN. Both contain mainly DA neurons and regulate reward-processing, motivation and locomotion. LepR expression has been found in both SN and VTA, suggesting that leptin modulates behaviours enforced by the midbrain, such as motivation (89–91).

VTA

The VTA contains DA (65%), inhibitory γ-aminobutyric acid (GABA, 30%) and excitatory glutamatergic neurons (5%). The major projection of the VTA is the mesolimbic DA circuit, which consists of VTA DA neurons that project to the striatum, which includes the nucleus accumbens (NAc). This projection is referred to as the mesolimbic DA system and is associated with reward-processing and motivational behaviour. In both rats and mice, the majority of LepR-expressing neurons in the VTA are DA neurons and some have been shown to express markers for GABA neurons (90, 92, 93).

Many studies point towards leptin induced inhibition of VTA DA neurons: peripheral leptin injections decrease VTA DA activity, leptin directly inhibits VTA DA neurons in slices and LepR deletion in DA neurons results in burst firing (90, 94–96). VTA GABA neurons also express LepR and are known to provide local inhibition of DA neurons (97). Whether indirect effects via VTA GABA-LepR neurons contribute to the inhibition of VTA DA neurons has not been studied thus far.

SN

The SN is divided into the SN pars compacta (SNc) and SN pars reticulata (SNr), which contain mainly DA and GABA neurons, respectively. The main DA output of the SN is to the dorsal striatum. This pathway is referred to as the nigrostriatal pathway and is the SN correlate of the mesolimbic DA pathway originating in the VTA. SN DA neurons are known for their role in motor control, particularly for the motor deficits following degeneration of SN DA neurons in Parkinson's disease. In addition to motor symptoms, psychiatric symptoms, such as depression, anxiety and motivational deficits, but also body weight loss and malnutrition are often observed in patients with Parkinson's disease (98–100). Recently, a study in rats showed that a loss of SN DA neurons resulted in motivational deficits, highlighting the role of SN DA neurons in other behaviours than motor control alone (101).

Other than that LepR are expressed in the SN, in both SNc and SNr regions and >95% of SN-LepR neurons co-localize with TH, not much is known about SN LepR neurons and the function of SN LepR in behaviour has been rather neglected (91, 93).

Midbrain LepR neurons

Increased midbrain DA activity is associated with enhanced motivation (102–104). Since VTA DA neurons express LepR and are inhibited by leptin, VTA DA-LepR neurons were thought to mediate leptin's decrease in motivation in fasted individuals. However, VTA LepR neurons are scarcely expressed in the mesolimbic DA pathway important for motivational behaviour. Instead, these neurons project heavily to the extended amygdala, which is important in fear behaviour (91, 92). These findings were supported by studies showing that direct injection of leptin into the VTA had no effect on motivational behaviour, whereas anxiety-like behaviour decreased with intra-VTA leptin injections (105, 106). However, knockdown of LepR in the midbrain neurons increased motivational behaviour, suggesting that midbrain LepR neurons modulate motivational behaviour (105). Together, these results suggest that SN LepR neurons may drive changes in motivation induced by leptin.

Midbrain LepR neurons have further been shown to modulate other aspects of energy homeostasis, such as feeding and locomotion. Intra-VTA leptin infusion and VTA leptin overexpression decrease body weight and feeding, while VTA leptin antagonist and VTA LepR knockdown using shRNA increase feeding (90, 107–109). These studies suggest that the VTA mediates at least part of leptin's anorexic effect. Furthermore, knockdown of midbrain LepR and deletion of LepR or STAT3 in DA transporter (DAT) neurons have no effect on feeding behaviour (95, 105, 110). Since the VTA mediates a decrease in feeding, but silencing LepR signalling specifically in DA neurons does not affect feeding, this suggests that non-DA-LepR neurons of the VTA underlie this behaviour. Whether SN LepR neurons also modulate feeding behaviour has not been examined yet.

Locomotor behaviour is modulated by midbrain LepR neurons, but experiments studying these neurons produced rather contradicting results. Mice are spontaneously active in the dark phase of the 12:12hr light cycle adapted in laboratories, while they rest during the light phase. Of interest, when studied in the light phase, locomotion is not affected by any strategy targeting midbrain LepR neurons: shRNA LepR knockdown in the VTA, LepR deletion in DAT neurons or STAT3 deletion in DAT neurons (90, 95, 110). When studied in the dark phase, VTA leptin injections in ad libitum fed rats do not affect locomotion, whereas shRNA LepR knockdown in the VTA or LepR deletion in DAT neurons increase locomotion (90, 95, 110). Taken together, these data

show that midbrain LepR neurons modulate dark phase locomotion, but intra-VTA leptin injections do not. This suggests a role for SN LepR neurons in mediating leptin's effect on locomotion.

To conclude, literature suggests that midbrain LepR neurons modulate motivational behaviour, locomotion and feeding and that VTA LepR neurons are likely not involved in motivational behaviour, but in the regulation of fear and feeding. These results imply that SN LepR neurons also mediate effects of leptin. However, behavioural effects of SN LepR neurons specifically have not yet been characterized.

Leptin in the lateral hypothalamus

In the 1950's, the LH emerged as the feeding centre, because in both cats and rats LH lesions resulted in starvation (111). Since then the number of behaviours known to be mediated by the LH has substantially increased and include feeding, drinking, arousal, stress and reward.

OX and MCH neurons

LH neurons are identified by their neurochemical phenotype. Two well-known cell types of the LH are hypocretin/orexin (OX) and melanin-concentrating hormone (MCH) neurons that have mostly complementary roles in physiological functions including sleep, activity and thermogenesis (112–119). For instance, MCH reduces thermogenesis and physical activity, whereas OX increases these parameters (120–124). Two behavioural aspects in which OX and MCH are similar is that they are both known to be orexigenic and promote feeding and that both are capable of modulating the mesolimbic dopamine system and reward related behaviour (125–129). Both MCH and OX expression in the LH increase with food restriction, suggesting that these neurons mediate adaptive changes in behaviour in response to energy state, for instance by changes in leptin levels (130, 131).

GABA neurons

Even though OX and MCH are the most well-known cell types of the LH, GABAergic neurons are the most predominantly expressed cell type population of the LH (132, 133). LH GABA neurons are regarded orexigenic, as activation promotes feeding, whereas inhibition suppresses feeding (134–139). These neurons are further associated with food reward and locomotion (136, 140). LH GABA neurons modulate the mesolimbic DA system and reward behaviour by projections that directly innervate the VTA, but also indirect LH->VTA projections via local OX neurons, which are known to stimulate VTA DA activity (128, 134, 137, 140).

LH LepR neurons

LepR expression in the LH is exclusively present in GABAergic neurons and not in OX or MCH neurons (130). Although LH GABA neurons are considered orexigenic, intra-LH leptin injections decrease feeding and body weight suggesting that leptin inhibits LH GABA mediated behaviours (130). Furthermore, intracerebroventricular leptin decreases the rewarding effect of LH stimulation, suggesting a role for LH leptin signalling in reward (141). LH LepR neurons densely innervate and inhibit local OX neurons (130, 142). LH LepR neurons further project to the VTA, periaqueductal grey, dorsal raphe, SN and dorsomedial hypothalamus (130). Intra-LH leptin injections regulate TH expression in VTA DA neurons and NAc DA content (130, 143). As for projections to the VTA, it has been suggested that LH LepR neurons directly innervate VTA DA and non-DA neurons, such as GABA neurons (144). Thus, LH LepR neurons modulate the mesolimbic DA system through both direct and indirect (via OX) projections, similar to LH GABA neurons.

LH LepR neurons have been shown to co-express neurotensin (Nts, ~60%), galanin (Gal, ~44%), the melanocortin-4 receptor (MC4R, ~27%) and cocaine- and amphetamine-regulated transcript (CART, ~24%) (145–147). These numbers do not add up to 100%, because there is a high overlap between Gal-LepR and CART expression, between MC4R and Nts neurons and between Nts and Gal expression (145). Interestingly, only 34% of LH LepR neurons are depolarized by leptin and 22% are hyperpolarized (130).

Neurotensin

Nts is a neuropeptide known to reduce feeding, induce drinking and promote weight loss (148, 149). Activation of LH Nts neurons decreases body weight and strongly induces locomotion and drinking, but not feeding, suggesting that Nts release from the LH is more involved in energy expenditure than feeding (150–152).

LH LepR-Nts neurons are mostly depolarized by leptin, inhibit local OX neuronal activity and project directly to the VTA (146). Loss of LepR in Nts neurons increases body weight, but not feeding and decreases locomotion and motivational behaviour (64, 146). Intra-LH leptin injections decrease motivational behaviour in food restricted mice, but this effect is blunted in mice with LepR deletion in Nts neurons (64). Furthermore, deletion of NtsR1 in the VTA resulted in a blunted response to leptin on motivation in mice (152). Together, these results suggest that Nts release by VTA projecting LH LepR-Nts neurons is necessary for the regulation of motivational behaviour by leptin.

Galanin

Gal is associated with increased dietary fat. Gal knockout mice decrease fat consumption and mice with Gal overexpression increase fat consumption (153, 154). Furthermore, LH Gal neuronal activation increases motivational behaviour for food reward and locomotor activity (140).

Intra-LH leptin stimulates Gal expression (155). LH LepR-Gal neurons innervate local OX neurons and the locus ceoruleus (LC), but rarely project to the VTA (155). Of note, there is a high overlap between Gal and Nts neurons and it is suggested that the majority of LH LepR-Gal also express Nts (145). OX neurons express Gal receptors but not Nts receptors and, as such, LH LepR neuron-mediated inhibition of OX neurons is via release of Gal (155). Similar to OX neurons, the LC is also associated with arousal, reward and nutrient intake (156, 157). In line with this, knockout of LepR in Gal neurons in the LH increases body weight gain, modulates nutrient intake and increases motivation for sugar reward (155). So, LH LepR-Gal neurons modulate motivation and nutrient preference possibly through both OX and LC projections.

MC4R

MC4R-expressing neurons in the LH are involved in regulating appetite, demonstrated by the fact that MC4R antagonism in the LH leads to hyperphagia when mice are placed on an obesogenic diet (158). The majority of MC4R-LepR neurons are hyperpolarized by leptin (147). Thus, leptin defends body weight gain in part by controlling caloric intake by hyperpolarizing MC4R-expressing LH neurons.

CART

CART is a neuropeptide involved in reward, feeding and stress. Activation of LH CART neurons increases locomotion, body temperature and feeding (159). Furthermore, CART release in the VTA is rewarding, while blocking CART-CART receptor binding decreases motivational behaviour (160). CART neurons of the LH project to the VTA and may thus modulate motivation (160). However, behavioural effects of LH LepR-CART neurons have not been studied thus far.

To summarize, LH LepR neurons mainly co-express Nts and Gal and seem to modulate energy expenditure and motivational behaviour. The latter is likely driven by regulation of the mesolimbic DA system through both direct and indirect projections via local OX neurons. Besides intra-LH Leptin injections or permanent deletion of LepR from particular LH subpopulations, behavioural effects of LH LepR neurons as a group have not yet been studied.

Leptin in the dorsomedial hypothalamus

The DMH is known to be an orexigenic centre and to regulate energy expenditure, most prominently BAT thermogenesis. Rodents with DMH lesions are hypophagic, hypoactive and show disturbed thermogenesis (161–163).

Previous studies indicate that leptin action in the DMH controls thermogenesis. Even in obese mice that are resistant to the anorexigenic effect of leptin, intra-DMH leptin injections increases body temperature (71, 72). Similarly, chemogenetic activation of LepR neurons in the DMH increases BAT thermogenesis and body temperature and also increases physical activity, resulting in body weight loss (71, 72). DMH LepR mediated thermogenesis is thought to occur via prolactin-releasing peptide (PrRP) neurons, since LepR deletion from these neurons inhibits leptin-induced thermogenesis (164). In line with this, DMH LepR neurons are known to project to brain regions involved in thermoregulation, such as the periaqueductal grey, paraventricular hypothalamic nucleus and preoptic area, but have also been suggested to directly project to BAT (165, 166).

Few studies link DMH LepR neurons to feeding, but studies that do make this link showed that intra-DMH leptin injections decrease feeding, leptin hyperpolarizes 40% of DMH GABA neurons that promote feeding and stimulation of DMH GABA-LepR neurons that project to ARC decrease feeding (71, 167, 168). However, chemogenetic activation or inhibition of DMH LepR, ablation of DMH LepR or intra-DMH leptin injections in *ob/ob* mice does not affect feeding (72, 168). These studies suggest that although DMH LepR neurons are capable of decreasing food intake, they are not necessary for control of feeding.

In conclusion, DMH LepR neurons control energy expenditure primarily via thermogenesis, but also through changes in physical activity. Of particular interest for weight loss therapies, DMH LepR stimulation has been shown to increase energy expenditure resulting in weight loss in mice (72). Thus, DMH LepR signalling mediates changes in energy expenditure and ultimately regulates body weight, but it remains to be determined whether weight loss can also be enhanced in obese or dieting individuals.

To summarize, our knowledge of the behavioural effects exerted by LepR neurons of the brain regions discussed (VTA, SN, LH and DMH) remains incomplete. The experiments presented in this thesis hope to increase this knowledge by transiently stimulating LepR neurons in these regions in mice using chemogenetics.

Chemogenetics

A relatively novel tool developed by the Roth lab in 2007 enables investigating the behavioural effects of modulating neuronal activity (169). The technique is based on the modification of cell-surface G-protein coupled receptors (GPCRs), so that activation by endogenous ligands is attenuated and instead activation occurs by small synthetic inert molecule drugs. Already in the late 1990's GPCRs were modified to activate upon synthetic ligand binding, but the ligands used had high affinity with a native receptor (170, 171). With the finding of an inert ligand that lacks pharmacological activity at other targets began an exciting new era in which modulation of neuronal activity is possible.

Designer receptors exclusively activated by designer drugs (DREADDs) are modified muscarinic receptors that exclusively respond to a designer drug, often clozapine-N-oxide (CNO) (169). CNO was chosen because i) its parent compound, clozapine, has high affinity to muscarinic receptors (172), ii) CNO has high bioavailability in rodents and humans and penetrates the CNS (although recent studies showed that compared to clozapine, CNO only has limited access to brain tissue) (173, 174) and iii) the lack of affinity of CNO for non-DREADD receptors (172).

To target specific neuronal subpopulations, such as LepR-expressing neurons, DREADDs can be designed to be cell-type specific by using recombination technology (figure 1). To do so, DREADDs are manufactured to be Cre-recombinase-dependent by incorporating DREADD into a viral vector using a double-floxed inverse open reading frame (DIO), which means that the DREADD sequence is inverted and flanked by two Cre-recombinase target sequences, called loxP sites. The Cre-recombinase enzyme binds to the DIO-loxP sites, brings the two together and inverts the loxP flanked DREADD segment. This allows for the appropriate reading direction of the DREADD sequence and expression thereof. In this thesis, we use transgenic mice, referred to as LepR-cre mice, in which Cre-recombinase is present in cells expressing the LepR. By injecting a Cre-dependent viral vector containing the excitatory DREADD-receptor, hM3DGq, into a brain area, such as the lateral hypothalamus, we are able to specifically activate LepR neurons in a particular brain region with CNO.

In experiments presented in this thesis we used adeno-associated viral vectors (AAVs) encoding DREADDS, primarily AAV5-hSyn-DIO-hM3DGq-mCherry. AAV is a single-stranded DNA virus with a small protein capsule. AAVs are considered to be non-toxic efficient vehicles for gene transfer and are capable of inducing long-term expression of that gene (175). Many serotypes of AAVs exist which differ in transduction efficiency and tropism. In mice and non-human primates, **AAV5** is one of the most efficient vectors that also transduces neurons (176, 177). Since AAV5 also efficiently transduces other non-neuron cells of the brain, such as microglia, the human synapsin (**hSyn**) gene promoter has been added to the viral vector to target AAV5 transduction exclusively to neurons (178). **DIO**, as explained in the previous paragraph, enables Cre-dependent expression of the excitatory DREADD, **hM3DGq**. Finally, the hM3DGq is fused with **mCherry**, which is a fluorescent protein that is used as a marker to determine the localization of functional DREADD expression after behavioural experiments are completed.

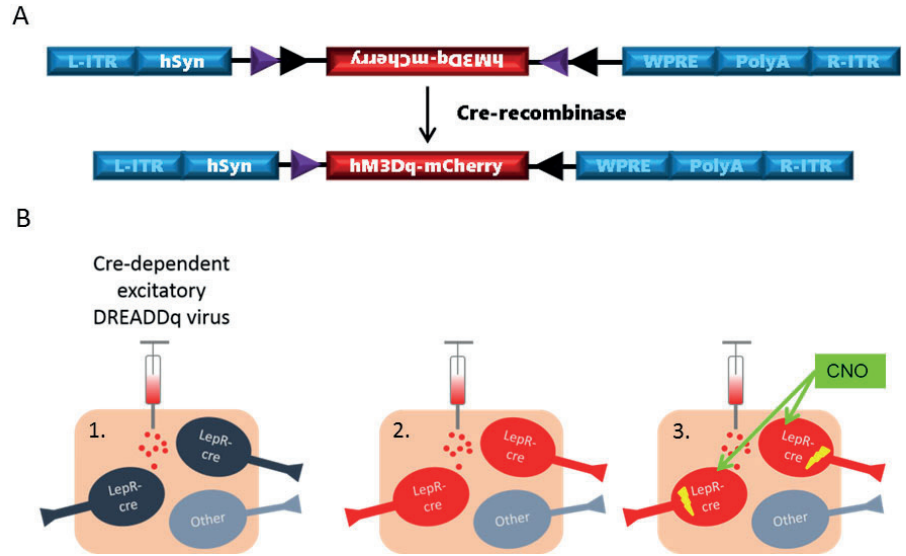

Figure 1. Using Cre-dependent DREADD to selectively target LepR neuronal subpopulations. (A) DREADDs are made Cre-recombinase dependent by incorporating DREADD-receptors (in figure: hM3Dq) into a viral vector in a double-floxed inverse manner. The Cre-recombinase enzyme is then necessary to recombine the sequence and allow expression of the receptor. (B) Schematic of chemogenetics.
1: Injection of a Cre-dependent excitatory DREADD (DREADDq) virus in a specific brain region of a LepR-cre mouse, which expresses Cre-recombinase in LepR-expressing neurons.
2: Cre-recombinase allows for the expression of DREADD. Since Cre is present in LepR-expressing neurons, DREADD will only be expressed in LepR neurons of the injected brain region.
3: Systemic CNO injection enhances neuronal activity of DREADD expressing neurons.

Chemogenetics has several advantages to older commonly used techniques, such as lesions, electrical stimulation or gene knockdown. First, chemogenetics, when used in combination with Cre-recombinase, enables us to manipulate neuronal activity of specific cell types, such as LepR-expressing neurons. Second, chemogenetics has a transient effect on neuronal activity (179). As such, chemogenetics makes it possible to compare baseline and CNO induced behaviour within the same animal. This not

only increases the power of the study, but because of this within-subjects design, also less animals are needed, since large variability between animals would otherwise urge you to use more animals. Third, behavioural effects of chemogenetics can last up to 10h (179). Since leptin is considered a long-term signal, it is of interest to study the behavioural effects of prolonged neuronal manipulation of LepR neurons. Because chemogenetic effects last up to 10h, this allows us to study the long-term effects of neuronal manipulation on behaviour, which is in contrast to the short-term effects (msec to sec) of optogenetics with which neuronal activity is acutely modulated by light stimulation. Fourth, DREADDs exhibit no endogenous activity and do not disrupt normal brain function (169, 179). This is in contrast to often used techniques, such as lesions or gene knockout, which are permanent adaptations and can induce downstream circuit changes. Finally, chemogenetics is a relatively non-invasive and undemanding technique. With chemogenetics, neuronal manipulation is possible with a systemic CNO injection and extra equipment, such as lasers necessary for optogenetics or external syringe pumps necessary for cannulation studies, are not required.

In conclusion, by using chemogenetics we are able to measure changes in behaviour after transiently activating neuronal activity of specific LepR neuronal populations in an otherwise intact animal.

Thesis outline

Body weight is regulated by balancing energy intake and energy expenditure. Leptin promotes weight loss by decreasing food intake and increasing energy expenditure, making it an ideal candidate to study body weight regulation. To date, the most extensive knowledge of leptin function comes from studies based on the ARC. However, leptin signalling in the ARC does not explain leptin's broad spectrum of behavioural effects. Therefore, this thesis focuses on LepR regions that have been less studied and explores how these regions contribute to body weight regulation by leptin.

In the following chapters, summarized below, we present the results of behavioural effects of peripheral leptin injections and chemogenetic activation of LepR neurons. In our chemogenetic experiments, we assessed the effects of enhancing LepR neuronal activity in the LH, DMH, VTA and SN. We studied behaviours that are known to be mediated by leptin, are relevant for body weight homeostasis and easily translatable to the human setting: feeding, motivation for food reward, thermogenesis and locomotion. With these experiments we hope to gain further insight into which brain regions mediate which aspects of leptin-mediated regulation of body weight. Ultimately, we hope to better understand mechanisms underlying both weight gain and weight loss and contribute to the finding of new therapeutic targets.

Chapter 1: Highly variable sensitivity to anorexic effect of leptin in mice

Leptin is known for reducing food intake. However, rat studies have showed that the feeding response to leptin (leptin sensitivity) is highly variable and the feeding response to leptin correlated with subsequent body weight gain on an obesogenic diet. This suggests that the sensitivity to leptin may explain differences in the susceptibility to become obese on an obesogenic diet. To test whether this relation is generalizable to another rodent species, chapter 1 reports the variability in leptin sensitivity and the correlation thereof to body weight gain on an obesogenic diet in mice. One specific aspect of feeding that leptin is known to reduce is the motivation for food in dieting rodents and humans. Therefore, we also examined whether leptin sensitivity correlated to the effect of leptin on motivational behaviour for food reward. If the correlation between leptin sensitivity and subsequent body weight gain exists in multiple rodent species, the likelihood that this phenomenon is generalizable to humans increases. Furthermore, if this is the case, leptin sensitivity may prove to be an interesting target for identifying individuals that are susceptible to obesity.

Chapter 2: Leptin receptor-expressing neurons in the substantia nigra regulate locomotion, and in the ventral tegmental area they regulate motivation and feeding

Many studies on leptin focus on the role of hypothalamic regions. A region far less studied is the midbrain, which consists of the VTA and SN and is known for its role in motivation. Previous studies remain inconclusive about the role of VTA LepR and SN LepR neurons on motivational behaviour and have further reported contradictory results regarding locomotion and feeding. In an attempt to decipher the role of VTA LepR neurons versus SN LepR neurons, in chapter 2 we chemogenetically activated SN LepR or VTA LepR neurons in LepR-cre mice to assess behavioural effects on motivational, feeding and locomotion.

Chapter 3: Do lateral hypothalamic leptin receptor-expressing neurons modulate motivational behaviour?

Leptin attenuates the increase in motivational behaviour for food reward in food restricted rodents and humans. Which LepR neurons mediate this behaviour remains largely unknown. VTA DA-LepR neurons were suggested to mediate this behaviour, but have been associated with regulating fear instead. Therefore, we wondered whether the LH, which is a LepR-containing region with dense VTA projections and is known to modulate VTA DA activity, mediates changes in motivation for food by leptin. Chapter 3 reports effects of permanently silencing LH LepR neurons and of chemogenetically activating or inhibiting LH LepR neurons on motivational behaviour for food.

Chapter 4: Effects of GABA and leptin receptor-expressing neurons in the lateral hypothalamus on energy homeostasis

LH GABA neurons are orexigenic neurons known to modulate various aspects of energy homeostasis, such as feeding and locomotion, which ultimately affect body weight. LH LepR neurons are a subpopulation of LH GABA neurons, which we hypothesized to have a more specific effect on energy homeostasis to promote a negative energy balance. In chapter 4, we assessed how chemogenetically activating LH GABA or LH LepR affect different aspects of energy homeostasis.

Chapter 5: Gnawing induced by LH GABA activation differently affects consumption dependent on experimental settings

In chapter 4, gnawing behaviour was repeatedly observed upon LH GABA stimulation. In chapter 5, we studied how different experimental settings and consistencies of food affect gnawing behaviour. We further determined whether gnawing behaviour is specifically directed at food or also non-food items and whether gnawing behaviour is affected by hunger status.

Chapter 6: Differential effects of short- and long-term stimulation of dorsomedial hypothalamic leptin receptor-expressing neuron activation on energy balance

DMH LepR neurons have been shown to enhance energy expenditure by increasing thermogenesis and physical activity. This results in a decrease in body weight. Therefore, we hypothesized that DMH LepR stimulation would enhance body weight loss in obese dieting mice by increasing energy expenditure, which would imply that DMH LepR neurons are an interesting target for weight loss therapies. To test this, in chapter 6, we chemogenetically activated DMH LepR neurons in both normal weight mice and mice previously exposed to an obesogenic diet to assess effects on energy homeostasis and weight loss.

Finally, in the ***discussion*** of this thesis we summarize and evaluate the findings of the experimental chapters. We also address limitations, future considerations and the societal significance of our results.

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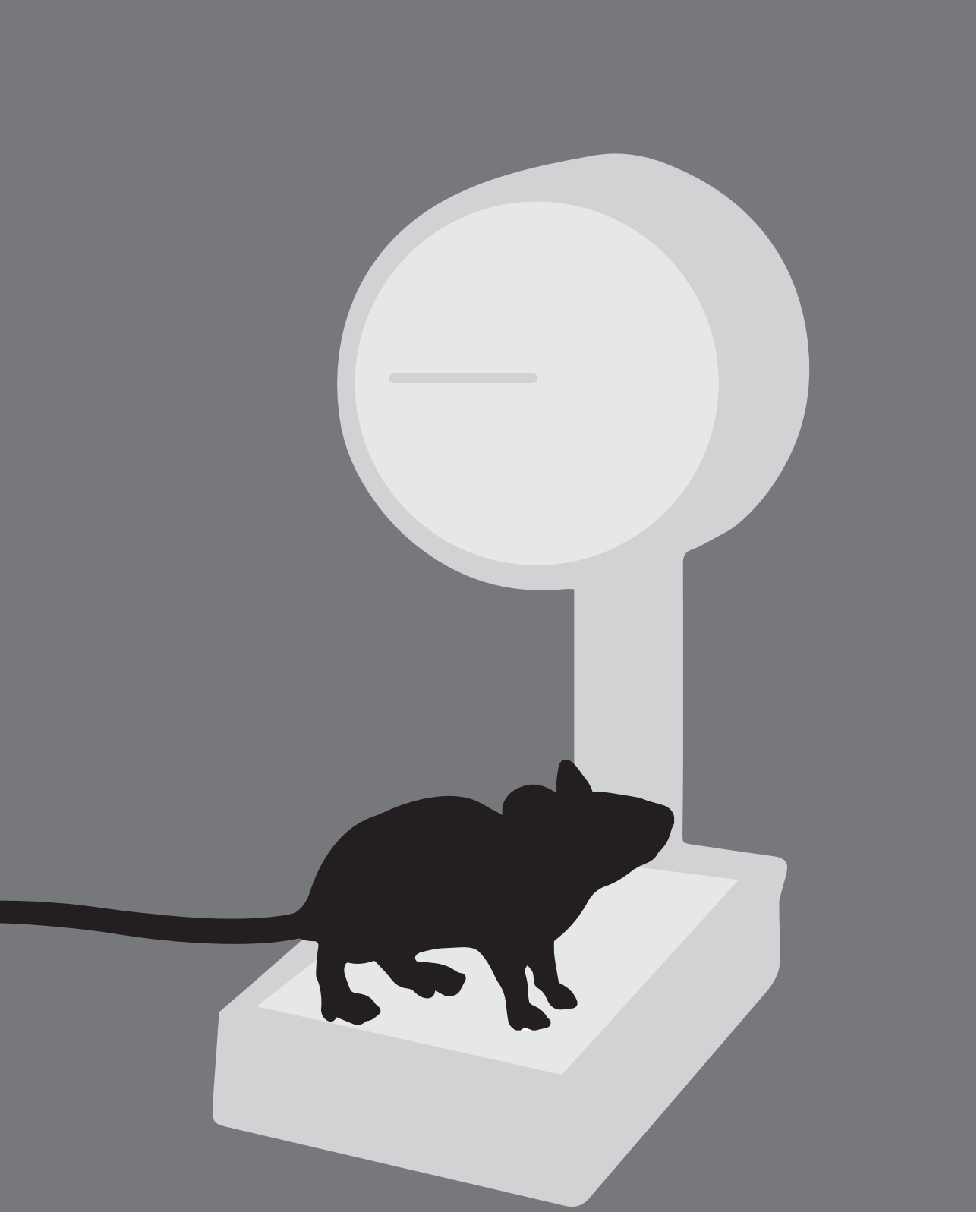

Chapter 1

Highly variable sensitivity in mice to anorexic effect of leptin

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Abstract

In humans and rodents, high variability exists in the tendency to become obese and it remains largely unclear why some become obese, whereas others remain lean when exposed to the same environment. Leptin has a significant role in body weight regulation and is well-known for decreasing appetite in both rodents and humans. In rats, it has been shown that not all animals respond to leptin by decreasing food intake, as some even increase food intake the first hours after leptin injections. This early feeding response to leptin ("leptin sensitivity") has been shown to predict weight gain on an obesogenic diet. These results suggest that leptin sensitivity may, in part, explain differences in susceptibility to obesity. We here examined leptin sensitivity in mice and whether leptin sensitivity predicts subsequent weight gain on a free choice high-fat high-sucrose (fcHFHS) diet. We also tested whether leptin sensitivity correlates with the response to leptin on motivation to work for a food reward. We found that leptin sensitivity correlated with both the response to leptin on motivation for food reward and the subsequent weight gain on a fcHFHS diet. Together, our results suggest that the relationship between leptin sensitivity and subsequent weight gain also exists in mice and may be generalizable to other species, perhaps even to humans.

Introduction

Overweight and obesity are major health risks affecting increasing numbers of the human population. In 2016, worldwide 39% of adults were overweight and 13% obese (WHO, 2018). While many people try to lose weight, on the long term this is effective in only ~20% of individuals (Wing and Phelan, 2005). Several environmental and biological factors are known to be related to weight gain (Bray, 2000) and it is unclear why some become obese, whereas others remain lean when exposed to the same environment. Thus, there is a need for deeper insights into the mechanisms of body weight regulation to explain differences in the susceptibility to obesity.

Leptin is an adipocyte-derived hormone, which has a significant role in body weight regulation in both humans and rodents. Leptin decreases appetite in both rodents and humans (Halaas et al., 1995; Hinkle et al., 2013; Rosenbaum et al., 2008) and increases energy expenditure by, for instance, increasing body temperature (Enriori et al., 2011). Recent studies in rats showed that leptin's effect on feeding is highly variable (de Git et al., 2018; Levin et al., 2004, 2003; Levin and Dunn-Meynell, 2002; Ruffin et al., 2004; van Dijk et al., 2005). Leptin sensitive (LS) rats responded with a strong anorexic response, while leptin resistant rats (LR) did not respond or even increased food intake after leptin injections (de Git et al., 2018; Ruffin et al., 2004).

In rats, the feeding response to leptin has been shown to predict weight gain on an obesogenic diet, i.e. animals that were sensitive to the anorexic effect of leptin gained less weight than those that were not sensitive (de Git et al., 2018; Ruffin et al., 2004). Similarly, in rats, baseline motivation for food reward correlates with obesity development and leptin administration has been shown to decrease motivation (Brown et al., 2017; Figlewicz et al., 2006; Hinkle et al., 2013; la Fleur et al., 2007; Rosenbaum et al., 2008; Sharma and Fulton, 2013; Woodworth et al., 2017). These results provide evidence that the sensitivity to the anorexic effect of leptin can explain differences in susceptibility to obesity in rats, and also that the effect of leptin on motivational behaviour may similarly predict weight gain. However, it remains unknown whether this relation is also present in another rodent species, the mouse. If this relation is present in more species, then perhaps this phenomenon is also generalizable to humans.

In mice, it has been reported that the susceptibility to obesity on an obesogenic diet varies (Enriori et al., 2007). However, no differences were found in the feeding response to leptin at 24h after leptin injection (Enriori et al., 2007). Differences in leptin sensitivity in rats were seen between 1 and 4h after injection, but not after 24h (de Git et al., 2018; Levin et al., 2004; Ruffin et al., 2004). Therefore, in this study we first assessed the feeding

response of individual mice at 1-24h after leptin injections. Furthermore, we analysed to what extent this feeding response predicts the effect of leptin on appetite-related motivational behaviour and subsequent weight gain on an obesogenic diet.

Material and methods

All experiments were approved by the Animal Experimentation Committee of the University Utrecht and were carried out in agreement with Dutch Laws (Herziene Wet op Dierproeven, Art 10.a2, 2014) and European regulations (Guideline 2010/63/EU).

Animals

Two cohorts of in house bred, 10-11 month old adult male homozygote LepRb-Cre transgenic mice (stock #008320, B6.129-Leprtm2(cre)Rck/J, The Jackson Laboratory, USA) were used for experiments. Cohort 1 consisted of 15 mice in which the feeding response to leptin and body weight gain on a free choice high-fat high-sucrose (fcHFHS) diet were assessed. Cohort 2 consisted of 11 mice, in which we similarly determined the feeding response to leptin and body weight gain on the fcHFHS diet, but we also tested for effects of leptin on motivational behaviour. Mice were housed individually (Type II L, 365x207x140mm, 530cm2, Tecniplast, Italy) and maintained in a temperature (21±2°C) and humidity (60-70%) controlled room on a reversed day/night (9:00 AM lights OFF, 9:00 PM lights ON) schedule with ad libitum chow and water, unless stated elsewise.

Drugs and foods

Recombinant mouse leptin (5mg/kg, NHPP, USA) was dissolved in 1x phosphate-buffered saline (PBS) and injected intraperitoneally. All animals received both leptin and PBS injections in a latin-square design.

Standard rodent chow (3,1kcal/gr, Standard Diet Service, United Kingdom), 10% (w/v) granulated sugar solution (4kcal/gr, Jumbo, the Netherlands) and lard (9.1kcal/gr, Ossewit/Blanc de Boeuf, Belgium) were used.

Leptin sensitivity

To test for leptin sensitivity, mice were food restricted 18h prior to the start of feeding measurements. Feeding measurements started 30min prior to the onset of the dark phase. Mice were injected with either leptin or vehicle and chow was presented ±25min after injections. Food intake was measured at 1, 2, 3, 4, 7 and 24h after injections. During the first 7h, mice were placed in an empty mouse cage, to which they were habituated prior to testing, with only 3 tissues, a water bottle and a pre-weighed amount of chow placed on the cage floor (no saw dust). Thereafter, mice were returned to their home cage with a pre-weighed amount of chow on the cage floor. In between leptin sensitivity tests, mice were allowed to recover for 1 week.

Progressive ratio operant behaviour

Mice operant boxes (ENV-307W, Med Associates Inc., USA) were fitted with two levers, a cue light above the active lever (AL), a house light, a speaker and a liquid receptacle. Throughout all sessions, when the number of AL presses to complete a ratio was reached, the house light and a tone were presented for 5sec, after which a sucrose reward was delivered for 2sec (38ul, 20% w/v sugar solution in tap water). During the training phase, animals were food restricted to ~90% of their original body weight. The first day animals were habituated for 15min to an operant box, in which we placed a droplet of sugar solution (20% w/v in tap water) in the receptacle. The next day, operant training started with a fixed ratio (FR) 1 paradigm for 30min/session. Once mice learned to press on the active lever >20 times and <10% on the inactive lever, animals were switched to FR3 (30min/session) and then FR5 (60min/session). Then, once >60 rewards were earned and <10% of the presses were made on the inactive lever, training was switched to the progressive ratio (PR, 60min/session) and animals were returned to ad libitum feeding. The amount of ALP necessary for each ratio in the PR task was based on 5*e(x*0.2)-5, rounded to the nearest integer, where x is the position in the ratio sequence (Richardson and Roberts, 1996). Injections started when mice had stable performance (over 3 consecutive days of training no more than ±1 reward from average).

Effects of leptin on PR response were measured by injecting leptin/vehicle after 6 or 18h food restriction. The effect is reported as ALP on injection day as a percentage of ALP the day before, on which mice were ad lib fed. When food restricting for 6h, food was removed 1-2h prior to dark phase onset and testing started 4-5h into the dark phase. When food restricting for 18h, food was removed, except for 1 piece of chow (~1.3gr/piece), halfway through the dark phase Operant testing was done at the onset of the dark phase the following day. In between operant tests with leptin/vehicle injections, mice were allowed to recover for 1 week.

Free choice high-fat high-sucrose diet

Previously, we found that a warm environment enhanced body weight gain in mice on a fcHFHS diet (fig. 1). Prior to the presentation of the fcHFHS, mice were habituated to a warmer 27±1°C room and a carton house in the cage for one week. Then, next to regular chow and water, a 10% sugar solution bottle and lard were presented on a suspended spoon in the cage. Cohort 1: After two weeks of the fcHFHS diet in the

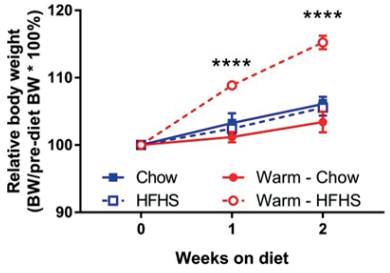

Figure 1. Mice gained weight within 2 weeks on a free choice high-fat high-sucrose diet when housed in a warm (27±1°C) room, but not when housed at normal room temperature (21±1°C).
Chow n=6, HFHS n=6, Warm-Chow n=5, Warm-HFHS n=8. Mean±SEM. **** p<0.0001: Warm-HFHS vs all other groups.

27±1°C room, the diet was continued for one week at 21±1°C. Thereafter the diet was removed and only chow and water were provided for one week until the end of the experiment. Cohort 2: Mice were given the fcHFHS diet at 27±1°C for two weeks and then at 21±1°C for 1,5 weeks. At the end of the experiment, both cohorts of mice were anesthetized with an intraperitoneal injection of Euthanimal (Euthanimal, Alfasan BV, the Netherlands). Epididymal and subcutaneous white adipose tissue were unilaterally dissected and weighed.

Data Analysis and Statistics

Behavioural data was analyzed and visualized using Microsoft Excel, Graphpad Prism (version 7.04, Graphpad Software Inc., USA) and SPSS (version 23, SPSS Inc., USA). Outliers or animals that ate <1gr chow over 7 hours (average: 1.9gr ±0.1, n=15) after injections were removed from analyses. Independent samples t-tests and two-way repeated-measures ANOVAs were used where applicable with Bonferroni adjusted post hoc tests. When in two-way repeated measures ANOVA the assumption of sphericity was violated, corrected degrees of freedom are reported. A significance criterion of $p<0.05$, two-tailed, was adopted in all statistical analyses.

Results

Cohort 1: leptin sensitivity and free choice high-fat high-sucrose diet

We tested leptin sensitivity by measuring food intake after intraperitoneal leptin (5mg/kg) and vehicle injections in mice that were food restricted during the late dark phase and the light phase. We normalized food intake after leptin injections to food intake after vehicle injections (leptin/vehicle*100%) to determine leptin sensitivity (defined as the percentage of reduction of food intake following leptin administration compared to vehicle). Individual plots of leptin sensitivity revealed two types of responders in mice: 8/14 mice suppressed feeding, while 6/14 mice increased feeding after leptin injections compared to vehicle (fig. 2A). Similar to de Git et al (de Git et

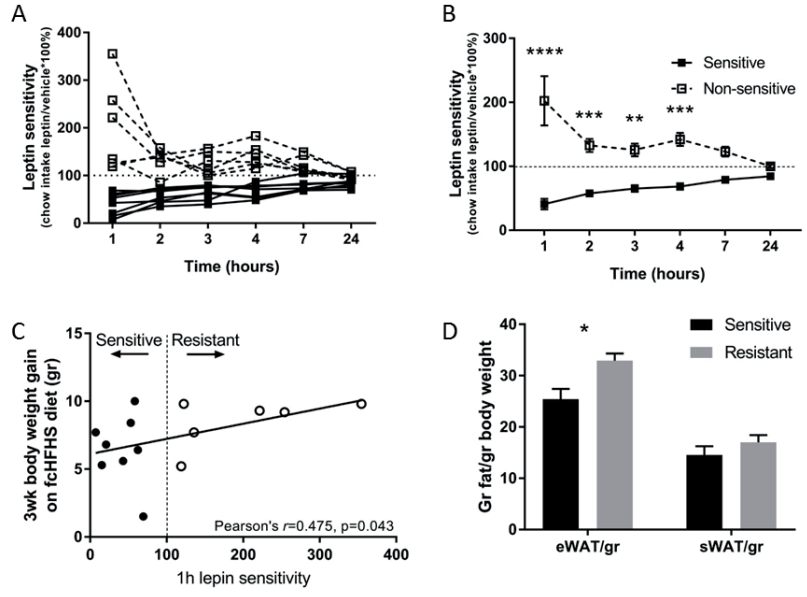

Figure 2. Leptin sensitivity in mice is highly variable. (A) Individually plotted leptin sensitivity. (B) Mice were grouped based on 1h leptin feeding response: mice that decreased feeding ('leptin sensitive', n=8/14) and mice that increased feeding ('leptin resistant', n=6/14) (C) Leptin sensitivity prior to an obesogenic diet correlated to body weight gain after 3 weeks of diet. (D) Leptin resistant mice had greater storages of epididymal white adipose tissue (eWAT), but not subcutaneous white adipose tissue (sWAT) than leptin sensitive mice. Mean±SEM. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

al., 2018), we grouped the mice based on the suppression of feeding during the first hour after injections: mice that ate less compared to vehicle were leptin sensitive (LS, n=8/14) and those that ate more were leptin resistant (LR, n=6/14). Leptin sensitivity at 1h was on average 40.8±8.3% in LS mice and 202.4±38.4% in LR mice. One animal could not be assigned to a group since it did not eat during the first hour after the vehicle injections and, therefore, we could not normalize its feeding. Comparing leptin sensitivity over the course of the feeding test of LS and LR mice showed that even though we based the groups on their difference in 1h leptin sensitivity, the difference persisted throughout the test, but at 24h there was no significant difference anymore (fig. 2B, interaction effect Injection x Time $F(5,60)=12.03$, $p=0.000000044$, n=14; post hoc 1h $p=0.0031$; 2h $p=0.00010$; 3h $p=0.0025$; 4h $p=0.00014$; 7h $p=0.053$; 24h $p=0.074$). Thus, we grouped mice into LS mice that are sensitive to leptin's anorexic response and decrease feeding up to 24 hours and LR mice that are not sensitive and even increase feeding.

We then exposed LS and LR mice to a free choice high-fat high-sucrose (fcHFHS) diet, by providing mice with lard and a 10% sucrose solution on top of usual chow and

water. Before exposure to the fcHFHS diet LS and LR mice had similar body weights (LS: 34.5±1.0gr, LR: 35.8±1.0). Total caloric intake over three weeks of fcHFHS diet was similar between LS (267.9±8.1kCal) and LR (261.5±8.2kCal) mice. Even though food intake was similar, there was a tendency for LS mice to gain less weight (6.5±0.9gr or 118.6±2.5% of pre-diet body weight) than LR mice (8.5±0.7gr or 123.8±2.0%; $t(12)=1.68$, p=0.12). Feeding efficiency was calculated as total caloric intake / body weight gain during fcHFHS diet. Feeding efficiency was higher in LS mice (7.85±2.5) (i.e. required higher caloric intake to gain 1gr of body weight) compared to LR mice (4.40±0.4), but this did not reach statistical significance ($t(12)=1.15$, p=0.27). Finally, after three weeks of fcHFHS diet and one week regular chow, fat pads were excised and weighed. Despite there being no significant difference in body weight gain, LR mice had a higher amount of epididymal white adipose tissue (normalized to body weight) than LS mice ($t(12)=2.865$, p=0.014) (fig. 2D).

Previously in rats, a strong positive correlation between leptin sensitivity and body weight gain on an obesogenic diet was found (Pearson's r=0.71, Levin & Dunn-Meynell, 2002; Spearman's r=0.53, Ruffin et al., 2004). In our experiment, leptin sensitivity (prior to the fcHFHS) moderately correlated to body weight gain on the fcHFHS (Pearson's r=0.475, p=0.043) (fig. 2C).

Cohort 2: leptin sensitivity, progressive ratio task and free choice high-fat high-sucrose diet

In a separate group of mice (n=11) we repeated the experiments above, but also tested mice for motivation to lever press to obtain a sucrose solution reward. Similar to the mice in cohort 1, 7/11 mice responded to leptin with a reduction in feeding (LS mice) and 4/11 increased feeding (LR) during the first hours after injections (fig. 3A). LS and LR mice significantly differed in their leptin sensitivity (feeding response) during the first hour only (fig. 3B; Interaction effect $F(5,45)=3.52$, p=0.0090; post hoc 1h: p=0.0018; 2-24h p>0.05). Next, we determined the response to leptin on motivational behaviour assessed by the amount of active lever presses (ALP) made during the progressive ratio task. To do so, we food restricted mice for 6 and for 18h and injected leptin or vehicle 1h prior to the start of the task. When food restricted for 6h, LS mice decreased the amount of ALP after leptin injection and LR mice increased ALP, but this did not reach statistical significance (fig. 3C). However, there was a strong correlation between the response to leptin on motivation and previously tested leptin sensitivity (fig. 3D; Pearson's r=0.744, p=00087). Leptin had no effect on ALP in either LS or LR mice when food restriction was extended to 18h (fig. 3E), nor was there a correlation with leptin sensitivity (fig. 3F).

Subsequently, mice from cohort 2 were exposed to the fcHFHS diet. Prior to the fcHFHS diet LS and LR mice had similar body weights (LS: 32.6±1.3gr; LR: 33.3±1.8gr). During

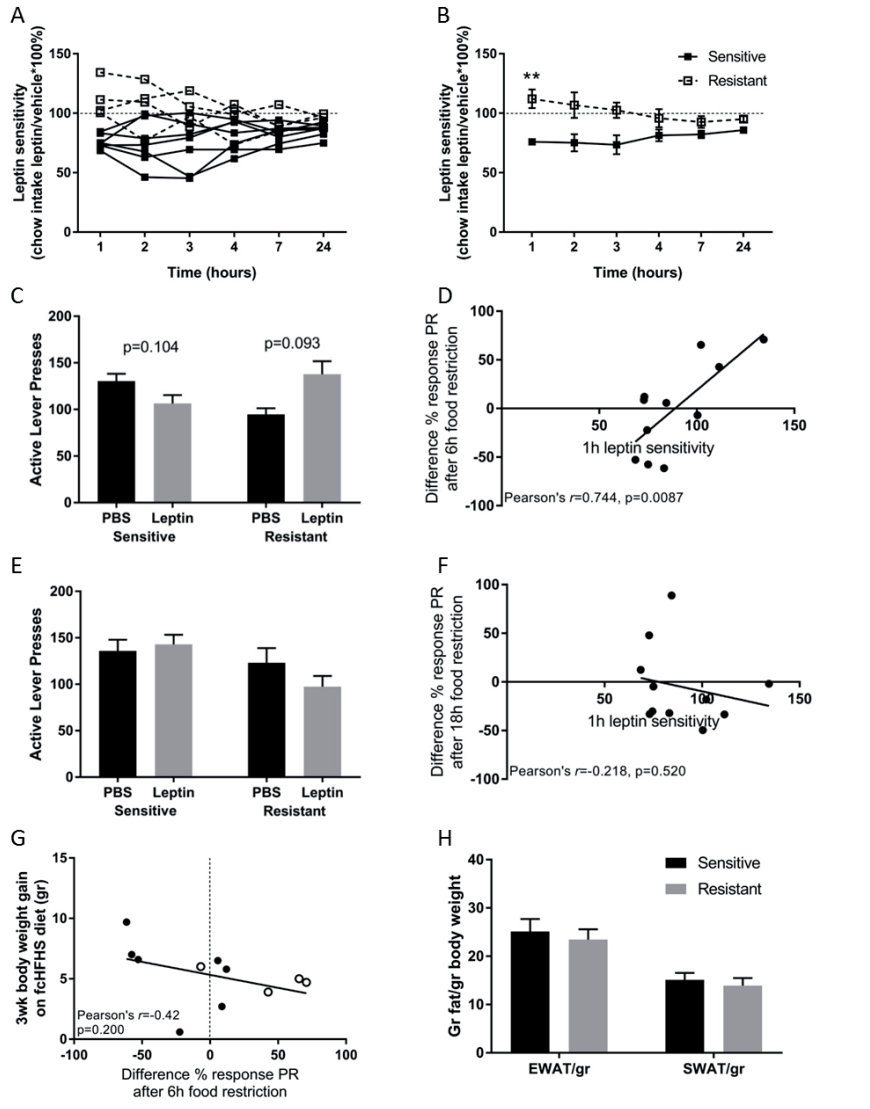

Figure 3. Motivational behaviour after peripheral leptin injections in mice. (A) Individually plotted leptin sensitivity showed highly variable responses. (B) Animals were grouped based on 1h leptin feeding response, i.e. leptin sensitivity: mice that decreased feeding ('leptin sensitive', n=7/11) and mice that increased feeding ('leptin resistant', n=4/11). (C) LS mice tended to decrease active lever presses upon leptin administration after 6h food restriction, while LR mice tended to increase active lever presses. (D) The response to leptin on motivation after 6h food restriction correlated with leptin sensitivity. (E) Leptin did not affect active lever presses in mice after 18h food restriction. (F) No correlation between response to leptin on motivation after 18h food restriction and leptin sensitivity. (G) The response to leptin on motivation after 6h food restriction did not correlate with body weight gain on a fcHFHS diet. (H) LS and LR mice had similar amounts of epididymal and subcutaneous white adipose tissue (EWAT and SWAT, respectively). Mean±SEM. ** p<0.01.

the fcHFHS diet, LS and LR mice ate the same amount of calories (LS: 255.3±6.0kCal; LR: 255.8±8.0kCal), gained a similar amount of weight (LS: 5.6±1.1gr or 118.0±3.2%; LR: 4.9±0.4gr or 116.9±1.3%) and, as a result, feeding efficiency of LS and LR mice were alike (LS: 47.8±10.2; LR: 53.6±5.4). Finally, removing epididymal and subcutaneous white adipose tissue after three weeks of fcHFHS revealed no difference in fat pads between LS and LR mice (fig. 3H).

We found no correlation between body weight gain on the fcHFHS diet and leptin sensitivity (Pearson's *r*=-0.067, p=0.84), PR response after 6h food restriction (fig. 3G; Pearson's *r*=-0.42, p=0.20) or PR response after 18h food restriction (Pearson's *r*=-0.0059, p=0.99).

Discussion

Previous studies reported that leptin sensitivity, defined as the percentage of food intake following leptin administration compared to vehicle, was highly variable in normal weight rats and that leptin sensitivity predicts weight gain on an obesogenic diet (de Git et al., 2018; Levin et al., 2004; Levin and Dunn-Meynell, 2002; Ruffin et al., 2004). Here, we showed that also in mice leptin sensitivity is variable and correlated with body weight gain on an obesogenic diet. Furthermore, we found that the response to leptin on feeding correlated to the effect of leptin on motivational behaviour after 6h food restriction.

In our experiments, we divided mice based upon the feeding response to leptin into mice that were leptin sensitive (LS) and mice that were leptin resistant (LR). In the first hour after leptin injection, leptin sensitive mice (8/14) in the first cohort decreased food intake to 41% on average and leptin resistant mice (6/14) increased food intake to 202% on average. In rats, the numbers of LS and LR animals were alike, suggesting high similarity between species (de Git et al., 2018 (LS: 11/21, LR:10/21); Ruffin et al., 2004 (LS: 7/15, LR: 8/15)). Of note, another experiment in mice by Enriori et al. measured food intake 24h after injection of leptin and found no difference in leptin sensitivity (Enriori et al., 2007). Our results revealed that, in mice, LS and LR differences in leptin sensitivities persisted up to 7h, but were absent after 24h. In rats, the effects were present at 1 to 4h and similarly absent after 24h (de Git et al., 2018; Ruffin et al., 2004). Together, these results suggest that differences in leptin sensitivity in rodents are most pronounced during the first few hours after leptin injections.
Body weight gain of LR mice was not significantly higher than LS mice after 3 weeks on an obesogenic diet. An explanation for this may lie in the exposure time and duration of the obesogenic diet. We exposed mice to the diet for 3 weeks, while others who saw larger body weight differences exposed animals for 8, 12, 16 or 20 weeks (de Git et al., 2018; Enriori et al., 2007; Ruffin et al., 2004; Tulipano et al., 2004). Another explanation may be that we used relatively old mice (11-12 months at the start of diet). Others that found body weight differences used animals 6-7 weeks after birth. Since younger animals are still gaining body weight, the effect of an obesogenic diet will be more pronounced. Thus, perhaps, exposing mice to the diet for a longer period or starting the obesogenic diet earlier in life would have resulted in differences in body weight gain between LS and LR mice.

Although body weight gain was not different at the group level, leptin sensitivity positively correlated to body weight gain on an obesogenic diet in mice of cohort 1 (*r*=0.475). This correlation was also found in rats (*r*=0.532 (Ruffin et al., 2004), *r*=0.71(Levin and Dunn-Meynell, 2002)). Since we found a correlation between leptin sensitivity and body weight gain, but no effect on body weight gain at the group level, this suggests that grouping animals into LS or LR is a simple model that does not fully capture the individual leptin sensitivity as predicting factor for body weight gain after short term exposure to the fcHFHS diet. Nevertheless, even with a small number of older mice, we find similar results on an obesogenic diet as have been described in rats.

Since both leptin sensitivity and motivation for sugar reward were found to be predictors of body weight gain on an obesogenic diet (de Git et al., 2018; la Fleur et al., 2007; Ruffin et al., 2004), it was not surprising to find a correlation between leptin sensitivity and the response to leptin on motivational behaviour after 6h food restriction. This suggests that increased motivation for food reward may drive those that are leptin insensitive to become obese in an obesogenic environment. However, in this cohort of mice there was no correlation between leptin sensitivity or the response to leptin on motivation and body weight gain on the fcHFHS, nor did we observe differences in fat pads in LS and LR mice after the fcHFHS diet. On top of that, the feeding responses to leptin in both LS and LR mice of cohort 2 were smaller than those of cohort 1. One distinct difference between the cohorts is that cohort 2 had been continuously food restricted to 90% of their original body weight over three months during previous experiments and cohort 1 had never been on continuous food restriction. Therefore, we cannot exclude that this long-term food restriction may have affected the response to leptin on feeding or motivation and body weight gain on an obesogenic diet. Experiments in naïve animals will be necessary to determine whether the response to leptin on motivation may actually drive obesity on an obesogenic diet.

In this study we showed that leptin sensitivity in mice is highly variable. We showed that leptin sensitivity correlated to the response to leptin on motivation (cohort 2) and weight gain in mice on an obesogenic diet (cohort 1). Based on our results and previous reports (de Git et al., 2018; Ruffin et al., 2004), we conclude that leptin sensitivity is highly variable in both rats and mice, supporting that it may be a general phenomenon in mammals, perhaps including humans. Therefore, leptin sensitivity may be a biomarker that can be used to identify subpopulations of rodents that are more susceptible for weight gain. Further research should determine to what extent these results are translatable to humans. Better insights in the mechanisms involved in the development of obesity in humans may help identify subpopulations that are at risk of becoming obese, such that prevention strategies can be adequately deployed.

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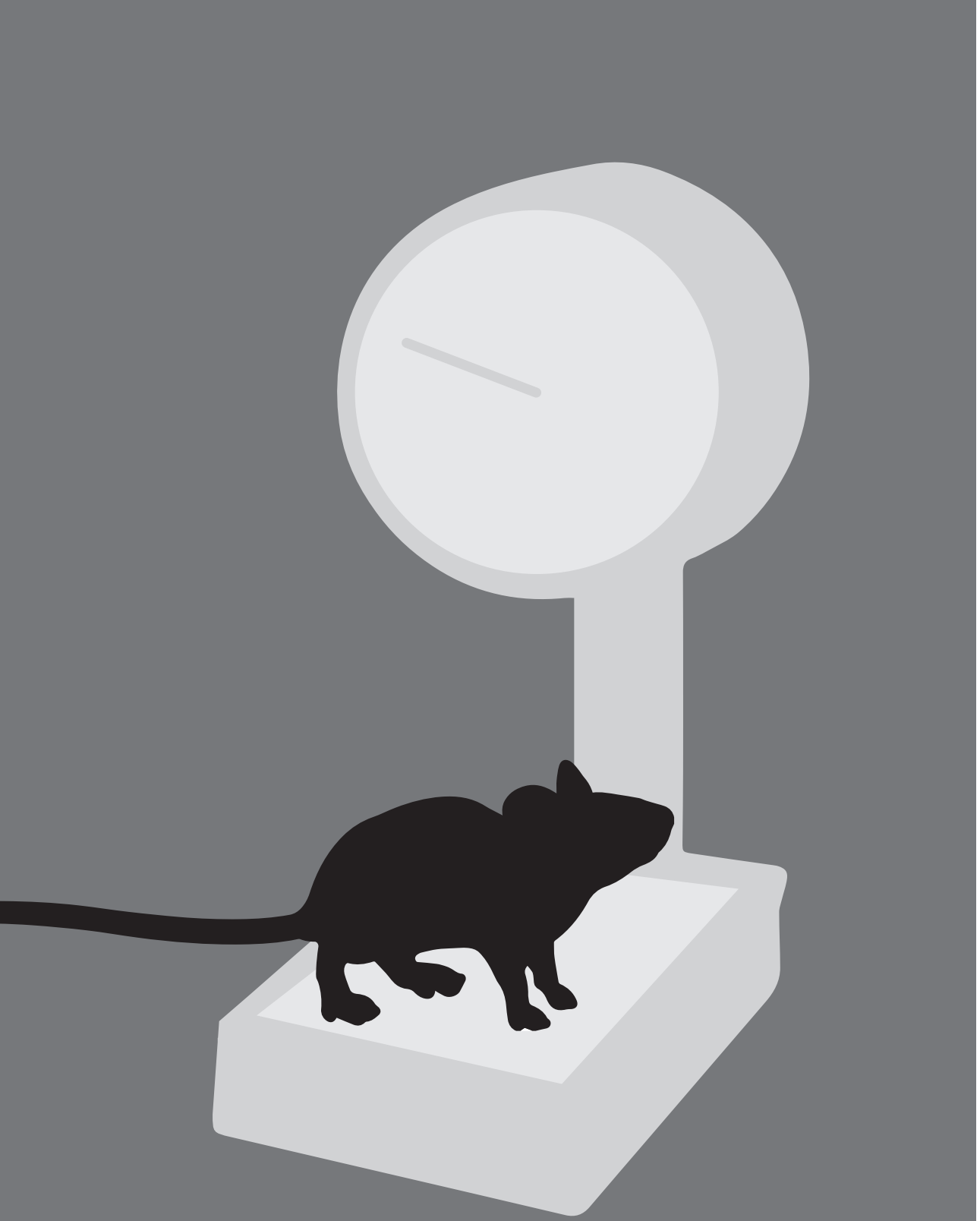

Chapter 2

Leptin receptor-expressing neurons in the substantia nigra regulate locomotion, and in the ventral tegmental area they regulate motivation and feeding

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Abstract

Leptin is an anorexigenic hormone, important in the regulation of body weight. Leptin plays a role in food reward, feeding, locomotion and anxiety. Leptin receptors (LepR) are expressed in many brain areas, including the midbrain. In most studies that target the midbrain, either all LepR neurons of the midbrain or those of the ventral tegmental area (VTA) were targeted, but the role of substantia nigra (SN) LepR neurons has not been investigated. These studies have reported contradicting results regarding motivational behaviour for food reward, feeding and locomotion. Since not all midbrain LepR mediated behaviours can be explained by LepR neurons in the VTA alone, we hypothesized that SN LepR neurons may provide further insight. We first characterized SN LepR and VTA LepR expression, which revealed LepR expression mainly on DA neurons. To further understand the role of midbrain LepR neurons in body weight regulation, we chemogenetically activated VTA LepR or SN LepR neurons in LepR-cre mice and tested for motivational behaviour, feeding and locomotion. Activation of VTA LepR neurons in food restricted mice decreased motivation for food reward ($p=0.032$) and food intake ($p=0.029$), but not locomotion. In contrast, activation of SN LepR neurons in food restricted mice decreased locomotion ($p=0.020$), but not motivation for food reward or food intake. Our results provide evidence that VTA LepR and SN LepR neurons serve different functions, i.e. activation of VTA LepR neurons modulated motivation for food reward and feeding, while SN LepR neurons modulated locomotor activity.

Introduction

The current obesity epidemic is a result of overconsumption that exceeds energy requirements. With no truly effective treatments, there is a need for further mechanistic insights into body weight regulation. Hedonic feeding is characterized by the increased consumption of palatable foods and implicates increased motivation for these foods, which ultimately results in obesity (la Fleur et al., 2007). Food deprivation or dieting is known to increase motivation for food, which counteracts weight loss (Pickering et al., 2009). In light of the obesity epidemic, it is of interest to gain further knowledge on mechanisms underlying increased motivation for (palatable) food.

Leptin is an anorexic adipose-tissue derived hormone with a central role in body weight regulation. Leptin is secreted in levels proportional to the amount of adipose tissue, i.e. fasting decreases leptin levels, while weight gain increases leptin levels (Frederich et al., 1995; Maffei et al., 1995; Masuzaki et al., 1995). Leptin is known to regulate body weight by reducing feeding and increasing energy expenditure (Halaas et al., 1995). Accumulating evidence shows that leptin is also involved in reducing motivation for food in both humans and rodents (Brown et al., 2017; Hinkle et al., 2013; Rosenbaum et al., 2008; Sharma et al., 2012; Woodworth et al., 2017). The midbrain, comprising of the ventral tegmental area (VTA) and substantia nigra (SN), is associated with motivational behaviour and expresses the leptin receptor (LepR), but it is unclear whether and which of these regions mediate leptin's effect on motivational behaviour for food reward (Ilango et al., 2014; Rossi et al., 2013; Wise, 2009).

Since VTA dopamine (DA) neurons are important for motivational behaviour, VTA DA neurons expressing LepR were proposed to mediate the effect of leptin on food reward (Figlewicz et al., 2003; Hommel et al., 2006; Leshan et al., 2010; van der Plasse et al., 2015). However, there are data that challenge this idea. An important reward associated pathway is that of VTA DA neurons projecting to the nucleus accumbens (NAc), but only few VTA-LepR neurons project to the NAc (Fulton et al., 2006). Instead, VTA LepR neurons primarily project to the central amygdala, and these neurons mediate effects of leptin on central amygdala associated behaviours, such as anxiety (Leshan et al., 2010). Indeed, leptin infusion into the VTA of mice decreased anxiety-like behaviour (Liu et al., 2016), but did not affect motivational behaviour in rats (Davis et al., 2011). Together, these data suggest that VTA (DA) LepR neurons modulate anxiety-like and not motivational behaviour. Yet, knockdown of LepR in the whole midbrain increased motivational behaviour (Davis et al., 2011). This suggests that another population of midbrain LepR neurons modulate motivation. In the SN, LepR are almost exclusively expressed on SN DA neurons (Leshan et al., 2010) and SN DA neurons have been implicated in motivational behaviour (Drui et

al., 2014). Thus, an involvement of SN LepR neurons in mediating the effect of leptin on motivational behaviour is possible, but has not yet been determined.

Besides the role of midbrain LepR neurons in motivation for food reward, this region has also been implicated in other aspects of leptin's behavioural effects such as feeding and energy expenditure. Leptin injection into the VTA decreased feeding (Hommel et al., 2006; Matheny et al., 2014; Scarpace et al., 2013; Trinko et al., 2011) and blocking or decreasing leptin signaling in the VTA increased feeding (Hommel et al., 2006; Scarpace et al., 2013). However, inhibiting leptin signaling in the whole midbrain (Davis et al., 2011) or specifically in DA neurons of the midbrain (Fernandes et al., 2015; Liu et al., 2011) did not impact on feeding. These results suggest that perhaps effects on feeding are mediated by non-DA neurons in the VTA. In addition, it remains unknown whether SN LepR neurons contribute to the anorexic effect of leptin.

Effects of midbrain LepR neurons on locomotor activity are contradicting. Whereas leptin infusion into the VTA had no effect on locomotion (Hommel et al., 2006), RNA interference knockdown of LepR in VTA LepR neurons or ablation of STAT3 in midbrain DA neurons increased locomotion (Fernandes et al., 2015; Hommel et al., 2006). While the latter studies suggest that VTA LepR neurons modulate locomotion, the fact that intra-VTA leptin had no effect on locomotion challenges this. Perhaps, SN LepR neurons underlie the effect on locomotion. Alternatively, since leptin levels correlate with body weight (Ahima et al., 1996), it is possible that endogenous leptin masked effects on locomotion in these experiments since intra-VTA leptin injections were given to ad libitum fed rats (Hommel et al., 2006). Thus, it remains unclear which midbrain LepR population modulates locomotion.

The aim of the current study was to gain further insight into the mechanisms underlying the effect of leptin in the midbrain and its involvement in body weight regulation. We started with characterizing the expression of LepR in the midbrain. Next, two questions were of particular interest for behavioural experiments : 1) whether SN LepR neurons modulate motivational, locomotor and feeding behaviour and 2) whether VTA LepR neurons mediate anxiety-like, feeding and locomotor behaviour. To address these questions, we directly compared behavioural effects of chemogenetically activating VTA LepR or SN LepR neurons on motivational behaviour for food reward, feeding, locomotion and anxiety in LepR-cre mice.

Material and methods

All experiments were approved by the Animal Experimentation Committee of the University Utrecht and were carried out in agreement with Dutch Law (Herziene Wet op Dierproeven, Art 10.a2, 2014) and European regulations (Guideline 2010/63/EU).

Subjects

In house bred, adult male homozygote LepRb-Cre transgenic mice (stock #008320, B6.129-Leprtm2(cre)Rck/J, The Jackson Laboratory, USA) were used for behavioural experiments. Mice were housed individually in plastic cages (Type II L, 365x207x140mm, 530cm2, Tecniplast, Italy) in a temperature (21±2^{o}C) and humidity (60-70%) controlled room on a reversed day/night (9:00 AM light OFF, 9:00 PM light ON) schedule. We tested all mice first in ad libitum situations and then under food restriction, because leptin levels are correlated with the amount of fat tissue in mice (Frederich et al., 1995; Maffei et al., 1995) and leptin influences midbrain DA activity (Hommel et al., 2006; Murakami et al., 2018; van der Plasse et al., 2015). Ad libitum fed mice were given ad libitum access to chow (3,1Kcal/gr, Standard Diet Service, UK) and water. Food-restricted mice were given chow (~3 pieces of standard rodent chow of ±1.3 grams each) after behavioural tasks were performed and maintained on ~90% of the original body weight by adapting the amount of chow given per day. For analysis of DA (determined by staining for TH immunoreactivity) and LepR co-localization, LepRb-Cre mice were crossed with a mouse line that expresses robust tdTomato fluorescence following Cre-mediated recombination (stock # 007914, B6.Cg-G*t*(ROSA)26Sortm14(CAG-tdTomato)Hze/J, The Jackson Laboratory, USA). Male mice homozygote for the LepRb-Cre allele and heterozygote for the TdTom allele ("LepR/tdTomato" mice, n=3) were socially housed on a normal day/night cycle (7:00AM lights ON) with ad libitum access to chow and water.

Surgical procedures

At least 30min prior to anesthetization, mice were given carprofen (5mg/kg, subcutaneous (s.c.), Carporal, AST Farma BV). Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (75mg/kg, Narketan, Vetoquinol BV) and medetomidine (1mg/kg, SedaStart, AST Farma BV). Mice were given eye cream (CAF, CEVA Sante Animale BW) and placed in a stereotactic frame (Kopf Instruments, USA). An incision was made in the skin along the midline of the skull and additional analgesia was applied by spraying Xylocaine (lidocaine 100mg/ml, AstraZeneca BV) on the skull. Microinjections of AAV5-hSyn-DIO-hM3DGq-mCherry (0.3ul/side, 3.0*109vp/ul, UNC Vector Core, USA) or AAV-Ef1a-DIO-hChR2-eYFP ("control" mice, 0.3ul/side, 3.0*10^9vp/ul, UNC Vector Core, USA) were performed bilaterally in the substantia nigra (-3.3 AP, +1.5 ML, -4.4 DV, 0° angle, n=6) or the ventral tegmental area (-3.2 AP,

+1.5 ML, -4.8 DV, 10° angle, n=6) at a rate of 0.1ul/min per side followed by a 10min period before retracting the needles. For assessment of SN LepR projection sites mice were injected with AAV-Ef1a-DIO-ChR2-eYFP (0.3ul/side, 3.0*109vp/ul, UNC Vector Core, USA) in the SN (n=4). After the operation, mice were given atipamezole (2.5mg/kg, i.p., SedaStop, AST Farma BV) and saline for rehydration. The following 2 days, mice were given carprofen (5mg/kg, s.c.) and were allowed to recover for at least 1 week. To ensure viral expression, testing commenced 3 weeks after virus injection.

Drugs

Clozapine N-oxide (CNO 99%, AK Scientific, Inc., USA) was dissolved in 0.9% saline and injected i.p. with a dose of 1.0mg/kg (and 3.0mg/kg for operant testing). For all experiments, each mouse received saline and CNO injections in a latin-square design.

Behavioural procedures

***Progressive ratio operant behaviour*:** Mouse operant boxes (ENV-307W, Med Associates Inc., USA) fitted with 2 levers, a cue light above the active lever (AL), a house light, a speaker and a liquid receptacle were used. Throughout all sessions, when the number of AL presses to complete a ratio was reached, the house light and a tone were presented for 5sec, after which a sucrose reward was delivered for 2sec (38ul, 20% w/v sugar solution in tap water). During the training phase, mice were food restricted to ~90% of their original body weight. The first day, mice were habituated for 15min to an operant box, in which we placed a droplet of sugar solution (20% w/v) in the receptacle. The next day, operant training started with a fixed ratio (FR) 1 paradigm for 30min/session. Once mice learned to press on the active lever >20x and <10% on the inactive lever, mice were switched to FR3 (30min/session) and then FR5 (60min/session). Then, once >60 rewards were earned and <10% of the presses were made on the inactive lever, training was switched to the progressive ratio (PR, 60min/session) and mice were returned to ad libitum feeding. PR schedule was based on the formula: 5*e(x*0.2)-5 , rounded to the nearest integer, where x is the position in the ratio sequence (Richardson and Roberts, 1996). Testing with saline/CNO started when PR performance appeared stable, i.e. over 3 days of training no more than ±1 reward from average and no incremental increase or decrease. Operant training and testing were performed during the first 3 hours of the dark phase. Injections were given 30min prior to the start of the PR task.

Free access 20% sucrose consumption: Mice were trained to lick for 20% (w/v) granulated sugar solution in an operant box fitted with a spout connected to a pump which delivered 8ul 20% sugar solution upon every detected lick. Mice were trained 4 times prior to testing. Training and testing was performed 3-5h into the dark phase and lasted 30min. On test days, mice were injected with CNO/saline 30min before the start of the test.

Feeding: To simplify finding pieces of chow at measurements, all mice were habituated to a second mouse cage, in which no bedding, but only 3 tissues and a water bottle were present, hereafter referred to as 'feeding cage'. Mice were injected with saline/CNO 30min prior to the onset of the dark phase and were directly placed in the feeding cage with a pre-weighed amount of chow on the cage floor. Food intake was measured at 1, 2, 3, 5 and 7h post injection, after which the mice were placed back in their home cage.

Locomotion: All mice were habituated 2x1h to an individual plastic cage (Type III H, 425x266x185mm, 800cm2, Tecniplast, Italy) prior to testing on separate days. The cages were surrounded by white carton to prevent interaction between mice. On the test day, mice were injected with either saline/CNO and placed in the behavioural testing room. 30min later, mice were placed in their own locomotion cage and horizontal movement was tracked using a camera placed above the cages that was coupled to a computer running Ethovision 7 (Noldus Information Technology, the Netherlands). Locomotion tests commenced 4-5h into the dark phase and lasted 1h.

Anxiety: Mice were injected with either saline/CNO 30min prior to testing and placed in the front room of the behavioural room where tests would be performed. Mice were lifted by their tails and placed in the center of the elevated plus maze in a brightly lit room and tracked using Ethovision (Noldus, Wageningen) for 5min. Anxiety tests were performed 6-7h into the dark phase. A previous study reported that longer intervals between anxiety tests led to reliable retesting of anxiety (Schneider et al., 2011), so we separated 2 test days by at least 3 weeks.

Tissue preparation and immunohistochemical analysis

Mice were anesthetized with Euthanimal (Euthanimal, Alfasan BV, the Netherlands) and transcardially perfused with 1x phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 1xPBS (PFA). Brains were dissected and kept in 4%PFA for 24h at 4°C, after which they were transferred to 30% sucrose in 1xPBS for at least 48h at 4°C. Using a cryostat, the brains were then sectioned to 40um slices and stored in 1xPBS with 0.01% sodium azide. Slices were washed 3x10min in 1xPBS and then blocked for 1h in 1xPBS containing 10% normal goat serum and 0,25% Triton-X100. Slices were then placed in 1xPBS containing the primary antibodies (Rabbit anti-dsRed 1:500, #632496, Clontech, Takara Bio USA Inc, USA; Mouse anti-Th 1:500, MAB318, Milipore) and 2% normal goat serum overnight at 4°C. At room temperature, slices were washed 3x10min in 1xPBS and placed in 1xPBS containing the secondary antibodies (Goat anti-Rabbit 568, 1:500, #ab175471, Abcam plc, UK; Goat anti-Mouse 488 1:500, ab150113, Abcam plc, UK) and 2% normal goat serum for 2h. Finally, slices were washed in 1xPBS and mounted onto slides, dried and covered using Fluorsave

(EMD Millipore Corporation, USA) and a coverslip. Images were collected on an epifluorescent microscope (Axio Scope A1, Zeiss, Germany).

Data Analysis

Behavioural data was analyzed using Microsoft Excel and GraphPad Prism (version 7.05, GraphPad Software, Inc., USA). Paired t-tests and one- or two-way repeated measures ANOVA and Bonferroni's multiple comparisons tests were used where applicable. A significance criterion of $p<0.05$, two-tailed, was adopted in all the statistical analyses.

Results

The majority of midbrain LepR neurons are dopaminergic

To verify previous reports of co-localization of LepR on DA neurons, we assessed LepR expression in the midbrain. To identify LepR neurons in the midbrain, we crossed LepR-cre mice onto the ROSA26-tdTomato reporter mouse line, which allowed robust tdTomato fluorescence following Cre-mediated recombination. Tyrosine hydroxylase (TH) is the rate limiting enzyme in DA production and is used as marker for DA neurons. TH- and LepR-tdTomato immunoreactivity revealed that in the VTA 67±1.0% and in the SN 90±2.7% of LepR/tdTomato neurons co-localized with TH (figure 1B). Of all TH neurons, only few co-localized with LepR/tdTomato in either region: 16±1.3% in the VTA and 15±0.8% in the SN (n=3, figure 1C). Thus, the majority of LepR-tdTomato co-

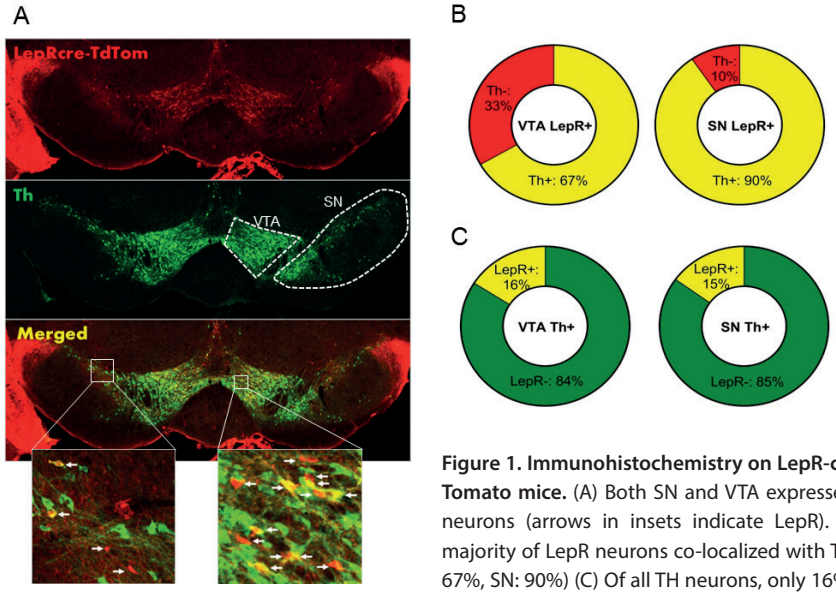

Figure 1. Immunohistochemistry on LepR-cre x Td-Tomato mice. (A) Both SN and VTA expressed LepR neurons (arrows in insets indicate LepR). (B) The majority of LepR neurons co-localized with TH (VTA: 67%, SN: 90%) (C) Of all TH neurons, only 16% in the VTA and 15% in the SN expressed the LepR.

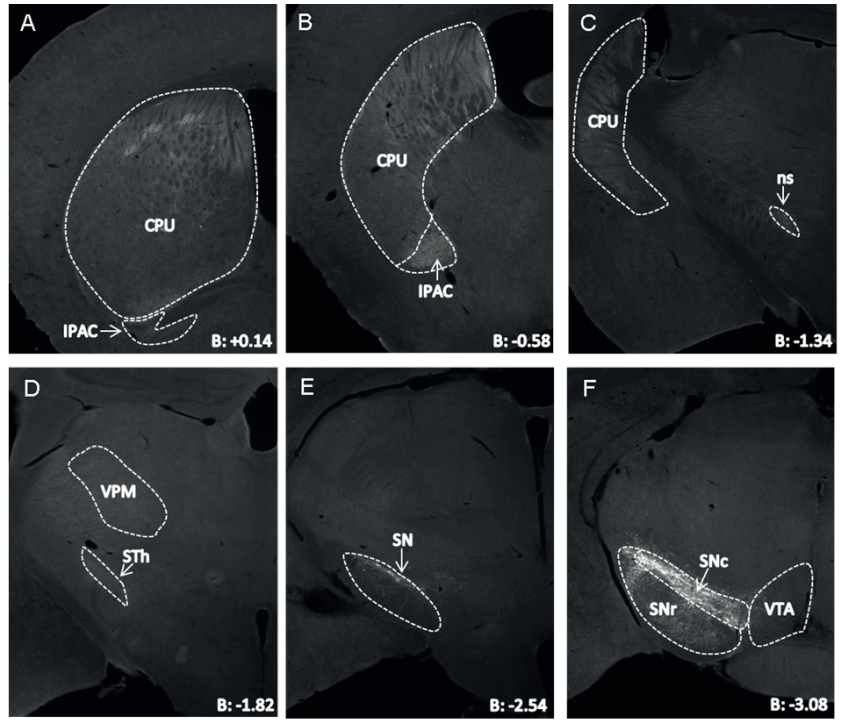

Figure 2. Representative AAV-DIO-ChR2-eYFP tracing of projections from SN LepR neurons in LepR-cre mice (n=4). (A-G) eYFP expression in CPU ++, IPAC ++, STh +, VPM + and VTA +/- to which SN LepR neurons sent detectable projections (++ strong eYFP, + eYFP present, +/- scarce eYFP). In E-G: eYFP soma and tracks within the SN. CPU: caudate putamen; IPAC: interstitial nucleus of the posterior limb of the anterior commissure; ns: nigrostriatal bundle; SN: substantia nigra; SNc: SN pars compacta; SNr: SN pars reticulate; STh: subthalamic nucleus; VPM: Ventral posteromedial thalamus; VTA: ventral tegmental area.

localized with DA neurons, but DA neurons that express LepR represented a minority of all DA neurons, which is consistent with previous reports (Figlewicz et al., 2003; Hommel et al., 2006; Leshan et al., 2010; Scott et al., 2009).

We next aimed to assess the projection areas of midbrain LepR neurons. It has been established that VTA LepR neurons primarily project to the central amygdala, while very few project to the NAc (Fulton et al., 2006; Leshan et al., 2010). SN LepR neurons have been shown to project to the caudate putamen (Leshan et al., 2010) and we sought to further extend on this knowledge. We identified projections of SN LepR neurons by

injecting LepR-cre mice with AAV-DIO-ChR2-eYFP into the SN, which also labels axonal projections. We found that SN LepR neurons projected heavily to the caudate putamen and further observed projections to the interstitial nucleus of the posterior limb of the anterior commissure (IPAC) (figure 2A-C). Some eYFP expression was found in the thalamus and in the VTA (figure 2D, F). Finally, labeling of axons with eYFP was also observed within SN pars compacta, SN pars reticula and lateral SN (figure 2D-G).

For behavioural experiments we injected AAV-DIO-hM3DGq-mCherry in the VTA (n=6) or SN (n=6) of LepR-cre mice to generate VTA LepR-Gq and SN LepR-Gq mice. We examined whether expression of hM3DGq-mCherry was representative for SN LepR or VTA LepR neurons. hM3DGq-mCherry was found mainly in the targeted region, but due to viral spread, some expression was seen in surrounding regions which express LepR-cre: of all hM3DGq-mCherry expressing neurons 68±1.1% were localized in the VTA of VTA LepR-Gq mice and 96±1.1% were localized in the SN of SN LepR-Gq mice (figure S1). The percentage of TH neurons expressing hM3DGq-mCherry or the percentage of hM3DGq-mCherry neurons expressing TH were similar to the numbers presented above of TH/LepR co-localization in LepR-tdTomato mice. Thus, hM3DGq-mCherry targeted neurons were representative for LepR neurons of the VTA or SN.

Motivation for sugar reward decreased upon activation of VTA LepR-Gq neurons in food restricted mice

To test for motivation, we trained mice to press for a 20% sugar solution reward in the PR task. Once stable, mice were injected with saline and 2 doses of CNO (1.0 and 3.0mg/kg). Ad libitum fed VTA LepR-Gq mice did not alter operant responding after CNO injections compared to saline (figure 3A), but when food restricted, VTA LepR-Gq mice decreased the amount of active lever presses made during the PR task ($F(1.110,5.549)=7.920$, p=0.032, figure 3C). Further analyses revealed that both CNO doses significantly decreased performance compared to saline (CNO1.0mg/kg, p=0.0007; CNO 3.0mg/kg, p=0.019). Operant behaviour was not affected upon chemogenetic activation of SN LepR-Gq neurons (figure 3B, D).

Free consumption of sucrose solution was not affected by VTA LepR or SN LepR activation

To dissociate motivational behaviour from consumption, we assessed free consumption of a 20% sucrose solution. Free consumption was not affected by activation of either VTA LepR or SN LepR, indicating that decreased motivation was driven by motivational behaviour without affecting consummatory behaviour (figure 4).

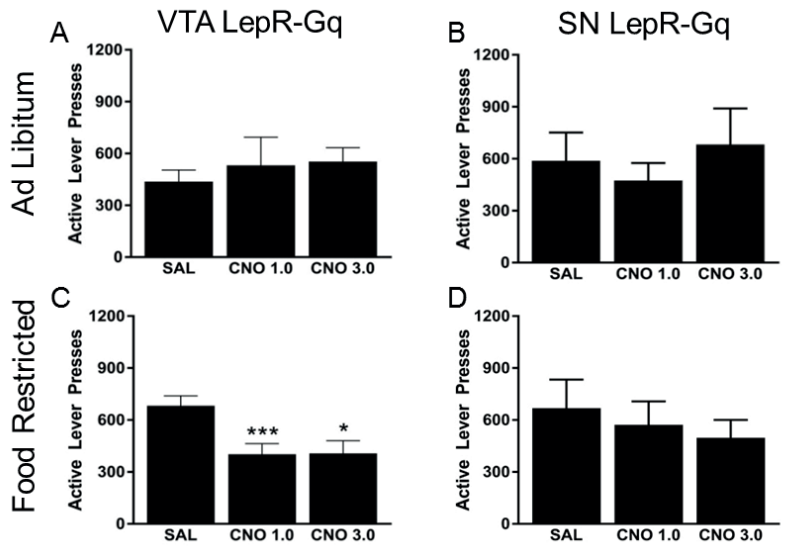

Figure 3. Active lever presses upon chemogenetic activation of VTA LepR or SN LepR neurons. In ad libitum fed mice, CNO did not affect active lever presses made in (A) VTA LepR-Gq or (B) SN LepR-Gq mice. (C) Food restricted VTA LepR-Gq mice decreased active lever presses after CNO compared to saline injections. (D) CNO did not affect active lever presses in food restricted SN LepR-Gq mice. Mean ± SEM. * p<0.05 , ***p<0.001.

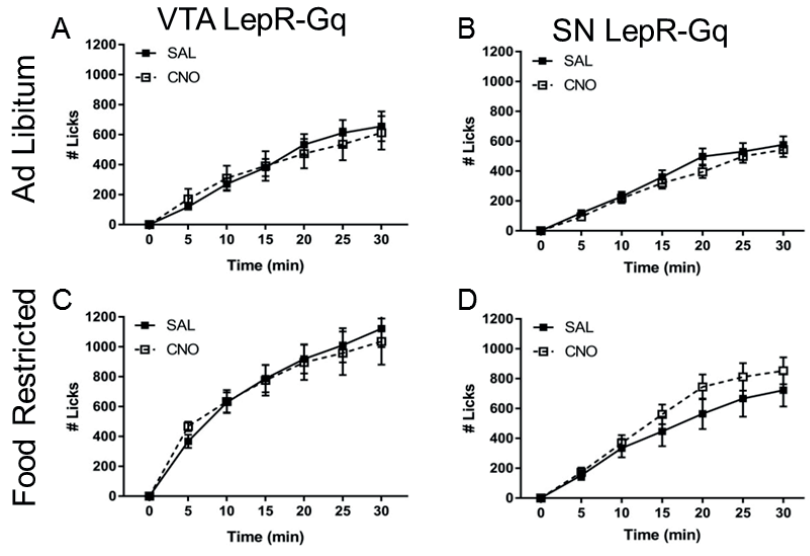

Figure 4. Free 20% sucrose solution consumption upon chemogenetic activation of VTA LepR or SN LepR neurons. CNO did not affect sucrose solution consumption in ad libitum fed (A) VTA LepR-Gq and (B) SN LepR- Gq or food restricted (C) VTA LepR-Gq and (D) SN LepR-Gq. Mean ± SEM.

Chow consumption decreased upon activation of VTA LepR-Gq neurons in food restricted mice

We next tested whether midbrain LepR neurons modulated feeding by measuring chow intake over 7h. Ad libitum fed mice did not alter consumption of chow after chemogenetically activating VTA LepR-Gq (figure 5A) or SN LepR-Gq (figure 5B) neurons. In food restricted VTA LepR-Gq mice, CNO decreased cumulative chow intake compared to saline (main effect of Injection $F(1,5)=9.138$, p=0.029, figure 5C). but food intake was unaffected in food restricted SN LepR-Gq mice (figure 5D).

Locomotion decreased upon activation of SN LepR-Gq neurons in food restricted mice

Locomotor activity was assessed by automatically tracking horizontal movement of mice for 1h. CNO injections did not affect locomotor activity in ad libitum fed VTA LepR-Gq mice (figure 6A) or SN LepR-Gq mice (figure 6B). Activating VTA LepR-Gq neurons in food restricted mice did not modulate locomotion (figure 6C), but activation of SN LepR-Gq neurons decreased locomotor activity (interaction effect of Time x Injection $F(1.184,5.920)=9.380$, p=0.020, figure 6D). Further analyses revealed that CNO decreased locomotion after 40 and 60min in SN LepR-Gq mice (40min, p=0.031; 60min, p=0.025).

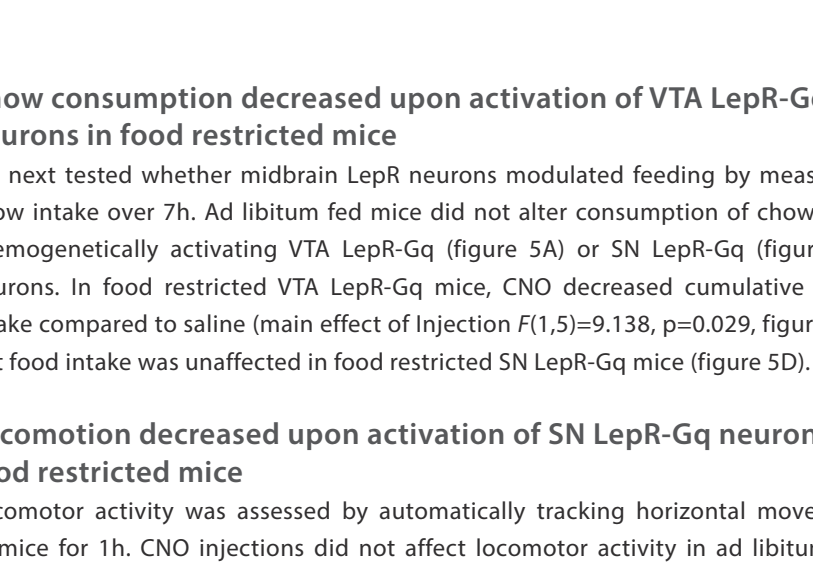

Figure 5. Consumption of chow upon chemogenetic activation of VTA LepR or SN LepR neurons. Chow intake was not affected by CNO injections in ad libitum fed (A) VTA LepR-Gq or (B) SN LepR-Gq mice. (C) CNO decreased cumulative chow intake in food restricted VTA LepR-Gq mice. (D) CNO did not affect feeding in food restricted SN LepR-Gq mice. Mean ± SEM. * p<0.05.

Chemogenetically activating VTA LepR or SN LepR did not affect anxiety-like behaviour

Finally, because previous studies showed that VTA LepR modulated anxiety (Liu et al., 2011, 2016), we aimed to verify this in our experiments and determined effects of activating VTA LepR or SN LepR on anxiety-like behaviour. To test for anxiety-like behaviour, we examined behaviour in the elevated plus maze (EPM) for 5min. This test is based on the natural aversion of mice to brightly lit, open and elevated spaces and we therefore report time spent in the avoided part of the EPM, i.e. the brightly lit and elevated open arms. Chemogenetic activation of VTA LepR or SN LepR in either food restricted or ad libitum fed mice did not affect the amount of time spent in the open arm of the EPM (figure 7). Although we increased the time between 2 test days to 3 weeks, anxiety-like behaviour may be reduced with repeated exposure to the tasks, so we also analyzed individual test days. However, we did not observe reduced anxiety-like behaviour on the second test day. When analyzing the data from the first test day only (comparing between subjects effects), we found no effect of CNO either (data not shown).

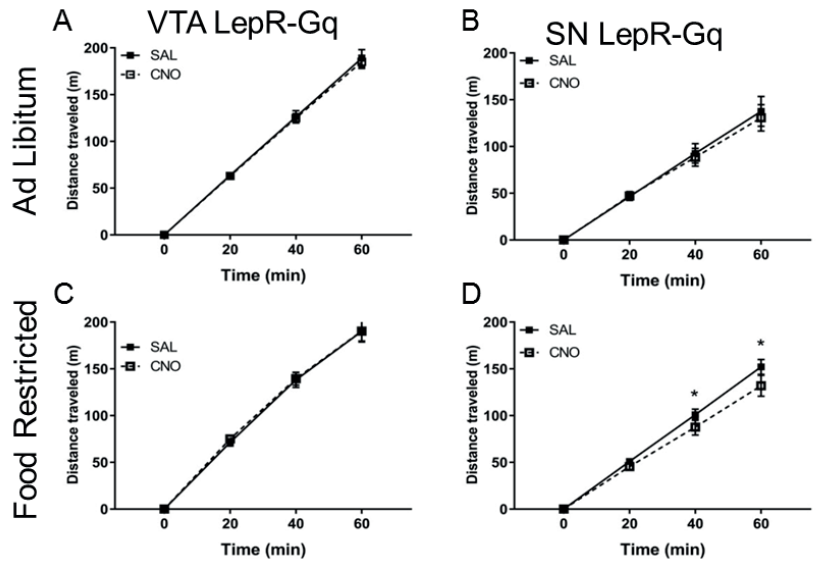

Figure 6. Locomotor activity upon chemogenetic activation of VTA LepR or SN LepR neurons. Locomotion was not affected by CNO injections in ad libitum fed (A) VTA LepR-Gq and (B) SN LepR-Gq or (C) food restricted VTA LepR-Gq mice. (D) CNO decreased locomotion in food restricted SN LepR-Gq mice. Mean ± SEM. * p<0.05.

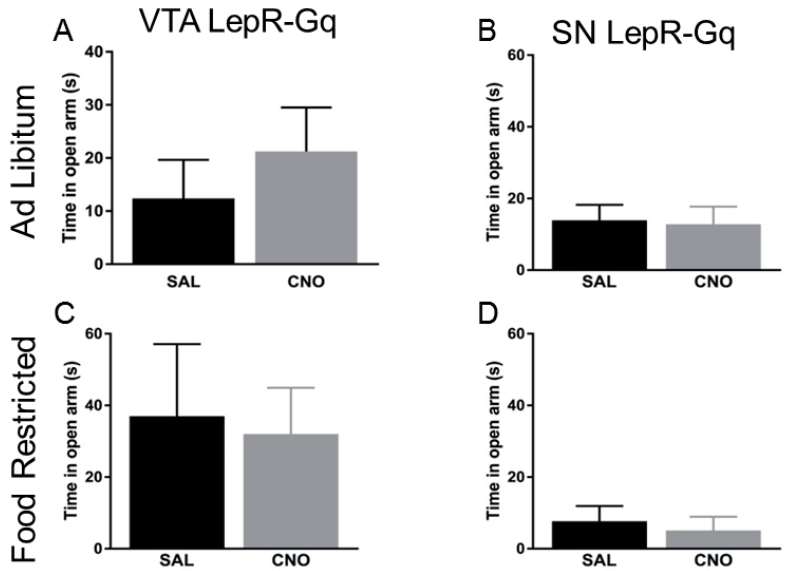

Figure 7. Anxiety-like behaviour upon chemogenetic activation of VTA LepR or SN LepR neurons. Anxiety-like behaviour tested in the elevated plus maze was not affected by injecting CNO in (A, C) VTA LepR-Gq or (B, D) SN LepR-Gq mice in either (A, B) ad libitum or (C, D) food restricted state. Mean±SEM.

To control for non-specific effects of CNO due to reverse-metabolism to its parent compound clozapine (Manvich et al., 2018), we injected AAV-Ef1a-DIO-hChR2-eYFP into the VTA or SN of LepR-cre mice. This results in expression of channelrhodopsin, which does not respond to CNO or clozapine. Indeed, CNO injections in control mice had no effect on any parameter tested (fig. S2, n=6). Therefore, we conclude that behavioural effects observed in Gq-injected mice are the result of enhanced neuronal activation in VTA LepR-Gq or SN LepR-Gq mice.

Discussion

In this study, novel insights into the behavioural effects of SN LepR and VTA LepR neurons were obtained using chemogenetics. Chemogenetic activation of VTA LepR neurons modulated motivation and feeding, whereas activation of SN LepR neurons modulated locomotion. Activation of neither population altered anxiety-like behaviour.

Chemogenetic activation of VTA LepR neurons decreased motivational behaviour for food reward and activation of SN LepR neurons did not impact on motivation. The decrease in motivation was a surprising result as most VTA LepR and SN LepR

neurons are DAergic, increased midbrain DA activity is associated with enhanced motivation (Ilango et al., 2014; Rossi et al., 2013; Wise, 2009) and chemogenetic activation of VTA DA neurons increases motivation (Boekhoudt et al., 2016). First of all, our results suggest that VTA LepR and not SN LepR neurons modulate motivation. This is contrary to our hypothesis, which was based on a previous study that found no effect on motivation after leptin infusion into the VTA of ad libitum fed rats (Davis et al., 2011). Since leptin levels correlate with body fat (Frederich et al., 1995; Maffei et al., 1995), in ad libitum fed rats endogenous leptin levels are high and may have masked the effect of intra-VTA leptin on motivation. Secondly, as we see a decrease in motivation, this supports that VTA DA-LepR neurons, most of which project to the amygdala (Leshan et al., 2010), do not play a role in motivation for food reward. VTA GABA neurons are known to provide local inhibition of DA neurons and modulate reward behaviour (Creed et al., 2014). One interpretation of our results is, therefore, that decreased motivation is the result of chemogenetic activation of VTA GABA-LepR neurons that reduce VTA DA activity.

Our data further show that activating LepR neurons in the VTA decreased feeding in food restricted mice. This is in line with previous studies that reported decreased feeding upon intra-VTA leptin injections (Bruijnzeel et al., 2013, 2011; Hommel et al., 2006; Morton et al., 2009; Trinko et al., 2011). Interestingly, previous reports suggest that non-DA neurons of the VTA mediate leptin effects on feeding: strategies targeting only DA LepR neurons, i.e. LepR deletion in DAT neurons (Liu et al., 2011) or STAT3 deletion in DAT neurons (Fernandes et al., 2015), did not affect feeding and mice with STAT3 deletion in DAT neurons remained responsive to leptin's anorexic effect upon intra-VTA leptin infusions (Fernandes et al., 2015). Our data support the idea that VTA GABA-LepR neurons modulate feeding, as the decrease in feeding in our experiments is most likely mediated by the activation of these inhibitory neurons.

Given that most SN LepR neurons are DA neurons and chemogenetic activation of SN DA neurons increased locomotion (Boekhoudt et al., 2016), it was unexpected to find that activating SN LepR neurons decreased locomotion. This suggests that activation of one fifth of DA neurons in the SN is not sufficient enough to increase locomotion and also that the few inhibitory SN LepR neurons reduce locomotion. Furthermore, we found no effect of VTA LepR activation on locomotion. However, increased locomotion observed after LepR knockdown in VTA neurons (Hommel et al., 2006) or loss of STAT3 in VTA DA neurons (Fernandes et al., 2015) suggest that VTA DA neurons that express LepR modulate locomotion. Since activating one fifth of SN DA neurons was not capable of increasing locomotion, perhaps activating one fifth of VTA DA neurons was similarly not sufficient to modulate locomotion.

We did not observe changes in anxiety-like behaviour upon chemogenetic activation of VTA LepR. However, previous studies report that leptin reduces anxiety by inhibition of VTA DA-LepR neurons that project to the extended central amygdala (Leshan et al., 2010; Liu et al., 2011; Liu et al., 2016). Furthermore, increased anxiety was seen after deletion of LepR in DA neurons and this was accompanied by increased burst firing of VTA DA neurons (Liu et al., 2011). Other studies have also suggested that burst firing in VTA DA neurons results in anxiogenic behaviour (Coque et al., 2011; Sagheddu et al., 2015). Together, these results imply that chemogenetic activation of VTA LepR neurons did not activate VTA DA neurons to the extent of burst firing that is capable of increasing anxiety.

Interestingly, behavioural effects of chemogenetic activation of VTA LepR or SN LepR were only observed in food restricted mice, which have lowered leptin levels (Ahima et al., 1998). As such, we assume that the neurons responsible for changing behaviour show decreased activity when leptin levels are low. Since neuronal stimulation resulted in the reduction of the behaviour tested, the observed effects were likely driven by the activation of inhibitory non-DA neurons in the VTA or SN, such as GABA neurons. Together, this suggests that GABA neurons mediated the observed reductions in behaviour and that GABA neurons are less active with lower leptin levels. Therefore, we propose that leptin stimulates activity of these LepR neurons to contribute to the suppression of motivation, feeding and locomotion. Future studies will be needed to verify this.

To conclude, our results show that activating SN LepR neurons decreased locomotion, whereas activating VTA LepR neurons decreased feeding and motivation for food reward. Although both SN LepR and VTA LepR neurons are predominantly DA neurons, which are inhibited by leptin, the effects described here support involvement of inhibitory non-DA neurons in feeding, locomotion and food reward. This is an open question that needs to be addressed to more fully understand the mechanism underlying leptin's effect on motivation for food reward.

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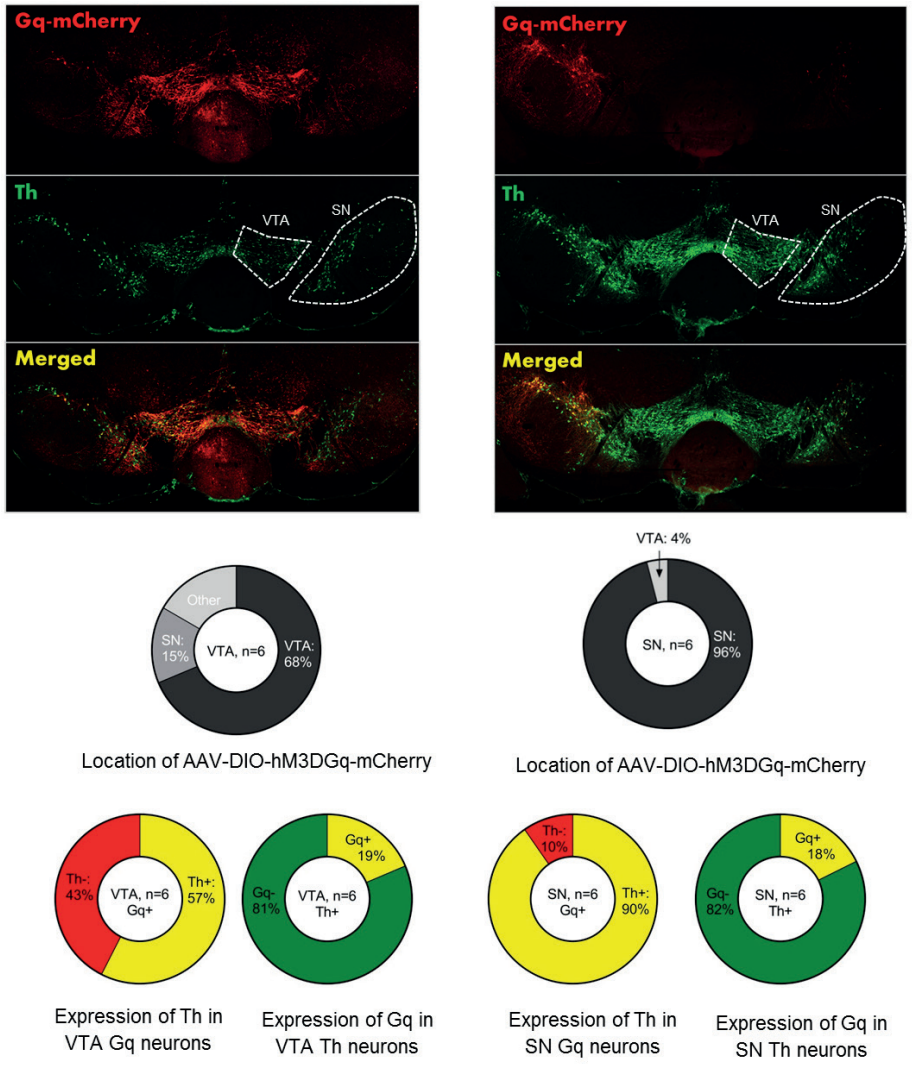
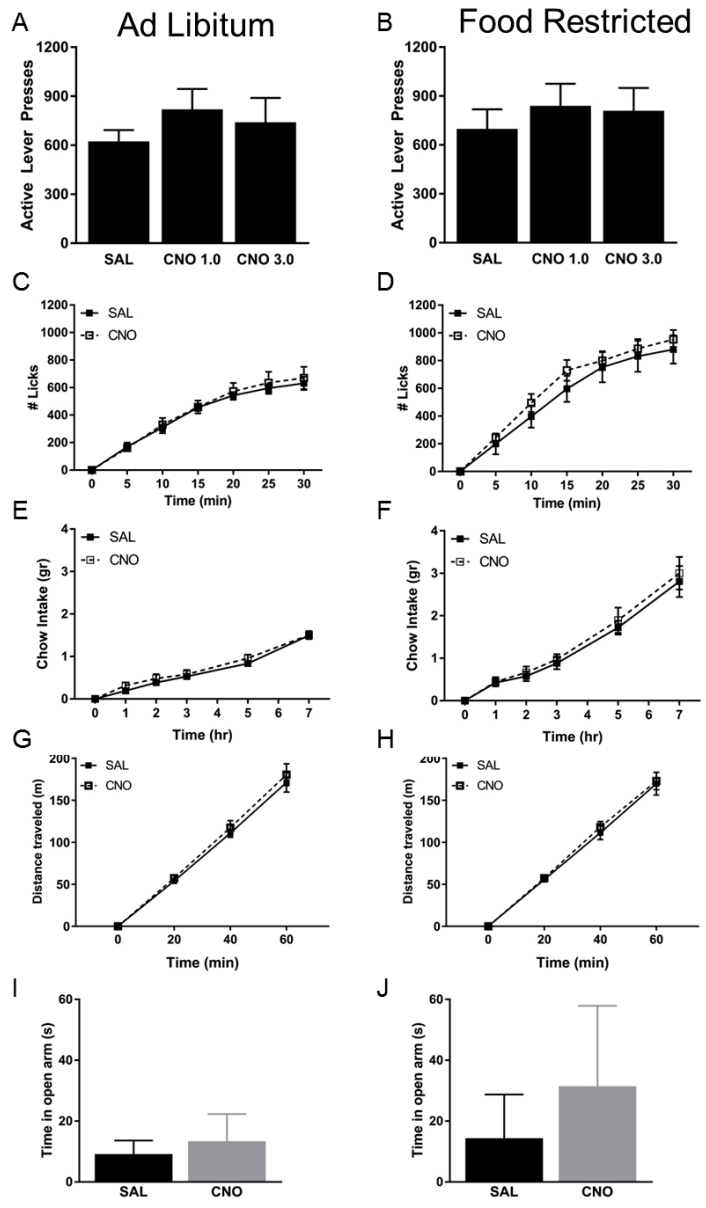
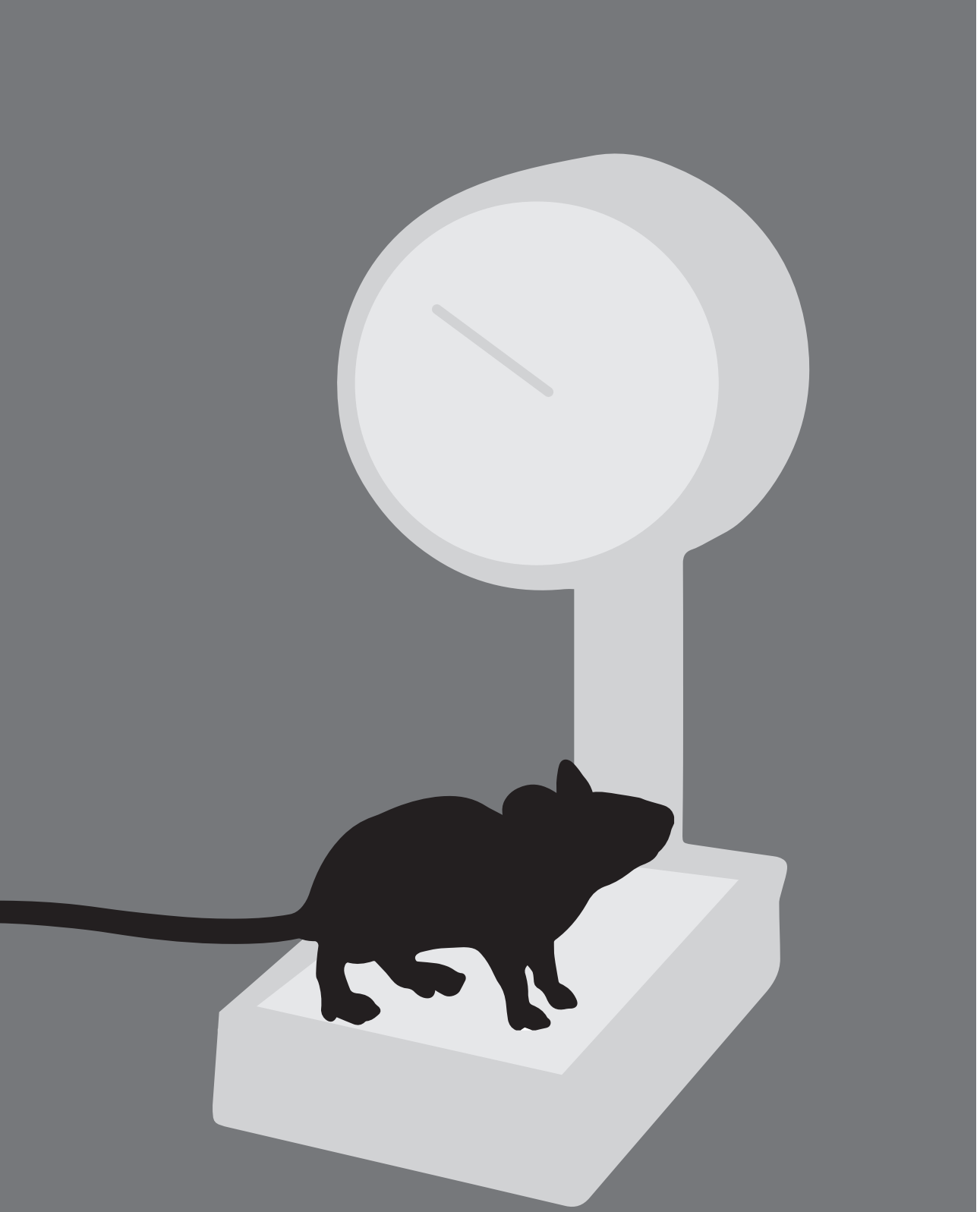


Supplemental figure 1. Expression of AAV-DIO-hM3DGq-mCherry in behavioural animals. Representative example of AAV-DIO-hM3DGq-mCherry expression in (A) VTA LepR-Gq mice (n=6) and (B) SN LepR-Gq mice (n=6). (C,D) Analysis of the location of Gq-mCherry expression showed that the majority of Gq-neurons were found in the targeted region. (E) Analysis of Gq and TH in VTA LepR-Gq mice showed that 57% of VTA Gq neurons co-localized with TH-immunoreactivity. (F) 19% of all VTA TH neurons co-localized with Gq. (G) 90% of SN Gq neurons co-localized with TH-immunoreactivity. (H) Of all SN Th neurons, 18% co-localized with Gq-mCherry.



Supplemental figure 2. Behavioural effects of CNO in control mice. In control LepR-cre mice injected with AAV-Ef1a-DIO-hChR2-eYFP CNO injections had no effect on (A, B) active lever presses, (C, D) free sucrose consumption, (E, F) chow intake, (G, H) locomotion or (I, J) anxiety-like behaviour. Mean ± SEM.



Chapter 3

Do lateral hypothalamic leptin receptor-expressing neurons modulate motivational behaviour?

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Abstract

Leptin has been shown to attenuate the increase in motivation for food reward in food restricted rodents. Which brain regions underlie this effect is largely unknown. Ventral tegmental area (VTA) dopamine (DA) neurons play an essential role in motivational behaviours. However, the subpopulation of VTA DA neurons that express the leptin receptor (LepR) are not likely involved in motivation and instead mediate fear behaviour. As such, we sought to determine the role of a densely VTA projecting LepR neuronal population situated in the lateral hypothalamus (LH) in mediating leptins effect on motivation. We trained LepR-cre mice to perform the progressive ratio task, which assesses motivation, and determined the role of LH LepR neurons on motivation by silencing LH LepR neurons using tetanus toxin light chain and by chemogenetic activation or inhibition of LH LepR neurons. Our results show that silencing LH LepR neurons decreased the amount of mice that are responsive to the reducing effect of leptin on motivational behaviour. Furthermore, we show that LH LepR stimulation increased motivation. Thus, we provide evidence that LH LepR neurons directly mediate changes in motivational behaviour.

Introduction

Overweight and obesity are major health issues concerning much of the human population. In 2016, worldwide 39% of adults were overweight and 13% obese (WHO, 2018). Dieting is largely unsuccessful on the long term (Wing and Phelan, 2005) and besides bariatric surgery, no truly effective therapies exist. Whilst dieting, physiological counter regulatory mechanisms defend higher weight by increased hunger feelings and increased motivation for food (Cameron et al., 2014; Miquelon et al., 2012; Sato et al., 2017). Therapies that aim to decrease motivation for food when dieting may prove to be beneficial in the future.

Leptin is an anorexigenic adipose-tissue derived hormone that has been shown to attenuate the increase in motivation for food reward caused by food restriction in rodents (Brown et al., 2017; Figlewicz et al., 2006; Sharma et al., 2012; Woodworth et al., 2017). Similarly, in humans that lost weight by dieting, leptin administration reduced the rewarding response to food (Hinkle et al., 2013; Rosenbaum et al., 2008). Despite this knowledge, it remains largely unknown which leptin responsive neurons mediate motivational changes by leptin.

Increased ventral tegmental area (VTA) dopamine (DA) activity is associated with enhanced motivation (Ilango et al., 2014; Rossi et al., 2013; Wise, 2009). While many leptin receptor expressing (LepR) neurons exist in the VTA, VTA DA-LepR neurons are known to primarily project to a region that is not involved in motivation (Leshan et al., 2010). As such, VTA DA-LepR neurons are suggested not to be involved in modulation of motivational behaviour, but instead are known to modulate fear behaviour (Leshan et al., 2010). Therefore, we wondered whether other regions projecting to the VTA mediate leptin's effect on motivational behaviour.

The lateral hypothalamus (LH) is a region known for its role in feeding, but also motivated behaviours (Tyree and de Lecea, 2017). The latter is likely mediated by dense LH projections to the VTA (Beier et al., 2015; Leinninger et al., 2009; Watabe-Uchida et al., 2012). LepR neurons in the LH have been shown to regulate DA content via both direct VTA projections and indirect projections via local orexin (OX) neurons (Leinninger et al., 2011, 2009; Louis et al., 2010; Opland et al., 2013). LH LepR are GABAergic neurons (Leinninger et al., 2009) and mainly co-express neurotensin (Nts) and galanin (Gal) (Brown et al., 2017; Laque et al., 2015; Leinninger et al., 2011). Deleting LepR in LH Nts neurons blunts the effect of leptin on decreasing motivation (Brown et al., 2017; Laque et al., 2015). Furthermore, motivational behaviour is decreased or increased upon deletion of LepR in LH Nts or LH Gal neurons, respectively (Brown et al., 2017; Laque et al., 2015).

Thus, contrasting effects were found on motivation after deleting LepR in different LH subpopulations, but there is evidence that LH LepR neurons modulate motivation.

Here, we further address the (direct) role of LH LepR neurons in motivation. As such, we first determined whether silencing of LepR signaling in the LH affects motivational behaviour. Since LH LepR deletion is permanent and may, as a result, alter downstream circuits, we next assessed the direct role of LH LepR neurons in motivational behaviour by chemogenetically activating or inhibiting LH LepR neurons in an otherwise intact neuronal circuitry. To test for motivation, we trained mice to perform a progressive ratio task in which animals have to press a lever in increasing amounts to earn a sucrose solution reward. To discriminate motivational behaviour for food from other behaviours that may influence performance in the operant task, we also determined the effects of our interventions on feeding and locomotion.

Materials and Methods

All experiments were approved by the Animal Experimentation Committee of the University Utrecht and were carried out in agreement with Dutch Law (Herziene Wet op Dierproeven, Art 10.a2, 2014) and European regulations (Guideline 2010/63/EU).

Mice

In house bred, adult male homozygote LepRb-Cre transgenic mice (stock #008320, B6.129-Leprtm2(cre)Rck/J, The Jackson Laboratory, USA) were used for behavioural experiments. Mice were housed individually in cages (Type II L, 365x207x140mm, 530cm2, Tecniplast, Italy) in a temperature (21±2^{0}C) and humidity (60-70%) controlled room on a reversed day/night (9:00 AM light OFF, 9:00 PM light ON) schedule. As stated in the text, we tested mice under ad libitum and/or food restricted feeding schedules. When ad libitum fed, mice were given ad libitum access to chow (3,1Kcal/gr, Standard Diet Service, UK) and water. When food restricted, mice were given chow (~3 pieces of standard rodent chow of ±1.3 grams each) after behavioural tasks were performed and maintained on ~90% of the original body weight.

Surgery

At least 30min prior to anesthetization, mice were given carprofen (5mg/kg, subcutaneous (s.c.), Carporal, AST Farma BV). Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (75mg/kg, Narketan, Vetoquinol BV) and medetomidine (1mg/kg, SedaStart, AST Farma BV). Mice were given eye cream (CAF, CEVA Sante Animale BW) and placed in a stereotactic frame (Kopf Instruments, USA). An incision was made in the skin along the midline of the skull and additional analgesia was applied by spraying Xylocaine (lidocaine 100mg/ml, AstraZeneca BV) on the skull. Microinjections of AAV1-CBA-DIO-GFP-TetTox-WPRE-pA (0.3ul/side, 3.0*109gc/ul), AAV5-hSyn-DIO-hM3DGq-mCherry (0.3ul/side, 3.0*109gc/ul, UNC Vector Core, USA), AAV1-hSyn-cDIO-HA-hM3DGq-biased (0.3ul/side, 2.2*109gc/ul) and AAV5-hSyn-cDIO-hM4DGi–mCherry (0.3ul/side, 0.8*109gc/ul) were performed bilaterally in the lateral hypothalamus (-0.12mm AP, +0.20mm ML, -.054mm DV, 10° angle) at a rate of 0.1ul/min per side followed by a 10min period before retracting the needles. For assessment of VTA projecting LepR neurons mice were injected with HSV-EF1a-LS1L-mCherry (0.3ul/side, 4×108gc/ml; McGovern Institute, USA) in the VTA. After the operation, mice were given atipamezole (2.5mg/kg, i.p., SedaStop, AST Farma BV) and saline for rehydration. The following 2 days, mice were given carprofen (5mg/kg, s.c.) and were allowed to recover for at least 1 week. To ensure viral expression, testing commenced 3 weeks after virus injection.

Drugs

Clozapine N-oxide (CNO 99%, AK Scientific, Inc., USA), was dissolved in 0.9% saline and injected i.p. with a dose of 1.0mg/kg (also other doses for operant testing). Recombinant mouse leptin (5mg/kg, NHPP, USA) was dissolved in 1x phosphate-buffered saline (PBS) and injected i.p. For all experiments, each mouse received saline/PBS and CNO/leptin injections in a latin-square design.

Experimental procedures

Progressive ratio operant behaviour: Mice operant boxes (ENV-307W, Med Associates Inc., USA) fitted with two levers, a cue light above the active lever (AL), a house light, a speaker and a liquid receptacle were used. Throughout all sessions, when the number of AL presses to complete a ratio was reached, the house light and a tone were presented for 5sec, after which a sugar reward was delivered for 2sec (38ul, 20% w/v sugar in tap water). During the training phase, mice were food restricted to ~90% of their original body weight. The first day, mice were habituated for 15min to an operant box, in which we placed a droplet of sucrose solution (20% w/v) in the receptacle. The next day operant training started with a fixed ratio (FR) 1 paradigm for 30min/session. Once the mice learned to press on the active lever >20x and <10% on the inactive lever, mice were switched to FR3 (30min/session) and then FR5 (60min/session). Then, once >60 rewards

were earned and <10% of the presses were on the inactive lever, training was switched to the progressive ratio (PR, 60min/session) and mice were returned to ad libitum feeding. The PR schedule was based on the formula: 5*e(x*0.2)-5, rounded to the nearest integer, where x is the position in the ratio sequence (Richardson and Roberts, 1996). Testing with saline/CNO started when PR performance appeared stable, i.e. over three days of training no more than ±1 reward from average and no incremental increase or decrease. Operant training and testing was performed during the first 4h of the dark phase. Injections were given 30min before the start of the PR task. When testing the effect of leptin/PBS on motivational behaviour, mice were food restricted for 6h prior to the start of the PR task.

Free access 20% sucrose consumption: Mice were trained to lick for 20% (w/v) granulated sugar solution in an operant box fitted with a spout connected to a pump which delivered 8ul 20% sugar solution upon every detected lick. Mice were trained 4 times prior to testing. Training and testing was performed 3-5h into the dark phase and lasted 30min. On test days, mice were injected with CNO/saline 30min before the start of the test.

Feeding: To simplify finding pieces of chow at measurements, all mice were habituated to a second mouse cage (Type II L, 365x207x140mm, 530cm2, Tecniplast, Italy), in which no bedding, but only 3 tissues and a water bottle were present, hereafter referred to as 'feeding cage'. Mice were injected with saline/CNO or PBS/leptin 30min prior to the onset of the dark phase and were directly placed in the feeding cage with a pre-weighed amount of chow on the cage floor. Food intake was measured at 1, 2, 3, 5 and 7h post injection, after which the mice were placed back in their home cage. When testing the effect of leptin/PBS on feeding behaviour, mice were food restricted for 18h prior to the start of the feeding task.

Locomotion: All mice were habituated 2x1h to an individual cage (Type III H, 425x266x185mm, 800cm2, Tecniplast, Italy) prior to testing on separate days. The cages were surrounded by white carton to prevent interaction between mice. On the test day, all mice (except TetTox) were injected with either saline/CNO and placed in the behavioural testing room. 30min later, mice were placed in their own locomotion cage and horizontal movement was tracked using a camera placed above the cages that was coupled to a computer running Ethovision 7 (Noldus Information Thechnology, the Netherlands). Locomotion tests commenced 4-5h into the dark phase and lasted 1h.

Immunohistochemistry

Mice were anesthetized with Euthanimal (Euthanimal, Alfasan BV, the Netherlands) and transcardially perfused with 1x phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 1xPBS (PFA). Brains were dissected and kept in 4%PFA for 24h at 4°C, after which they were transferred to 30% sucrose in 1xPBS for at least 48h at 4°C. Using a cryostat, the brains were then sectioned to 40um slices and stored in 1xPBS with 0.01% sodium azide. Slices were washed 3x10min in 1xPBS and then blocked for 1h in 1xPBS containing 10% normal goat serum and 0,25% Triton-X100. Slices were then placed in 1xPBS containing the primary antibodies (Rabbit anti-dsRed 1:500, #632496, Clontech, Takara Bio USA Inc, USA) and 2% normal goat serum overnight at 4°C. At room temperature, slices were washed 3x10min in 1xPBS and placed in 1xPBS containing the secondary antibodies (Goat anti-Rabbit 568, 1:500, #ab175471, Abcam plc, UK) and 2% normal goat serum for 2h. Finally, slices were washed in 1xPBS and mounted onto slides, dried and covered using Fluorsave (EMD Millipore Corporation, USA) and a coverslip. Images were collected on an epifluorescent microscope (Axio Scope A1, Zeiss, Germany).

Data Analysis and Statistics

Behavioural data was analyzed and visualized using Microsoft Excel, Graphpad Prism and SPSS. Paired t-tests and one- or two-way repeated measures ANOVA tests were used where applicable and a significance criterion of $p<0.05$, two-tailed, was adopted in all the statistical analyses.

Results

Previous studies showed that LH LepR neurons projected to the VTA (Leinninger et al., 2009). To verify this in our LepR-cre mice, we injected mice with a retrogradely labeling AAV-HSV-mCherry in the VTA of LepR-cre mice. Similar to previous resports, we observed mCherry labeled neurons in the LH, demonstrating that LH LepR neurons project to the VTA (fig. 1).

We then assessed behavioural effects of permanent silencing of LH LepR neurons, by utilizing tetanus toxin light chain to prevent exocytosis of synaptic vesicles, i.e. blocking neurotransmitter release (Ahnert-Hilger et al., 1989; Cornille et al., 1997; Kim et al., 2009). All mice referred to as TetTox mice, had low (n=5) to moderate (n=3) TetTox expression in the LH. Mice with TetTox expression (fig. 2A, n=8) had a similar body weight (fig. 2B), locomotor activity (fig. 2C) and average daily food intake measured over 10 days in home cage (fig. 2D) compared to control mice (n=5). To determine whether the anorectic response to leptin was altered in TetTox mice, we injected mice with vehicle or leptin after 18h food restriction. This revealed that leptin decreased feeding 7h after injections irrespective of the group of mice (main effect of Leptin vs PBS $F(1,11)=6.99$, $p=0.0228$; fig 2E). Of note, all control mice (5/5) decreased feeding upon leptin injections, but not all (6/8) TetTox mice decreased feeding; there was no apparent TetTox expression pattern in the 2/8 that did not decrease feeding after leptin that could explain why these 2 did not decrease feeding. Next, we trained mice to press for a sucrose solution reward in the

progressive ratio (PR) task to measure motivational behaviour for food reward. When all mice performed stably on the PR task, TetTox mice made less active lever presses than control mice, but this did not reach statistical significance (*t*=1.21, p=0.30; fig. 2F). We then tested whether leptin decreased motivation in mice after 6h food restriction. Leptin decreased motivational behaviour compared to vehicle injections irrespective of the group of mice (main effect Injection *F*(1,10)=10.12, p=0.0098, fig. 2G). Similar to leptin's effect on feeding, all control mice, but only half (4/8) of TetTox mice decreased motivation after leptin injection; 2 of these had moderate LH TetTox expression and in the 2 with the largest decrease after leptin in the TetTox group showed low expression. Together, these results suggest that silencing LH LepR neurons decreased the number of mice that are responsive to the decreasing effect of leptin on both feeding and motivational behaviour.

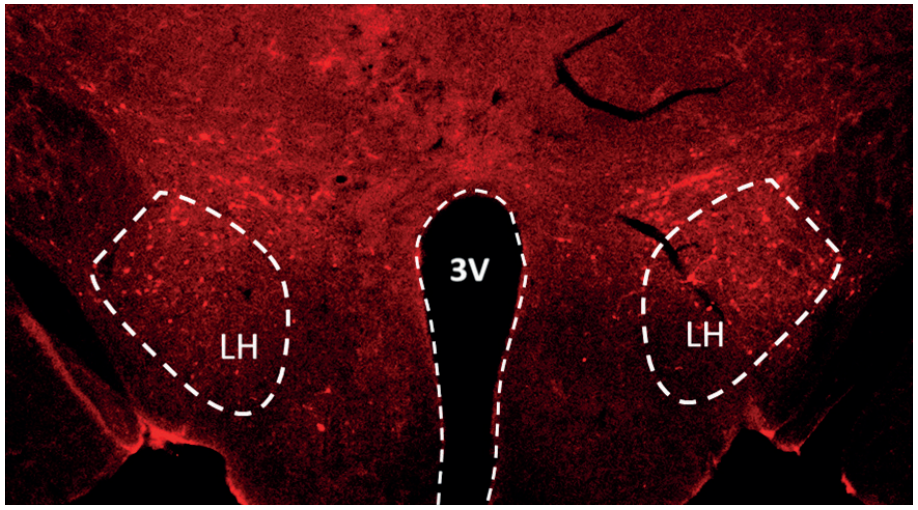

Figure 1. LH LepR neurons project to the VTA. HSV-mCherry injected into VTA of LepR-cre mice, showed mCherry fluorescence in LH neurons demonstrating that LH LepR neurons project to the VTA.

Since TetTox is a permanent intervention and may disrupt downstream circuits, we further tested the involvement of LH LepR neurons in motivational behaviour using chemogenetics. First, we injected LepR-cre mice with excitatory DREADD-Gq into the lateral hypothalamus ("LH LepR-Gq mice", n=7, fig. 3A). When ad libitum fed, activating LH LepR neurons increased the amount of active lever presses (*F*(2,12)=4.491, p=0.0350) after both CNO 1.0mg/kg (p=0.0584) and CNO 3.0mg/kg (p=0.0055) injections compared to saline (fig. 3B). The amount of inactive lever presses, pressing of which does not result in a reward, were unaffected by CNO (fig. 3C). We also assessed free consumption of the sucrose solution (which is the reward obtained in the PR), free consumption of chow and locomotor activity. These tests revealed that activating LH LepR neurons did not affect consummatory behaviour (fig. 3D and 3E) and increased locomotor behaviour (*t*(6)=-2.454, p=0.0494, fig. 3F). Since activating LH LepR neurons did not affect consumption,

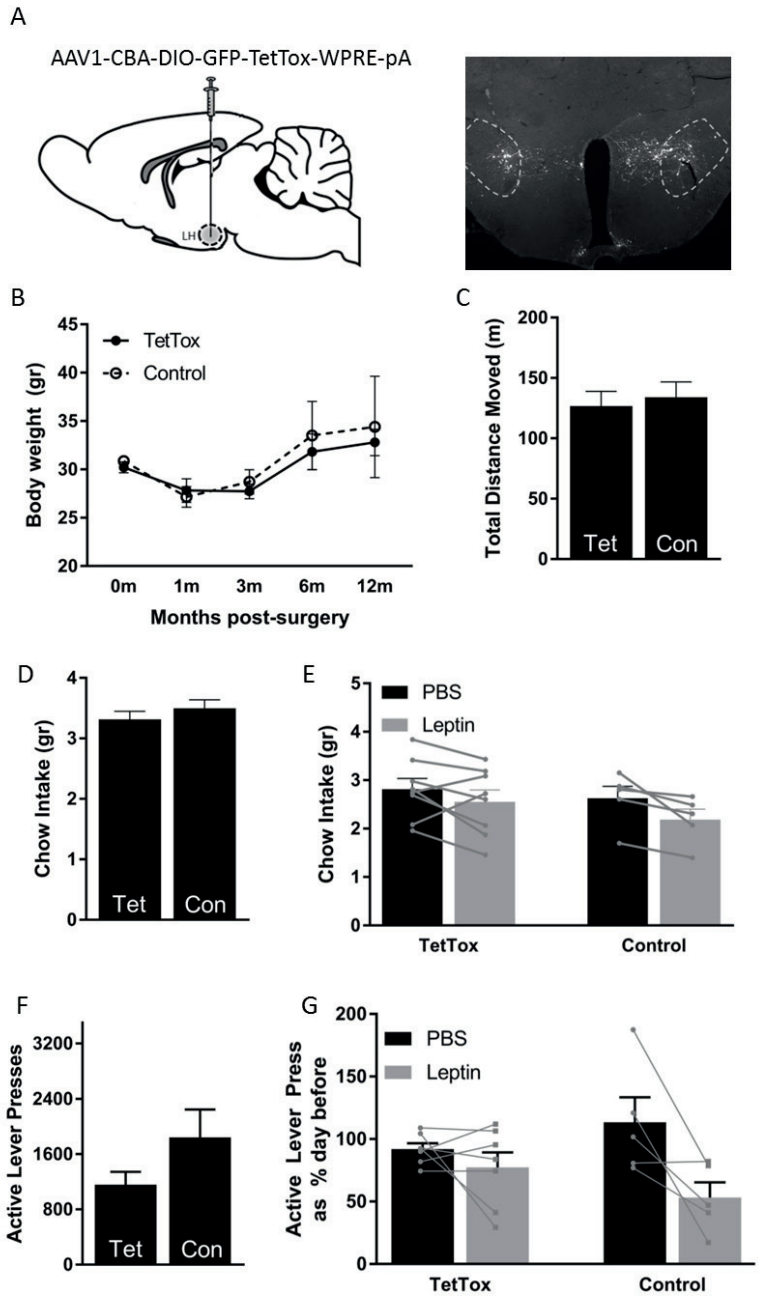

Figure 2. Behavioural effects of silencing LH LepR neurons in mice. (A) Representative example of LepR-cre mouse injected with AAV-DIO-TetTox in the LH. Silencing of LH LepR neurons using TetTox had no effect on (B) body weight, (C) locomotor activity, (D) home cage feeding and (F) baseline motivation. The number of mice that reduced (E) feeding or (G) motivational behaviour after leptin injections was reduced by silencing LH LepR neurons, but remained intact in control mice. Mean ± SEM.

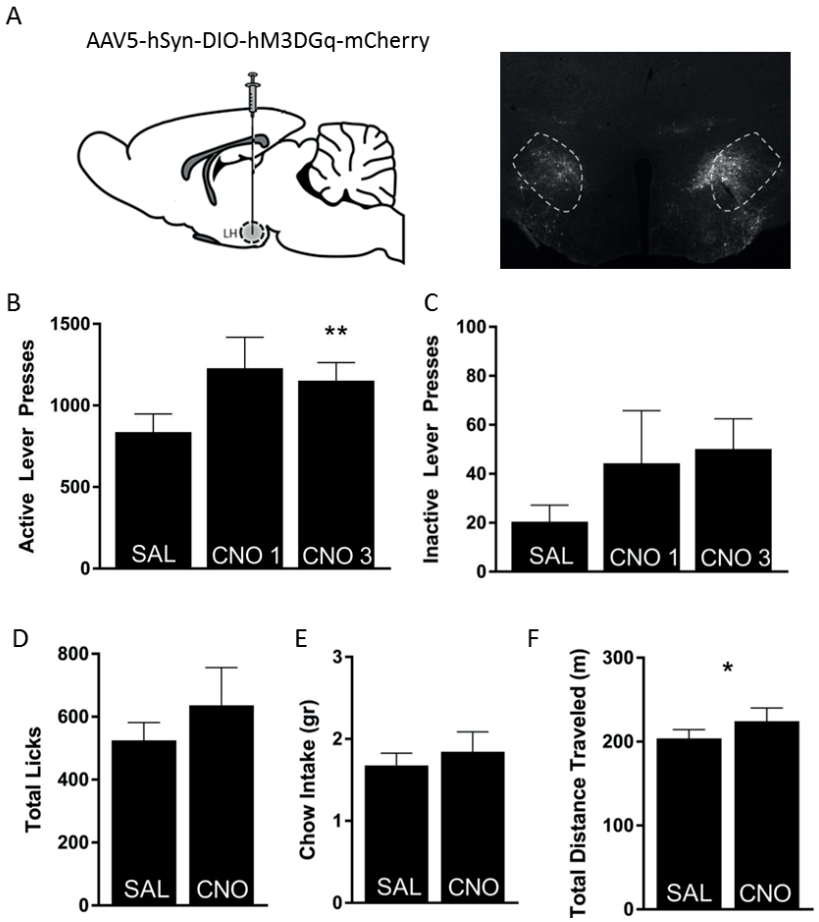

Figure 3. Behavioural effects of chemogenetically activating LH LepR neurons in ad libitum fed mice. (A) Representative example of LepR-cre mouse injected with AAV-DIO-hM3DGq in the LH. (B) Chemogenetically activating LH LepR neurons increased (B) active lever presses, but not (C) inactive lever presses. Stimulating LH LepR neurons did not affect (D) sucrose solution consumption or (E) consumption of chow and (F) increased locomotor activity. Mean ± SEM. 'CNO' corresponds to CNO 1.0mg/kg, otherwise the numbers next to CNO indicate the used dose. * p<0.05, ** p<0.01.

increased motivation seen in the PR task was not likely driven by changes in hunger. Increased locomotor activity may have enhanced lever pressing. Since only active lever presses (and not inactive lever presses) were increased, this suggests that the increase in active lever presses was reward driven.

The previous experiments were performed in ad libitum fed mice. We wondered whether food restriction, and thus lower fat mass and lower leptin levels would increase the efficacy of LH LepR neurons to stimulate reward seeking. In this setting, activating LH LepR

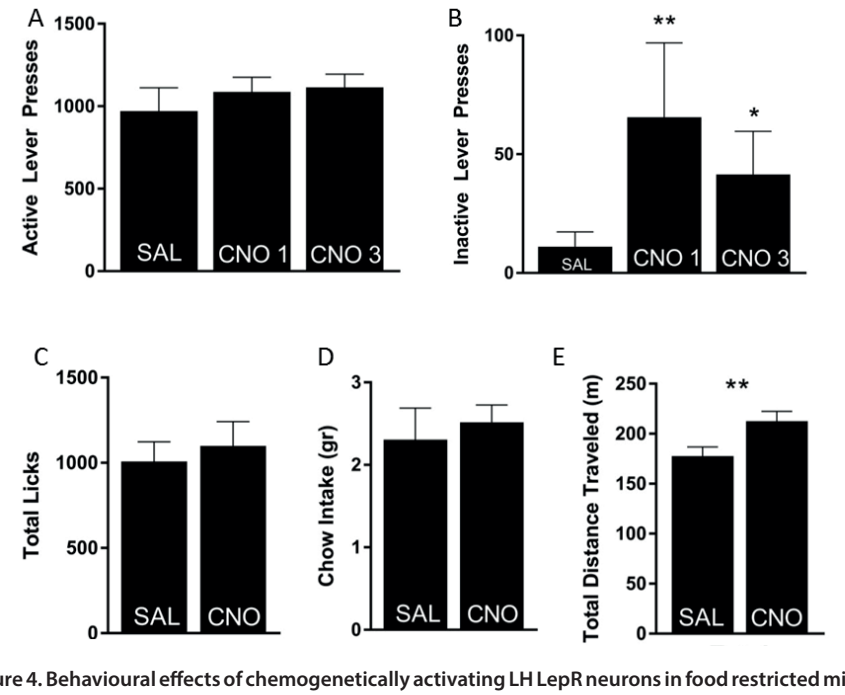

Figure 4. Behavioural effects of chemogenetically activating LH LepR neurons in food restricted mice. Chemogenetically activating LH LepR neurons had (A) no effect on active lever presses and (B) increased inactive lever presses. Stimulating LH LepR neurons did not affect (C) sucrose solution consumption or (D) consumption of chow and (E) increased locomotor activity. Mean ± SEM. "CNO' corresponds to CNO 1.0mg/kg, otherwise the numbers next to CNO indicate the used dose. * p<0.05, ** p<0.01.

neurons did not affect active lever presses (fig. 4A), free consumption of sucrose solution (fig. 4C) or free consumption of chow (fig. 4D). LH LepR-Gq mice increased inactive lever presses with both CNO doses ($F(2,12)=13.280$, p=0.0009; CNO 1.0: p=0.0033, CNO 3.0: p=0.0124, fig. 4B) and increased locomotion ($t(6)=-3.894$, p=0.0080, fig. 4E). Increased locomotion may have resulted in the increase in inactive lever presses.

Excitatory DREADD-Gq not only stimulates neuronal activity, but also triggers β-arrestin-mediated signalling. The latter can result in desensitization and internalization of the receptor via clathrin coated vesicles (Hu et al., 2016). High activation of DREADD-Gq may thus lead to desensitization and we wondered whether this may have affected our LH LepR-Gq results. So, in a next group of mice we injected DREADD-Gq-biased, which does not interact with β-arrestins due to point mutations in the C-terminal portion of the receptor ("LH LepR-Gq-biased", n=7, fig. 5A), (Hu et al., 2016). When tested in the PR task, ad libitum fed LH LepR-Gq-biased mice increased motivational behaviour upon CNO injections, but this did not reach statistical significance (fig. 5B, C). Similar to LH LepR-Gq

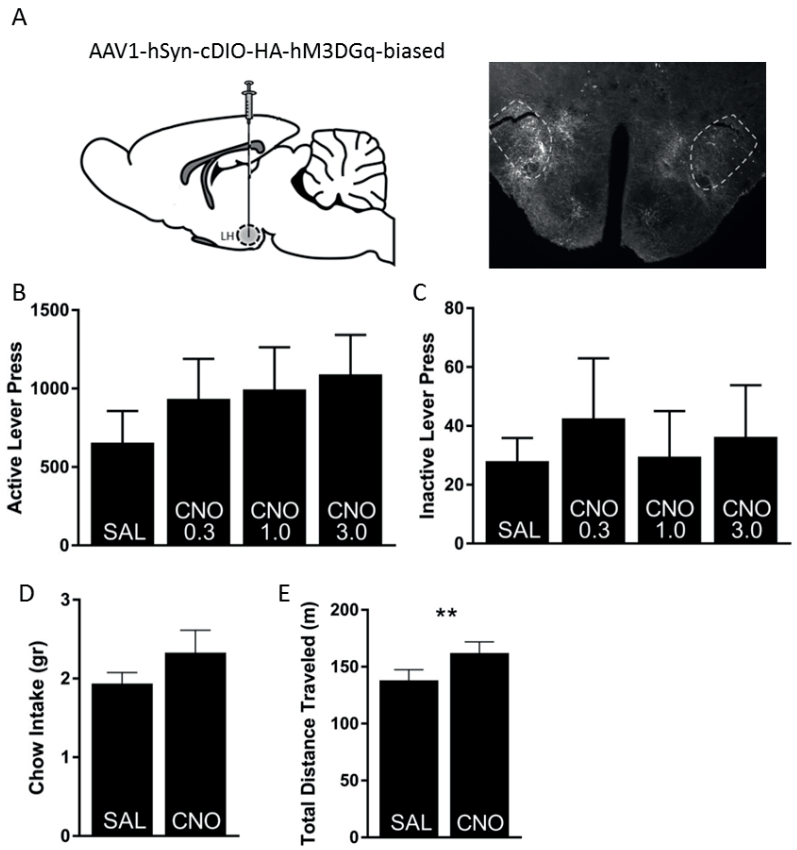

Figure 5. Behavioural effects of chemogenetically activating LH LepR neurons in ad libitum fed mice using Gq-biased. (A) Representative example of LepR-cre mouse injected with AAV-DIO-hM3DGq-biased in the LH. Activating LH LepR neurons did not affect (B) active lever presses, (C) inactive lever presses or (D) consumption of chow and (E) increased locomotor activity. Mean ± SEM. 'CNO' corresponds to CNO 1.0mg/kg, otherwise the numbers under CNO indicate the used dose. ** p<0.01

mice, CNO in LH LepR-Gq-biased mice had no effect on free consumption of chow (fig. 5D) and increased locomotion (fig. 5E, $t(6)=3.893$, p=0.0080).

Since LH LepR stimulation with DREADD-Gq increased motivation, we questioned whether LH LepR inhibition would decrease motivation. As such, we injected DREADD-Gi in mice to inhibit neuronal activity of LH LepR neurons ("LH LepR-Gi", n=7, fig. 6A). Because LH LepR-Gq mice increased motivation in ad libitum fed state, we reasoned that LH LepR neurons mediating this behaviour are less active in ad libitum fed state and thus would be less sensitive to chemogenetic inhibition. As a result, we tested chemogenetic inhibition of LH LepR neurons in food restricted mice only. However, this intervention did not affect motivational behaviour, free consumption of chow or locomotion (fig. 6B-E).

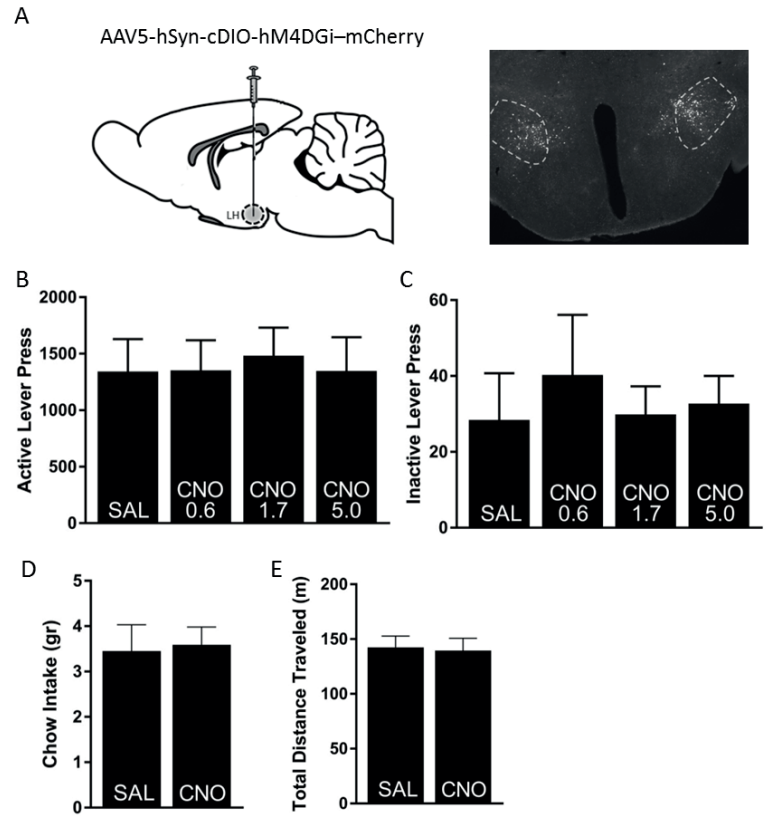

Figure 6. Behavioural effects of chemogenetically inhibiting LH LepR neurons in food restricted mice. (A) Representative example of LepR-cre mouse injected with AAV-DIO-hM4DGi in the LH. Inhibiting LH LepR neurons did not affect (B) active lever presses, (C) inactive lever presses, (D) consumption of chow or (E) locomotor activity. Mean ± SEM. "CNO' corresponds to CNO 1.7mg/kg, otherwise the numbers under CNO indicate the used dose.

To control for non-specific effects of CNO due to reverse-metabolism to its parent compound clozapine (Manvich et al., 2018), we injected control mice with ChR2, which is not a receptor for CNO. In these experiments we did not observe any behavioural changes upon CNO injections in food restricted or ad libitum fed situations in any task tested (fig. S1, S2). Thus behavioural changes upon CNO injections in LH LepR-Gq(-biased) mice were specific to DREADD-Gq induced neuronal stimulation.

Discussion

We determined whether LH LepR neurons modulate motivational behaviour using various techniques in LH LepR mice. Our most critical observation was that chemogenetically activating LH LepR neurons increased motivation for food in ad libitum fed mice, without affecting food intake per se. These results suggest that LH LepR neurons directly modulate motivational behaviour. Furthermore, silencing LH LepR neurons using tetanus toxin light chain did not alter baseline motivational behaviour, but decreased the amount of mice that were responsive to leptin's reduction in motivation.

It is of interest to consider the possible neurocircuitry by which stimulation of LH LepR neurons increased motivational behaviour. Given that VTA DA neuronal activity is associated with motivational behaviour, LH LepR neurons likely connect (in)directly to these neurons. The common denominator of all LH LepR neurons is that they are GABAergic and the main peptides co-expressed on LH LepR neurons are neurotensin (Nts) and galanin (Gal) (Brown et al., 2017; Laque et al., 2015; Leinninger et al., 2011, 2009). LH LepR-Nts neurons project to local OX and to the VTA, while LH LepR-Gal neurons project to local OX, but not to the VTA (Laque et al., 2015; Leinninger et al., 2011). Thus, the two main candidates for changing motivational behaviour are indirect VTA projections of LH LepR-Nts/-Gal neurons via local OX neurons and direct VTA projections by LH LepR-Nts neurons.

First, OX neurons, which have a role in motivation and excite VTA DA neurons (Harris et al., 2005; Liu et al., 2017; Mahler et al., 2014), are innervated by LH LepR-Nts and -Gal neurons. Both fasting and LepR deletion in LH Gal neurons increases OX neuronal activity, suggesting modulation by LH LepR neurons (Laque et al., 2015; Leinninger et al., 2011). Intra-LH leptin injections inhibit OX neurons via Galanin release (Bonnavion et al., 2015; Goforth et al., 2014). As such, chemogenetic stimulation of LH LepR neurons would inhibit OX neuronal activity and decrease motivation. Therefore, it is unlikely that this route explains the results we found, since chemogenetic activation of LH LepR neurons increased motivation for food reward.

Second, LH LepR(-Nts) neurons have been shown to project to both DA and GABA neurons of the VTA (Leinninger et al., 2011; Louis et al., 2010). GABA release by LH LepR neurons onto VTA DA neurons would decrease motivation (and thus could not explain our results), while GABA release onto VTA GABA neurons would increase motivation. Since VTA GABA neurons provide local inhibition of VTA DA neurons (Creed et al., 2014), inhibiting VTA GABA neurons would thus lead to a disinhibition of VTA DA neurons. This would increase VTA DA neuronal activity and may contribute to enhanced motivational behaviour. This LH -> VTA GABA -> VTA DA neurocircuitry has been established for LH GABA neurons, but remains to be determined for LH LepR neurons (Nieh et al., 2016). An alternative explanation for our results could be that release of Nts by VTA projecting LH LepR-Nts neurons increased motivation, since: 1) Nts in the VTA excites VTA DA neuronal activity (Seutin et al., 1989), 2) leptin mediated decrease in motivation is dependent on Nts-receptor expression in VTA DA neurons (Woodworth et al., 2017) and 3) stimulation of LH Nts promotes nucleus accumbens DA efflux via the VTA (Patterson et al., 2015). Thus, our finding that LH LepR neurons enhanced motivation could be explained by enhanced GABA release onto VTA GABA neurons or Nts release onto VTA DA neurons.

Our results suggest that leptin alters motivational behaviour at least in part via LH LepR neurons. Leptin has been shown to decrease motivation (Brown et al., 2017; Figlewicz et al., 2006; Sharma et al., 2012; Woodworth et al., 2017) and attenuates the rewarding effect of LH stimulation (Fulton et al., 2000), so it was relatively surprising to find that LH LepR stimulation increased motivation. However, LH LepR neurons are known to be differently regulated by leptin, with about 34% being depolarized and 22% hyperpolarized (Leinninger et al., 2009). Together, this implies that the LH LepR neurons that underlie increased motivation are most likely hyperpolarized by leptin. Previous studies showed that LH Nts neurons are mostly depolarized by leptin (Leinninger et al., 2009). Given that LH LepR-Nts neurons project to both local OX neurons and to the VTA (Leinninger et al., 2009), it is possible that those projecting locally are depolarized by leptin and those projecting to the VTA are hyperpolarized. Further studies will be necessary to determine which and how LH LepR projections underlie changes in motivation.

It is important to address the apparent differences in expression levels of the viruses used. In the TetTox group, 3/8 mice had moderate TetTox expression in the LH and 5/8 had low LH expression. Though we did not find clear differences in expression patterns that could explain why 4/8 reduced motivational behaviour and 6/8 reduced feeding after leptin injections, we cannot exclude that differences in viral expression may have affected behavioural results. Furthermore, TetTox expression was not localized exclusively to the LH, but also to nearby LepR expressing regions such as the dorsomedial hypothalamus and zona incerta. As such, it would be essential to reassess behavioural effects of silencing LH LepR neurons with viral expression localized to the LH and a more uniform expression across mice. As for the chemogenetic groups, although we did not quantify the level of viral vector expression, LH LepR-Gq-biased and LH LepR-Gi mice had noticeably lower expression levels than the LH LepR-Gq group. Lower expression levels may have resulted in the lack of effect on motivational behaviour upon chemogenetic activation of LH LepR neurons using Gq-biased or chemogenetic inhibition of LH LepR neurons. Moreover, inhibition of LH GABA neurons has been shown to decrease reward (Navarro et al., 2016;

Nieh et al., 2016, 2015). Therefore, follow-up studies will need to redetermine whether inhibiting LH LepR neurons affects motivation.

This chapter provides evidence for the direct modulation of motivational behaviour by LH LepR neurons. These findings are relevant for the increasing obesity epidemic in humans. Since the physiological response to dieting in both rodents and humans is to increase food reward, LH LepR neurons may be pivotal in new treatments that help to decrease motivation for food whilst dieting.

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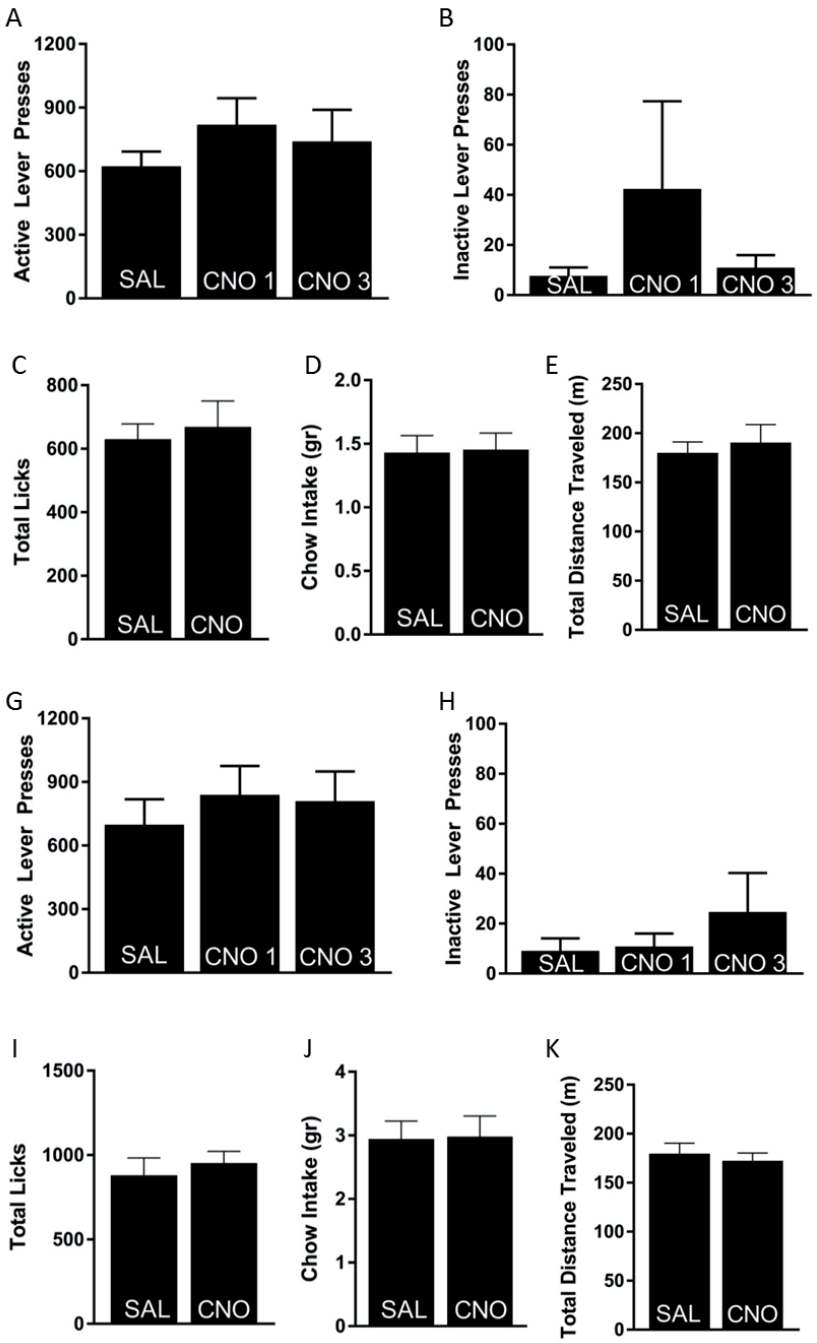


Figure S1. Behavioural effects of CNO in control mice injected with AAV-DIO-ChR2. CNO (1.0-3.0mg/kg) had no effect in ad libutum fed mice on (A) active lever presses, (B) inactive lever presses, (C) sucrose solution consumption, (D) consumption of chow or (E) locomotor activity. Similarly, CNO (1.0-3.0mg/kg) had no effect in food restricted mice on (F) active lever presses, (G) inactive lever presses, (H) sucrose solution consumption, (I) consumption of chow or (J) locomotor activity. Mean ± SEM. "CNO' corresponds to CNO 1.0mg/kg, otherwise the numbers next to CNO indicate the used dose.

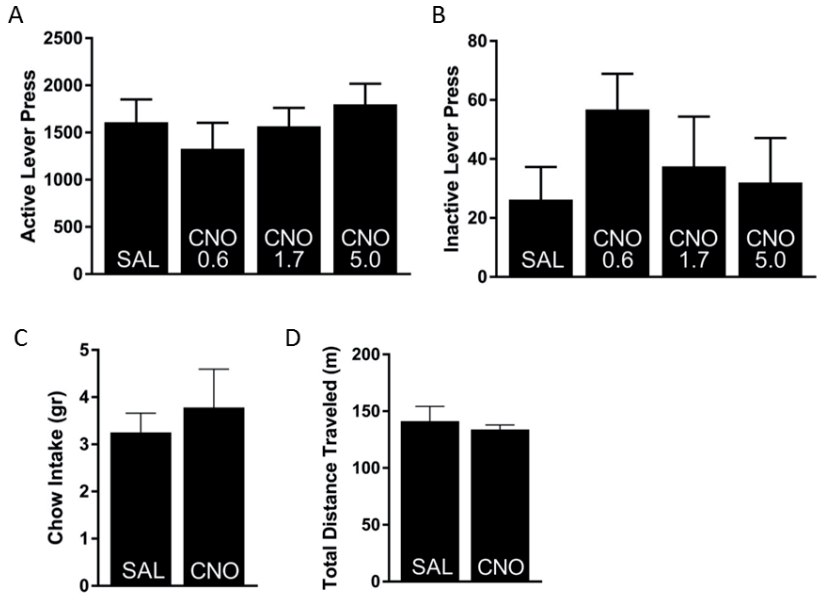
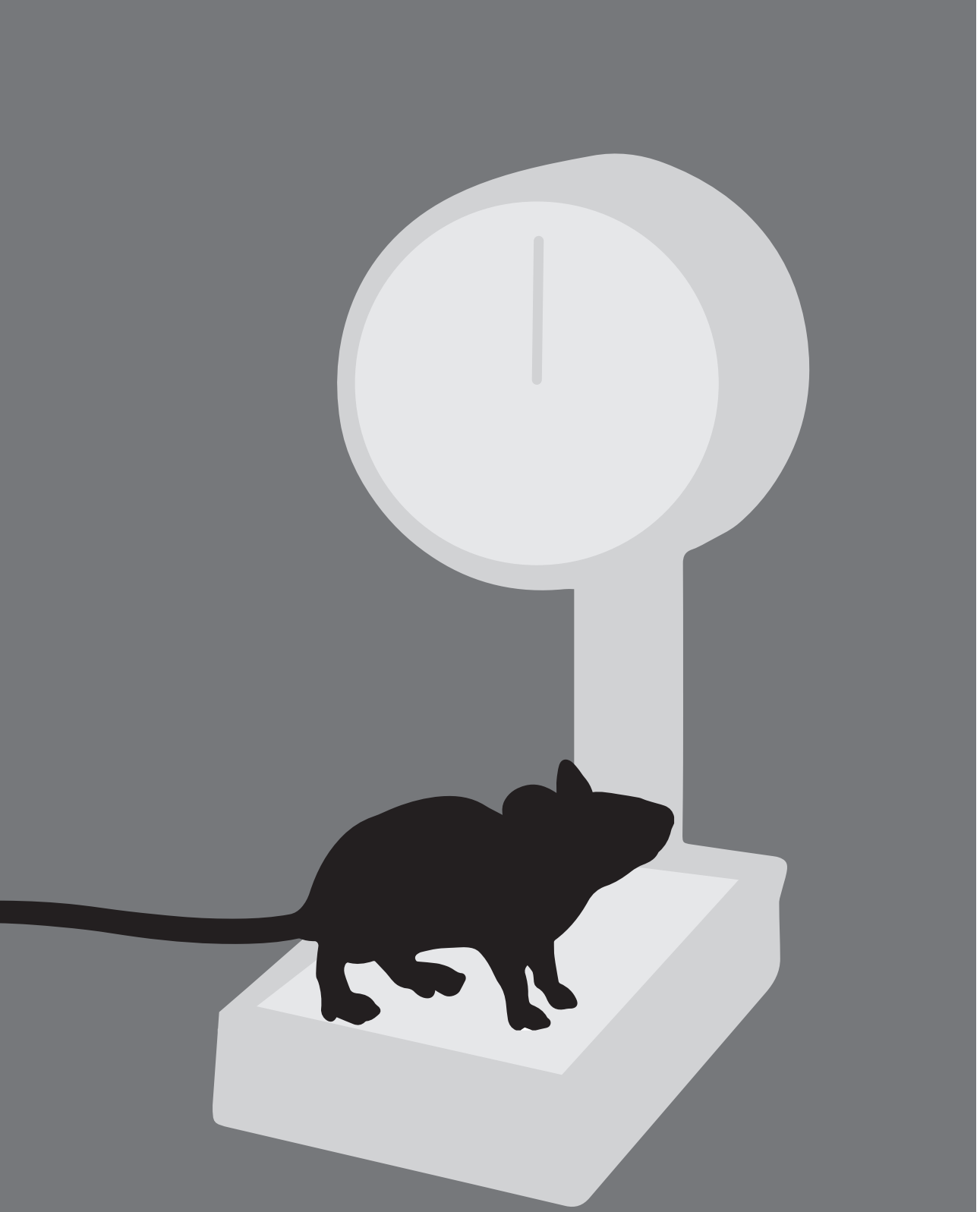


Figure S2. Behavioural effects of higher doses of CNO in control mice injected in AAV-DIO-ChR2. CNO (0.6-5.0mg/kg) had no effect in food restricted mice on (A) active lever presses, (B) inactive lever presses, (C) consumption of chow or (D) locomotor activity. Mean ± SEM. "CNO' corresponds to CNO 1.7mg/kg, otherwise the numbers under CNO indicate the used dose.



Chapter 4

Effects of GABA and leptin receptor-expressing neurons in the lateral hypothalamus on energy homeostasis

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Abstract

The lateral hypothalamus (LH) is known for its role in feeding and it also regulates other aspects of body weight homeostasis. How genetically defined LH neuronal subpopulations mediate LH effects on energy homeostasis remains poorly understood. We compared behavioural effects of chemogenetically activating LH GABA and the more selective population of LH GABA neurons that co-express the leptin receptor (LepR) on multiple aspects of energy homeostasis. To this end, LepR-cre and VGat-cre mice were injected with AAV5-hSyn-DIO-hM3DGq-mCherry in the LH. We assessed behavioural effects of chemogenetic activation of LH GABA or LH LepR neurons on feeding, locomotion, thermogenesis and body weight. Activation of LH GABA neurons increased body temperature and decreased body weight, despite decreased locomotor activity and transiently increased food intake. Also, similar to other studies studying LH GABA neurons, we found that activation of LH GABA neurons induced gnawing on both food and non-food items. Activation of LH LepR neurons decreased body weight and food intake, when presented on the cage floor, but not when presented in the cage top and increased locomotor activity and body temperature. Thus, LH LepR neurons are a subset of LH GABA neurons, which more specifically regulates energy homeostasis to promote a negative energy balance than LH GABA.

Introduction

Overweight and obesity affect millions of people worldwide and predisposes individuals to diseases, such as diabetes, hypertension, and certain forms of cancer (Farag and Gaballa, 2011; Masters et al., 2013). Except for bariatric surgery, there are no truly effective therapies and long-term weight loss as a result of dieting is largely unsuccessful (Wing and Phelan, 2005). Physiological responses to weight loss are increased hunger and decreased energy expenditure (Heilbronn et al., 2006; Kissileff et al., 2012; Soare et al., 2011), which counteract decreased body weight. Understanding how the brain regulates body weight may unravel novel mechanisms on which new therapies or therapeutic targets can be developed that support weight loss.

An interesting brain region with respect to body weight regulation is the lateral hypothalamus (LH). The LH emerged as the feeding center, because lesions of the LH in rats and cats attenuated feeding and even resulted in starvation (Anand and Brobek, 1951; Harrell et al., 1975; Keesey and Powley, 1973). The LH has also been associated with other aspects of body weight regulation, such as physical activity and thermogenesis (Cerri and Morrison, 2005; Inutsuka et al., 2014). Previous studies have explored whether the LH is a potential target for weight loss therapies in humans. LH lesions in humans induced a transient decrease in feeding and body weight and deep brain stimulation in both humans and rats led to long lasting decreases in feeding and body weight (Quaade et al., 1974; Sani et al., 2007; Soto-Montenegro et al., 2014; Whiting et al., 2013). Although the LH has gained attention for its role in body weight regulation, there is still a poor understanding of the role of specific LH neuronal subpopulations in this process.

The LH contains a heterogeneous assembly of cell populations, in which GABAergic neurons predominate (Kimura and Kuriyama, 1975; Vong et al., 2011). LH GABA neurons are known to mediate multiple aspects important for body weight regulation, such as promoting consumption of chow (Barbano et al., 2016; Gigante et al., 2016; Jennings et al., 2015; Navarro et al., 2016; Nieh et al., 2015; Qualls-Creekmore et al., 2017) and palatable solutions (Jennings et al., 2015; Navarro et al., 2016) and altering energy expenditure (Navarro et al., 2016; Qualls-Creekmore et al., 2017). LH GABA neurons differ in expression of neurochemical markers, such as neurotensin, galanin and the leptin-receptor (LepR). How different neuronal subgroups contribute to LH GABA mediated behaviours remains largely unknown. Here, we aimed to determine behavioural effects of LH GABA and LH LepR neurons, which have been shown to co-localize with LH GABA neurons (Leinninger et al., 2009). Leptin is a white adipose tissue derived hormone, which is well-known for decreasing food intake (Halaas et al., 1995) and increasing energy expenditure (Hwa et al., 1997; Ribeiro et al., 2011) via LepR signaling in the brain. Intra-LH leptin injections

resulted in decreased feeding and body weight (Leinninger et al., 2009) and deletion of LepR from specific LH neuronal subpopulations modulated energy expenditure and nutrient preference (Brown et al., 2017; Laque et al., 2015).

We hypothesized that LH LepR neurons are a distinct group of LH GABA neurons and that these neurons more specifically affect body weight homeostasis to promote a negative energy balance compared to all LH GABA neurons. To test this hypothesis, we compared effects of chemogenetically activating LH GABA and LH LepR neurons on multiple aspects of energy homeostasis: consumption of chow and palatable foods, locomotion, body temperature and body weight.

Material and methods

All experiments were approved by the Animal Experimentation Committee of the University Utrecht and were carried out in agreement with Dutch Laws (Herziene Wet op Dierproeven, Art 10.a2, 2014) and European regulations (Guideline 2010/63/EU).

Animals

In house bred, adult male homozygote LepRb-Cre and Vgat-cre transgenic mice (stock #008320, B6.129-Leprtm2(cre)Rck/J and stock #028862, B6J.129S6(Cg)-Slc32a1tm2(cre) Lowl/MwarJ, The Jackson Laboratory, USA) were used for experiments. Animals were housed individually in plastic cages (Type II L, 365x207x140mm, 530cm2, Tecniplast, Italy) and maintained in a temperature (21±2^{o}C) and humidity (60-70%) controlled room on a 12h reversed light/dark cycle (lights on at 9PM) with ad libitum access to chow (3,1kcal/gr, Standard Diet Service, UK) and water.

Surgeries

Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (75mg/kg, Narketan, Vetoquinol BV) and medetomidine (1mg/kg, SedaStart, AST Farma BV). Mice were given eye cream (CAF, CEVA Sante Animale BW) and placed in a stereotactic frame (Kopf Instruments, USA). An incision was made along the midline of the skull and additional analgesia was applied by spraying Xylocaine (lidocaine 100mg/ml, AstraZeneca BV) on the skull. Microinjections of AAV5-hSyn-DIO-hM3DGq-mCherry (0.3ul/side, 3.0*109 genomic copies/ul, UNC Vector Core, USA) or AAV-Ef1a-DIO-hChR2-eYFP (0.3ul/side, 3.0*10^9vp/ul, UNC Vector Core, USA) were performed bilaterally in the lateral hypothalamus (-1.2 AP, +2.0 ML, -5.4 DV, 10° angle) at a rate of 0.1 ul/min per side followed by a 10min waiting period before retracting the needles. Following surgery, mice were given carprofen (5mg/kg, subcutaneous (s.c.), Carporal, AST Farma BV), atipamezole (2.5mg/kg, i.p., SedaStop, AST Farma BV) and saline for rehydration. The following 2 days, mice were given carprofen (5mg/kg, s.c.) and were allowed to recover for at least 1 week. To ensure viral expression, testing commenced 3 weeks after virus injection.

Drugs

Clozapine N-oxide (CNO 99%, AK Scientific, Inc., USA), was dissolved in 0.9% saline and injected i.p. with a dose of 1.0mg/kg. For all experiments, each animal received saline and CNO injections in a latin-square design.

Experimental procedures

Feeding experiments. To simplify the search of food pieces during measurements, all animals were habituated to a second cage (Type II L, 365x207x140mm, 530cm2, Tecniplast, Italy), in which no bedding, but only 3 tissues (to retain comfort and environmental enrichment in the form of nest building) and a water bottle were present, from here on referred to as 'feeding cage'. All feeding tests were done in these cages and were initiated 30min prior to onset of the dark phase. Tests were done with standard rodent chow (3,1kcal/gr, Standard Diet Service, UK), sugar cubes (4kcal/gr, Van Gilse, Netherlands), lard (9.1kcal/gr, Ossewit/Blanc de Boeuf, Belgium) or a combination. Mice were habituated to novel foods overnight at least 3 days prior to testing to prevent neophobia. On test days, directly after saline/CNO injections mice were placed in the feeding cage with a pre-weighed amount of food present and a water bottle at all times. Chow or sugar cubes were present on the cage floor and lard was presented on a suspended spoon (Walking Dinner Amuse Spoon, Yong). Food intake was measured 1, 2, 3, 5 and 7h after injections, after which the mice were placed back in their home cage. If food was wet, food was replaced and wet food was dried before weighing.

20% Sucrose intake assessment. Mice were habituated to a bottle with 20% (w/v) granulated sugar solution (4kcal/gr, Jumbo, Netherlands) prior to testing. Tests were commenced 3h into the dark phase and lasted 2h, during which animals were placed in the feeding cages with only the sucrose bottle present.

Wood block exposure. During feeding experiments, we observed gnawing behaviour after LH GABA stimulation. To test whether this was non-specific (not directed at food) gnawing behaviour, we determined the extent of gnawing on a non-food item, a wood block. Mice were habituated to a wood block prior to testing. Tests started 4h into the dark phase and mice were placed in their feeding cage with a pre-weighed block of wood (~20gr). 2h later animals were removed and the wood blocks were dried and weighed.

Locomotion assessment. All animals were habituated to an empty cage (Type III H, 425x266x185mm, 800cm2, Tecniplast, Italy) for 1h on 2 separate days. Locomotion tests were performed 3-4h into the dark phase. 30min prior to the locomotion test, animals were injected with either saline/CNO and placed in the behavioural testing room. At the start of the task, animals were placed in their own locomotion cage and horizontal movement was tracked using a camera placed above the cages that was coupled to a computer running Ethovision 7 (Noldus, Wageningen, the Netherlands). Locomotion was tracked for 1h.

Temperature measurements. Temperature measurements started 4h into the dark phase. Prior to baseline temperature measurements, food, water and tissues were removed from the home cage. To measure baseline temperature, 15 and 30min after removal mice were placed in an empty smaller cage (Type 1144B, 331x159x132mm, 335cm2, Tecniplast, Italy) and a movie was made using a thermal camera (Flir T420, FLIR Systems, Inc., USA) and the accompanying software program ResearchIR (version 4.40.6.24, 64-bit, FLIR Systems, Inc., USA). Directly after the second baseline movie, mice were injected with either saline or CNO and 30, 60 and 90min after injections, thermal movies were made of each mouse. In between movies, mice were placed back in their home cage. ResearchIR was used to determine body temperature by directing a 3x3 sized pixel towards the center of the eyes of mice standing on their hind legs and looking upwards. The temperature was noted from the area of the eye with the least standard deviation. Temperature is displayed as a change in temperature from baseline (temperature - average of baseline temperatures).

Repeated injections. For repeated injections of saline or CNO, mice were injected twice per day for 3 consecutive days. Injections were given at 8.30AM and at 3.30PM. Directly after injections, the mice, chow in the cage top and water bottles were weighed. On the fourth day no injections were given, but mice, food and water were weighed at 8.30AM. Before repeating this task, animals were allowed to recover for at least 1 week.

Immunohistochemistry

Animals were anesthetized with an overdose of sodium pentobarbital (Euthanimal, Alfasan BV, The Netherlands) and transcardially perfused with 1x phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 1xPBS. Brains were dissected and kept in 4% PFA for 24h at 4°C, after which they were transferred to 30% sucrose in 1xPBS for at least 48h at 4°C. The brains were then sectioned to 40um slices on a cryostat and stored in 1xPBS with 0.01% sodium azide. Slices were washed 3x10min in 1xPBS and then blocked for 1h in 1xPBS containing 10% normal goat serum and 0,25% Triton-X100. Slices were then placed in 1xPBS containing primary antibody (Rabbit anti-dsRed 1:500, #632496, Clontech, Takara Bio USA Inc, USA) and 2% normal goat serum overnight at 4°C. At room temperature, slices were washed 3x10min in 1xPBS and then placed in 1xPBS containing secondary antibody (Goat anti-Rabbit 569, 1:500, #ab175471, Abcam Plc, UK) and 2% normal goat serum for 2h. Finally, slices were washed in 1xPBS and mounted onto slides, dried and covered using Fluorsave (EMD Millipore Corporation, USA) and a coverslip. Images were collected on an epifluorescent microscope (Axio Scope A1, Zeiss, Germany).

Data Analysis and Statistics

Behavioural data were analyzed using Microsoft Excel, Graphpad Prism (version 7.05, Graphpad Software Inc., USA) and SPSS (version 23, SPSS Inc., USA). Animals that ate <1gr chow over 7h after injections were removed from analyses (average chow intake after saline injections: 2.2±0.1gr). One mouse (out of 9 mice total) from the LH LepR group was removed due to the lack of viral expression. Paired t-tests and two-way repeated measures ANOVA tests were used where applicable with Bonferroni adjusted post hoc tests. On cumulative consumption and locomotion data main effects of Time are not reported, because time will by definition increase when analyzing cumulative data. A significance criterion of $p<0.05$, two-tailed, was adopted in all the statistical analyses.

Results

To test how LH GABA and LH LepR neurons affect behaviours important for energy homeostasis, we injected VGAT-cre (n=6) and LepR-cre mice (n=9) with AAV5-hSyn-DIO-hM3Dq-mCherry targeted at the LH region. One mouse from the LH LepR group was removed due to the lack of viral expression. All other LH VGAT (n=6) and LH LepR (n=8) mice had prominent viral expression in the LH (figure 1A and 2A). Due to viral spread, expression of hM3Dq-mCherry extended to a region dorsal to the LH, the zona incerta. As expected, expression in LH VGAT mice was clearly higher than in LH LepR mice, reflecting lower numbers of LepR GABA neurons.

Activation of LH GABA neurons acutely promotes consumption, whereas LH LepR activation decreases feeding

To test for effects of chemogenetically activating LH GABA and LH LepR neurons on feeding, we presented mice with regular chow and palatable foods and measured the change in the weight of food over 7h.

We first measured intake of standard laboratory chow. Chemogenetically activating LH GABA neurons increased the weight change of chow at 1h and 2h, but not at

other time points (fig. 1B, interaction of Time x Injection $F(5,25) = 5.651$, p=0.0013; 1h: p=0.00092, 2h: p=0.00086). Since previous studies reported increased consumption of palatable liquids (Jennings et al., 2015; Navarro et al., 2016), we also presented mice with a 20% sucrose solution injections, which revealed an increase in the weight change of the 20% sucrose solution in 5/6 mice, but this did not reach statistical significance (fig. 1C, $t(5)$=-1.714, p=0.15). Next, we tested intake of non-liquid palatable foods: sugar cubes and lard. LH VGAT mice did not alter the weight change of sugar cubes after CNO injections (fig. 1D). The weight change of lard was decreased at 3, 5 and 7h upon CNO compared to vehicle injections (fig. 1E, interaction effect Time x Injection $F(5,25)$=5.722, p=0.0012, 3h: p=0.0031, 5h: p=0.000017, 7h:

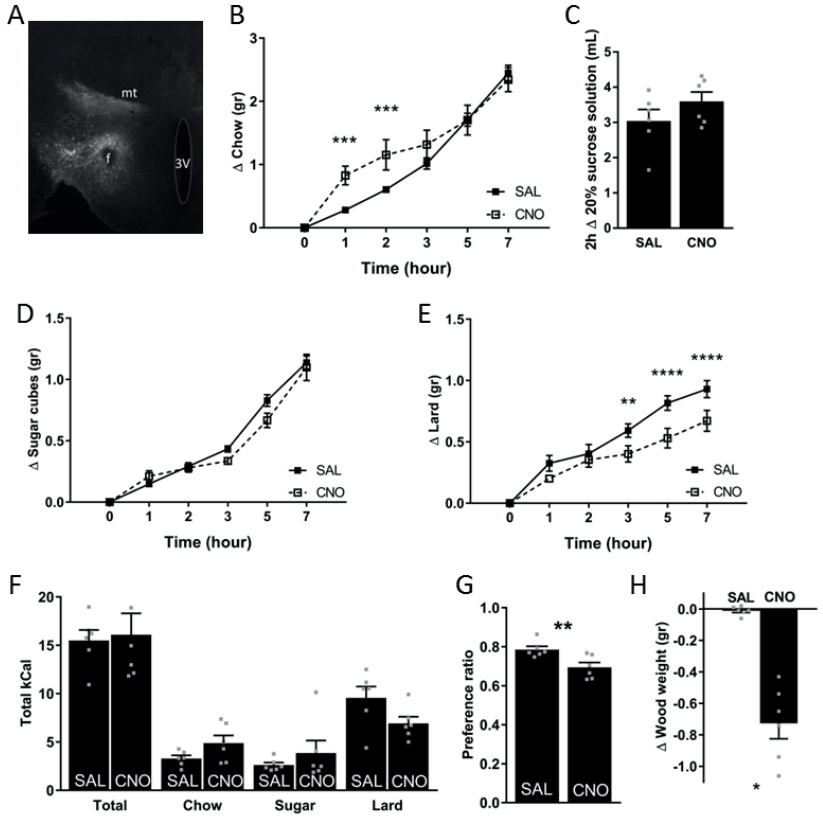

Figure 1. Feeding upon chemogenetic activation of LH GABA neurons. (A) Representative example of a VGAT-cre mouse injected with AAV5-hSyn-DIO-hM3DGq-mCherry targeted at the lateral hypothalamus. Compared to saline injections, CNO (1.0mg/kg) injections in VGAT-cre mice (n=6) (B) altered the feeding pattern of chow intake, (C) had no effect on sucrose solution consumption or (D) sugar cube intake, (E) decreased lard intake, (F) had no effect on total caloric intake or intake of separate foods when given free choice between chow, lard and sugar, (G) decreased preference for lard and sugar over chow and (H) decreased the weight of a wood block. Mean ± SEM. Individual points are plotted as grey squares. * p<0.05; ** p<0.01, *** p<0.001.

p=0.000077). Finally, we determined whether presentation of a combination of chow, sugar cubes and lard differently affected intake of the foods. CNO treated VGAT animals did not consume more calories over 7h ("Total" in fig. 1F). When taking the separate foods into account, paired t-tests revealed increased weight change of chow ($t(5)$=-2.661, p=0.045) and decreased weight change of lard ($t(5)$=3.100, p=0.027), but when corrected for multiple testing these values did not reach statistical significance. To assess whether the preference of animals for palatable foods was altered by CNO, we calculated the animals' preference ratios as caloric intake of sugar cubes+lard/ total caloric intake (fig. 1G). This analysis revealed that the preference for palatable foods was decreased in VGAT LH animals after CNO injections compared to vehicle injections ($t(5)$=5.248, p=0.003).

Throughout the feeding experiments, we anecdotally observed torn tissues, wet cages and chow dust (when chow was available) in CNO, but not saline injected VGAT mice. Specifically, when sugar cubes were presented in a petri dish, mice chewed on the petri dish, which was heard and evidenced by bite marks on the edges of the dish.

As it became apparent that LH VGAT mice had an urge to chew on chow, tissues and water bottle sippers when injected with CNO, we tested whether this was non-specific (non-food directed) gnawing behaviour. Therefore, we presented the animals with a block of wood as was previously done by Navarro et al. (Navarro et al., 2016). Indeed, CNO injected LH VGAT mice shredded the wood and decreased wood weight ($t(5)$=6.651, p=0.001, fig. 1H).

We performed the same experiments in LH LepR mice and measured intake of chow and palatable foods after CNO and vehicle injections. Activating LH LepR neurons decreased chow intake at all time points measured (fig. 2B, interaction Time x Injection $F(5,30)$=7.875, p=0.000078; 1h: p=0.041, 2h: p=0.0018, 3h: p=0.0000036, 5h: p=0.000013, 7h: p=0.000000053). Injecting CNO in LH LepR mice had no effect on intake of 20% sucrose solution, sugar cubes or lard (fig. 2C, D and E). Finally, when LH LepR mice were given free choice of chow, sugar cubes and lard, there was no effect of CNO on total caloric intake or of total caloric intake of the separate foods (fig. 2F). Similarly, the preference of palatable sugar cubes and lard over chow did not differ between CNO and vehicle injections (fig. 2G).

Since LH VGAT mice gnawed on wood blocks, we assessed whether the more specific group of LH GABA-LepR neurons contribute to this behaviour. We did not notice any urge to chew in CNO or vehicle treated LH LepR mice during feeding experiments. Similarly, LH LepR mice did not gnaw on wood after either vehicle or CNO injections (fig. 2H).

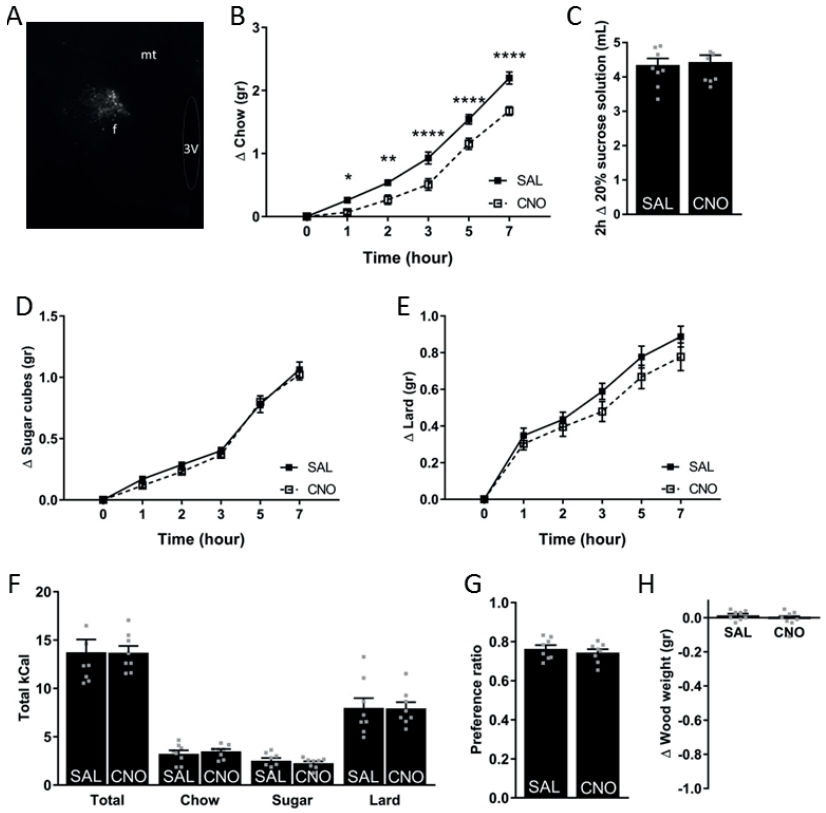

Figure 2. Feeding upon chemogenetic activation of LH LepR neurons. (A) Representative example of a LepR-cre mouse injected with AAV5-hSyn-DIO-hM3DGq-mCherry targeted at the lateral hypothalamus. Compared to saline injections, CNO (1.0mg/kg) injections in LepR-cre mice (n=8) (B) decreased chow intake, but did not affect (C) sucrose solution consumption, (D) sugar cube intake, (E) lard intake, (F) total caloric intake or intake of separate foods when given free choice between chow, lard and sugar, (G) preference for lard and sugar over chow or (H) the weight of a wood block. Mean ± SEM. Individual points are plotted as grey squares. * p<0.05; ** p<0.01, *** p<0.001.

Activating LH VGAT neurons decreases locomotion and increases body temperature, whereas activating LH LepR neurons increases both locomotion and body temperature

To test two aspects of energy expenditure, we assessed both locomotor activity and body temperature upon chemogenetic activation. Mice were habituated to and tested in standard rat cages above which cameras automatically tracked horizontal locomotor activity. As proxy for body temperature we used the temperature of the eye, which we found to be the highest temperature spot detected on images produced by a thermosensitive camera. Moreover, we found eye temperature to

increase the reproducibility of measurements, because temperature within the eye is more stable across different voxels compared to other regions that are used to detect temperature, such as brown adipose tissue.

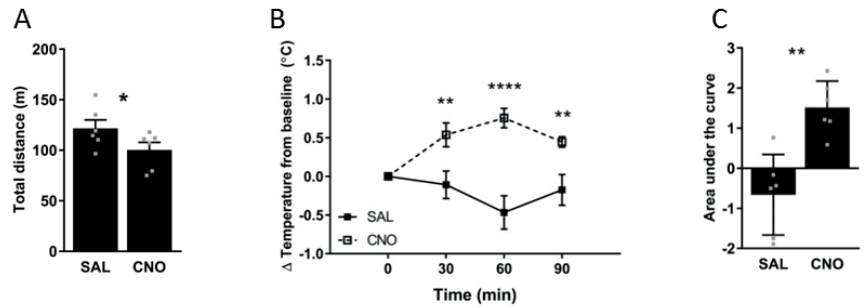

Figure 3. Energy expenditure upon chemogenetic activation of LH GABA neurons. CNO (1.0mg/kg) injections in VGAT-cre mice (n=6) decreased (A) locomotor activity and (B) increased eye temperature compared to saline injections (C) Area under the curve of B. Mean ± SEM. Individual points are plotted as grey squares. * p<0.05, ** p<0.01.

Figure 4. Energy expenditure upon chemogenetic activation of LH LepR neurons. CNO (1.0mg/kg) injections in LepR-cre mice (n=8) increased (A) locomotor activity and (B) increased eye temperature compared to saline injections. (C) Area under the curve of B. Mean ± SEM. Individual points are plotted as grey squares. * p<0.05, ** p<0.01.

LH VGAT mice decreased 1h locomotor activity after CNO injections (fig. 3A, $t(5)=3.031$, p=0.029). Furthermore, CNO injections increased eye temperature at all time points measured (interaction effect of Time x Injection $F(3,15)=9.043$, p=0.0012; 0-30min: p=0.0057, 30-60min: p=0.000009, 60-90min: p=0.0078 (fig. 3B).

Activation of LH LepR neurons increased both aspects of energy expenditure: LH LepR mice increased locomotor activity (fig. 4A, $t(7)=-4.820$, p=0.002) and increased eye temperature after CNO compared to vehicle injections (fig. 4B, main effect of Injection $F(1,7)=7.495$, p=0.029).

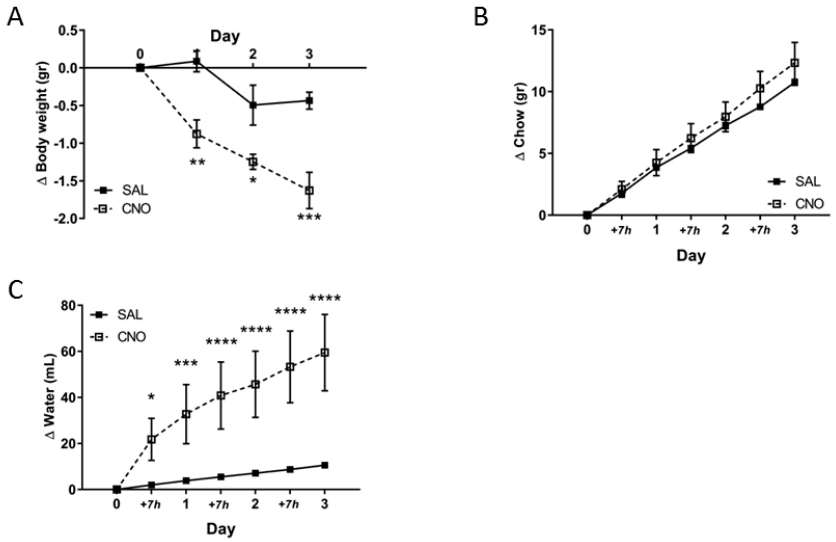

Figure 5. Body weight regulation upon repeated chemogenetic activation of LH GABA neurons. CNO (1.0mg/kg) injections compared to saline injections in VGAT-cre animals (n=6) (A) increased body weight loss, (B) had no effect on food intake and (C) increased water intake during a three day twice daily injection scheme. Mean ± SEM. * p<0.05, ** p<0.01.

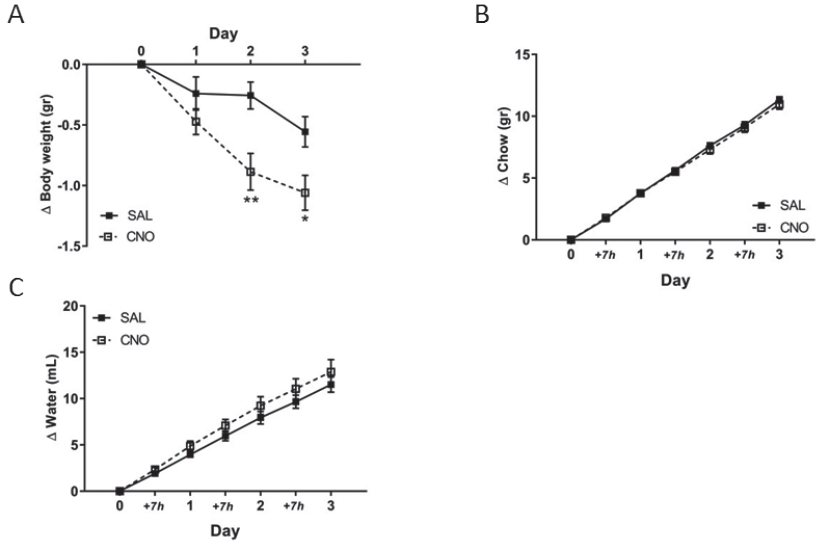

Figure 6. Body weight regulation upon repeated chemogenetic activation of LH LepR neurons. CNO (1.0mg/kg) injections compared to saline injections in LepR-cre animals (n=8) (A) increased body weight loss, (B) had no effect on food intake or (C) water intake during a three day twice daily injection scheme. Mean ± SEM. * p<0.05.

Activating LH VGAT and LH LepR neurons with twice-daily CNO injections for three days enhanced body weight loss

To test whether the behavioural effects on feeding and energy expenditure affected body weight, LH VGAT and LH LepR mice were subjected to two injections of CNO or saline during the dark phase for three consecutive days. Throughout these days we recorded body weight and changes in weight of the water bottle and cage top chow.

Repeated CNO injections in LH VGAT mice resulted in a larger decrease in body weight compared to vehicle injections (fig. 5A, interaction effect of Injection $F(3,15)=5.667$, p=0.0084; day 1: p=0.0019, day 2: p=0.014, day 3: p=0.00025). The weight change of chow was not affected (fig. 5B), but repeated CNO administration increased the weight change of water compared to vehicle (fig. 5A, interaction of Time x Injection $F(6,30)=7.848$, p=0.000039; day 0+7h: p=0.016, day 1: p=0.00025, day 1+7h: p=0.000011, day 2: p=0.0000026, day 2+7h: p=0.00000017, day 3: p=0.000000025).

Similar to LH VGAT mice, repeated CNO injections in LH LepR mice decreased body weight to a greater extent than vehicle injections (fig. 6A, interaction effect of Time x Injection $F(3,21)=3.489$, p=0.034; day 2: p=0.0017, day 3: p=0.0124). In LH LepR mice, repeated CNO injections had no effect on consumption of either chow (fig. 6B) or water (fig. 6C).

To control for non-specific effects of CNO due to reverse-metabolism to its parent compound clozapine (Manvich et al., 2018), we injected mice with a control virus that induces expression of channelrhodopsin, which does not respond to CNO or clozapine. CNO injections in control mice had no effect on any parameter tested (fig. S1, n=4). Therefore, we conclude that behavioural effects observed in Gq-injected mice are the result of enhanced neuronal activation in LH VGAT and LH LepR mice.

Discussion

Activating LH GABA neurons acutely and transiently increased feeding, decreased locomotion and induced gnawing of both food and non-food items. In contrast, activating LH LepR neurons decreased feeding, but only when chow was easily accessible, increased locomotion and did not induce gnawing behaviour. Both LH GABA as well as LH LepR activation increased body temperature and repeated stimulation led to body weight loss. Our results showed that all behavioural effects mediated by LH LepR neurons promoted a negative energy balance, which was not the case for LH GABA neurons. Thus, our data suggest that LH LepR neurons more specifically regulate parameters of energy balance compared to LH GABA neurons.

Recently, a study reported that LH GABA stimulation induced eating by low frequency optogenetic stimulation, but induced gnawing at higher frequencies that did not elicit feeding (Barbano et al., 2016). These results imply that LH GABA neurons modulate feeding and gnawing depending on the extent of stimulation and that we and others (Barbano et al., 2016; Jennings et al., 2015; Navarro et al., 2016; Nieh et al., 2015) who reported gnawing behaviour may not have examined true feeding effects. We observed that solid chow and water bottle sippers robustly induced gnawing behaviour upon LH GABA activation, but porous (sugar cubes) or soft (lard) foods did not. LH GABA stimulation increased the change of the weight of food and water bottles, which suggested that consumption of chow and water was increased. However, we also observed wet cages and chow dust in the cage, which suggests that LH GABA activation resulted in spillage due to gnawing. Therefore, we suspect that previous reports on ingestive behaviours (reporting intake of chow and palatable solution) upon activating LH GABA neurons may be overestimated due to the spillage as a result of gnawing.

We observed decreased consumption of chow when presented on the cage floor after activating LH LepR neurons, which is in line with studies reporting decreased feeding after intra-LH leptin injections (Leinninger et al., 2009). However, the LH LepR stimulation decrease in feeding was only observed when chow was presented on the floor and not during repeated CNO injections when chow was in the cage top. Interestingly, 7h after vehicle injections, mice ate more when chow was more easily accessible on the cage floor (2.0±0.1gr) compared to when chow was in the cage top (1.5±0.1gr). Thus, activation of LH LepR neurons decreased consumption of chow only when food is easily accessible.

To our knowledge we provided the first evidence that stimulating LH VGAT and LH LepR neurons increased body temperature. In LH LepR mice, increased locomotor activity may have contributed to the increase in body temperature. However, LH VGAT mice increased body temperature, but decreased locomotion, indicating an effect of LH GABA activation on thermogenesis, independent of locomotor activity. LH lesions have been shown to decrease body temperature (Clark et al., 1939; Refinetti and Carlisle, 1987, 1986) and disinhibition of neurons in the LH increased thermogenesis (Cerri and Morrison, 2005). Furthermore, the periaqueductal grey (PAG) is involved in thermogenesis (Chen et al., 2002) and both LH LepR and GABAergic neurons are known to substantially project to the PAG (Leinninger et al., 2009; Venner et al., 2016). Our data implicate both LH GABA and the more specific LH GABA-LepR neurons in LH mediated thermogenesis, perhaps via projections to the PAG.

Deep brain stimulation of the LH in humans and rats has been shown to promote weight loss (Quaade et al., 1974; Sani et al., 2007; Soto-Montenegro et al., 2014; Whiting et al., 2013). Our data reveal that repeated stimulation of both LH GABA and LH LepR neurons enhanced body weight loss and suggests that LH induced weight loss by deep brain stimulation is likely mediated by LH GABA neurons. Weight loss in LH LepR mice may have been the consequence of both increased locomotion and thermogenesis, while weight loss in LH VGAT mice seemed to be primarily driven by thermogenesis since locomotion was decreased.

Several limitations to this study exist. First, expression of DREADDGq was not exclusive to the LH. In LH VGAT mice there was copious expression of the DREADDGq in the zona incerta (ZI), which is very similar to expression patterns seen in other LH GABA targeted experiments (Jennings et al., 2015; Navarro et al., 2016; Qualls-Creekmore et al., 2017). ZI expression was also observed in LH LepR animals, but to a far lesser degree. Activation of ZI GABA neurons has been shown to increase body weight and feeding, also of palatable foods and did not affect locomotion (Zhang and van den Pol, 2017). In our study, activation of LH GABA neurons increased feeding, but decreased lard intake, locomotion and body weight. Although it cannot be excluded, this supports that it is unlikely that activation of ZI GABA neurons had major effects on the parameters that we assessed here. Secondly, we chemogenetically activated the majority of LH LepR neurons, but leptin depolarizes 33% and hyperpolarizes 22% of LH LepR neurons (Leinninger et al., 2009). Changes in chow intake by LH LepR were observed after intra-LH leptin injections (Leinninger et al., 2009), but not after LH LepR deletion, LepR deletion in neuronal subpopulations of the LH (Brown et al., 2017; Davis et al., 2011; Laque et al., 2015) or when we chemogenetically activated LH LepR neurons. This suggests that leptin-induced feeding in the LH is regulated by the intricate activation and inhibition of LH LepR neurons and perhaps effects on feeding are masked when increasing the activity of all LH LepR neurons.

To conclude, our results reveal that LH LepR neurons more specifically regulate aspects of energy balance to promote a negative energy balance compared to LH GABA neurons. Furthermore, we provided the first evidence that activation of LH GABA and LH LepR neurons modulated body temperature and when stimulated repeatedly enhanced body weight loss. The latter is especially interesting in light of the obesity epidemic and may aid in the discovery of new treatment strategies to combat obesity.

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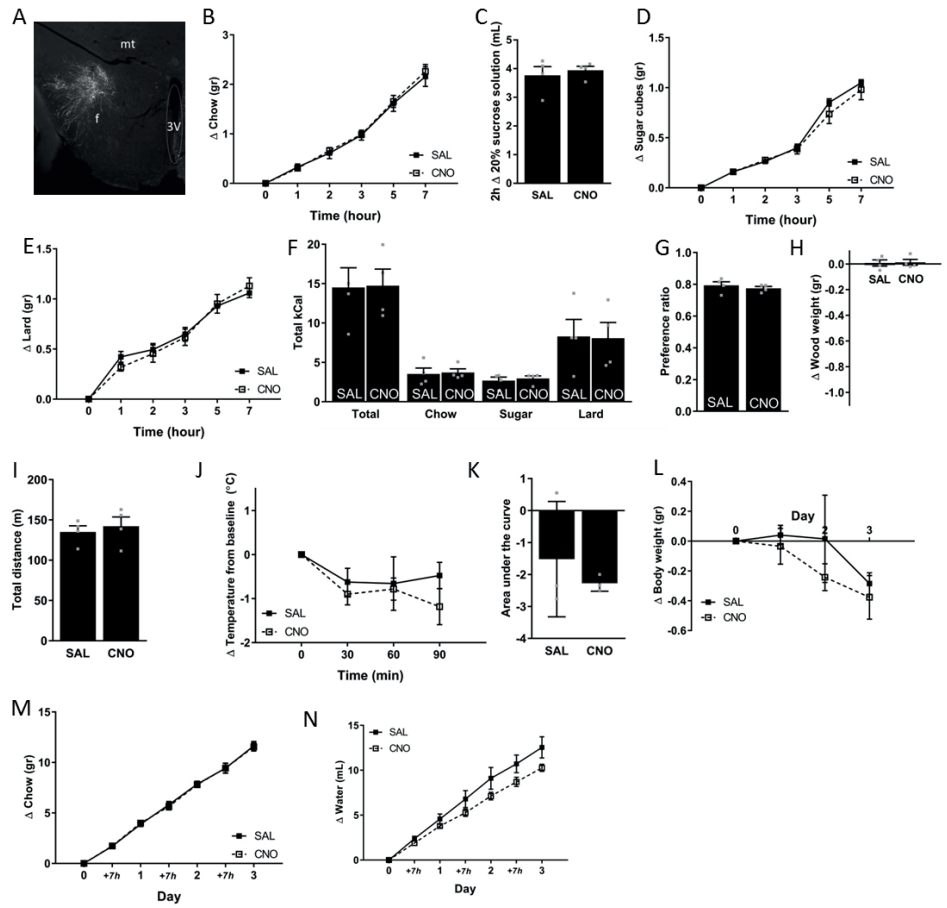
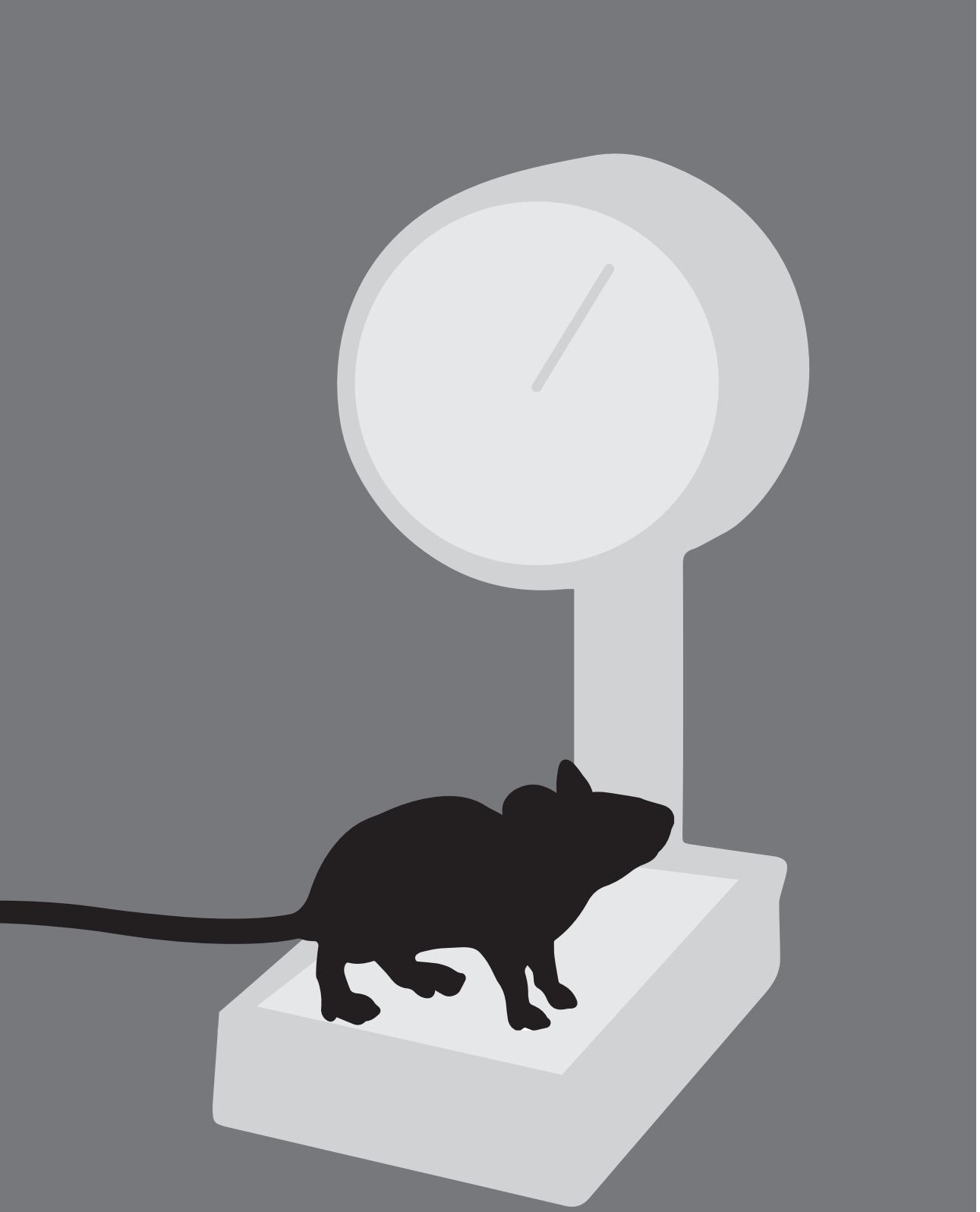


Figure S1. CNO injections in ChR2-injected control mice had no effect on behaviours tested. (A) Example of a LepR-cre mouse injected with ChR2 targeted at the lateral hypothalamus. Compared to saline injections, CNO (1.0mg/kg) injections in control mice (n=4) did not affect (B) chow intake, (C) sucrose solution consumption, (D) sugar cube intake, (E) lard intake, (F) total caloric intake or intake of separate foods when given free choice between chow, lard and sugar (free choice high-fat high-sucrose, fcHFHS), (G) preference for lard and sugar , (H) the weight of a wood block, (I) locomotor activity, (J) eye temperature or (K) area under the curve of eye temperature. Three days of twice daily CNO injections did not affect (L) body weight loss, (M) chow intake or (N) water intake compared to saline injections. Mean ± SEM. Individual points are plotted as grey squares.



Chapter 5

Gnawing induced by LH GABA activation differently affects consumption dependent on experimental settings

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Abstract

Many studies have reported gnawing behaviours upon LH GABA neuronal activation, which may compromise assessment of the role these neurons have on feeding. Stimulating LH GABA neurons induces gnawing directed at both food and non-food items. We here explored how gnawing induced by activation of LH GABA neurons affected caloric consumption with different foods and in different experimental settings. We exposed mice to calorie and non-calorie containing substances with different consistencies, i.e. solid (chow, wood block), porous (sugar cubes), soft (lard) and liquid (water, sugar solution). We find that activation of LH GABA neurons induced gnawing behaviour towards both food and non-food items in normal fed and hungry mice. For calorie containing foods, it seemed that the chewability of a food predicted gnawing upon LH GABA stimulation, since solid foods were gnawed on, whereas porous or soft foods were not.

Introduction

As far back as 1951, it was found that lesions to the lateral hypothalamus (LH) led to the attenuation of feeding and drinking in both rats and cats (Anand and Brobek, 1951; Grossman et al., 1978; Grossman and Grossman, 1982; Stricker et al., 1978). The behaviour went to an extent that when food was presented right before the animal's nose, it would push the food away. Contrarily, stimulation of the LH induced feeding and compulsive gnawing behaviours on food and non-food items, such as chow, wood and metal screws (Delgado and Anand, 1953; Roberts and Carey, 1965; Valenstein et al., 1968).

A large group of neurons within the LH are GABAergic. GABA neurons express many receptors and neuropeptides linked to feeding, such as the leptin-receptor and galanin peptide (Laque et al., 2015, 2013). In line with this, LH GABAergic neurons are involved in feeding behaviour: chemogenetic and optogenetic activation increased consumption (Barbano et al, 2016; Gigante et al, 2016; Jennings et al., 2015; Navarro et al., 2016; Nieh et al., 2015; Wu et al., 2015), while ablation, chemogenetic inhibition and optogenetic inhibition decreased consumption (Jennings et al., 2015; Navarro et al., 2016; Nieh et al., 2016; Wu et al., 2015). Similar to whole LH stimulation, activating LH GABA neurons induced gnawing or mouth movements without food present (Barbano et al., 2016; Jennings et al., 2015; Navarro et al., 2016; Nieh et al., 2015). Navarro et al. (Navarro et al., 2016) showed that gnawing was not per se directed to food as the animals also gnawed on wood blocks. Thus, stimulating LH GABA neurons directly induces gnawing and since gnawing is also directed at non-food items, previously reported effects of LH stimulation on food consumption may be compromised by gnawing that is not per se directed to ingestion of food.

We here explored how gnawing induced by the activation of LH GABA neurons affected caloric consumption of different foods in different settings. Since previous studies reporting LH GABA induced gnawing only reported short term effects (up to 2h), we evaluated behavioural effects up to 7h following chemogenetic stimulation. We exposed mice to calorie and non-calorie containing substances with different consistencies, i.e. solid (chow, wood block), porous (sugar cubes), soft (lard) and liquid (water, sugar solution). During the measurements we determined how different choices in experimental setup affected feeding and gnawing behaviour. We expected that hungry mice would gnaw less on non-food items, such as wood blocks, and that chewing would be redirected towards food. Therefore, we also compared gnawing of food and non-food items in food restricted and ad libitum fed mice.

Materials & Methods

All experiments were approved by the Animal Experimentation Committee of the University Utrecht and were carried out in agreement with Dutch Law (Herziene Wet op Dierproeven, Art 10.a2, 2014) and European regulations (Guideline 2010/63/EU).

Animals

In house bred, adult male homozygote vGAT transgenic mice (Vgat-ires-cre from JAX, C57BL/6J background) were used for experiments. Animals were housed individually in plastic cages (Type II L, 365x207x140mm, 530cm2, Tecniplast, Italy) and maintained in a temperature and humidity controlled room on a reversed day/night (9:00 AM light OFF, 9:00 PM light ON) schedule. Chow (standard rodent chow, Standard Diet Service, UK) and water were always present unless stated elsewise. When food restricted, all chow except one piece (~1.3g/piece) was taken away 4h into the dark phase and 24h prior to testing.

Surgery

At least 30min prior to anesthetization, mice were given carprofen (5mg/kg, subcutaneous (s.c.), Carporal, AST Farma BV). Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (75mg/kg, Narketan, Vetoquinol BV) and medetomidine (1mg/kg, SedaStart, AST Farma BV). Mice were given eye cream (CAF, CEVA Sante Animale BW) and placed in a stereotactic frame (Kopf Instruments, USA). An incision was made in the skin along the midline of the skull and additional analgesia was applied by spraying Xylocaine (lidocaine 100mg/ml, AstraZeneca BV) on the skull. Microinjections of AAV5-hSyn-DIO-hM3DGq-mCherry (excitatory DREADD, 0.3ul/side, 3.0*10^9gc/ul, UNC Vector Core, USA) or AAV-Ef1a-DIO-ChR2-eYFP (used as control virus, 0.3ul/side, 3.0*10^9gc/ul, UNC Vector Core, USA) were performed bilaterally in the lateral hypothalamus (-1.2 AP, +2.0 ML, -5.4 DV, 10° angle) at a rate of 0.1ul/min per side followed by a 10min period before retracting the needles. Following surgery, mice were given atipamezole (2.5mg/kg, i.p., SedaStop, AST Farma BV) and saline for rehydration. The following 2 days, mice were given carprofen (5mg/kg, s.c.) and were allowed to recover for at least 1 week. To ensure viral expression, testing commenced 3 weeks after virus injection.

Drugs

Clozapine N-oxide (1.0mg/kg, CNO 99%, AK Scientific, Inc., USA), was dissolved in saline. For all experiments, each animal received saline and CNO injections i.p. in a latin-square design.

Experimental procedures

For all experiments, to simplify finding pieces of food or wood during measurements, mice were placed in a separate plastic cage (Type II L, 365x207x140mm, 530cm2, Tecniplast, Italy), which was empty except for 3 tissues and to which they were habituated to twice prior to the first experiment, from hereon called "feeding cage".

Feeding experiments. Feeding tests were done in the feeding cage. Tests were done with standard rodent chow (3.31kcal/gr, Standard Diet Service, UK), sugar cubes (4.0kcal/gr, Van Gilse, the Netherlands) and lard (9.1kcal/gr, Ossewit/Blanc de Boeuf, Belgium). Mice were habituated to novel foods overnight at least 3 days prior to testing to prevent neophobia. On test days, directly after saline/CNO injections mice were placed in the feeding cage with a pre-weighed amount of food present and a water bottle at all times. Chow or sugar cubes, in or without petri dishes, were placed on the cage floor and lard was presented on a suspended spoon (Walking Dinner Amuse Spoon, Yong). Food intake was measured 1, 2, 3, 5 and 7h after injections, after which the mice were placed back in their home cage. If food was wet, food was replaced and wet food was dried before weighing. During the second round of sugar cube testing, sugar cubes were replaced at every weighing moment, to avoid that the sugar cubes became wet. Feeding experiments were initiated 30min prior to onset of the dark phase.

Home cage chow and water intake. Mice were injected 30min prior to onset of the dark phase. Cage top chow and water were weighed directly after injections and 7h after injections.

20% sugar intake assessment. 20% (w/v) sugar (4.0kcal/gr, Jumbo, the Netherlands) solution was tested in the home cage, in which cage top chow and the water bottle were removed and replaced by a bottle filled with 20% sugar solution. Habituation and testing commenced 3h into the dark phase and lasted 2h. For habituation, mice were presented the 20% sugar bottle three times for 2h prior to testing. On test days, chow and water bottles were removed ~15min before injections and the pre-weighed 20% sugar bottle was placed in the cage directly after the injections. 2h later the sucrose bottle was weighed again and water and cage top chow were returned.

Wood block test. Wood block tests were performed in the feeding cage. Mice were injected with either saline or CNO 3 to 4h into the dark phase and placed in their feeding cage with no tissues or water bottle, but only a pre-weighed block of wood. 2h later, mice were removed and the wood block was weighed at least 24h later to ensure that it was dry again.

Wood block and chow. The experiment above was repeated with the exception that animals had a choice between wood and chow. This test was also performed after 24h of food restriction.

Immunohistochemistry

Animals were anesthetized with an overdose of sodium pentobarbital (Euthanimal, Alfasan BV, The Netherlands) and transcardially perfused with 1x phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 1xPBS. Brains were dissected and kept in 4%PFA for 24h at 4°C, after which they were transferred to 30% sucrose for at least 48h at 4°C. Using a cryostat, the brains were then sectioned to 40um slices and stored in 1xPBS with 0.01% sodium azide. Slices were washed 3x10min in 1xPBS and then blocked for 1h in 1xPBS containing 10% normal goat serum (NGS) and 0,25% Triton-X100. Slices were then placed in 1xPBS containing primary antibody (Rabbit anti-dsRed 1:500) and 2%NGS overnight at 4°C. At room temperature, slices were washed 3x10min in 1xPBS and placed in 1xPBS containing secondary antibody (Goat anti-Rabbit 569, 1:500) and 2%NGS for 2h. The slices were then washed in 1xPBS, mounted onto slides, dried and covered using Fluorsave (EMD Millipore Corporation, USA) and a coverslip. Images were collected on an epifluorescent microscope (Axio Scope A1, Zeiss, Germany).

Data analysis and statistics

Behavioural data was analyzed and visualized using Microsoft Excel and Graphpad Prism (version 7.05)). Two-way repeated measures ANOVAs and paired t-tests with, where applicable, Bonferroni corrections were used. When the assumption of normality was violated, data was transformed prior to further testing. A significance criterion of $p<0.05$, two-tailed, was adopted in all the statistical analyses.

Results

To test behavioural effects of LH GABA activation on consumption and gnawing of various stimuli, we injected VGat-cre mice with AAV5-hSyn-DIO-hM3DGq-mCherry targeted at the LH. Due to viral spread, Gq-mCherry expression was extensive in the LH, but was also present in neighbouring cre-expressing regions, such as the zona incerta (fig. 1A).

We assessed the effect of LH GABA activation on the intake of different food and non-food items in different settings. First, we determined the effect of LH GABA activation over 7h on chow. When tested with chow on the cage floor, CNO increased the weight change of chow the first hour after injections, after which this was normalized to saline injections (interaction effect of Injection x Time $F(4,20)=7.549$, $p=0.0007$; post hoc 1 hour: $t(5)=5.188$, $p=0.00005$) (fig. 1B). After 7h, only one mouse had increased weight change of chow after CNO injections (saline 2.84gr, CNO 3.66gr). We next tested 7h chow intake in the home cage with chow in the cage top. Although one CNO injected mouse increased the weight change of chow after CNO (from 1.95gr to 5.79gr), at a group level, CNO injections (mean: 1.76±0.07gr) had no effect on the weight change of chow compared to saline (mean: 2.14±0.73gr). Thus, chemogenetic activation of LH GABA neurons increased chow consumption in the first hours following CNO injection.

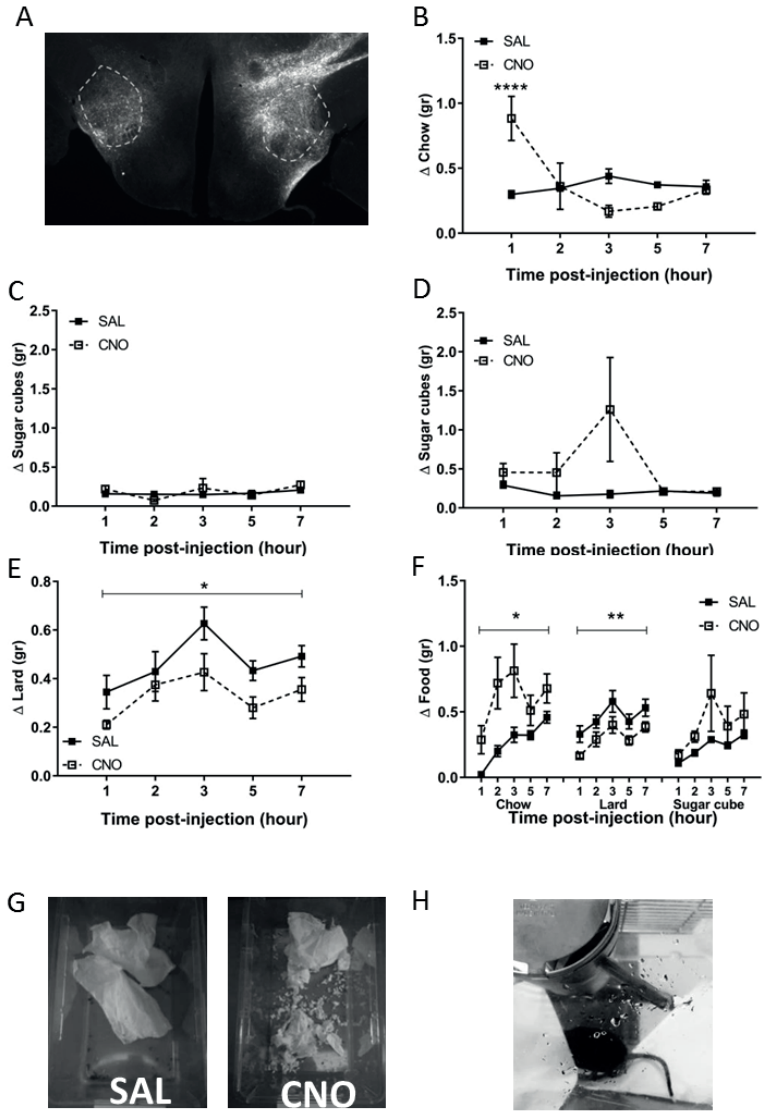

Figure 1. Effects of LH GABA stimulation on feeding. (A) Example of AAV5-DIO-hM3DGq targeted at the LH in a VGAT-cre mouse. The LH is outlined by dashed lines. (B) Chow intake. (C) Sugar cube intake in petri dish. (D) Sugar cube intake without petri dish. (E) Lard intake. (F) Chow, sugar cube and lard intake when presented simultaneously. (H) Representative example of torn tissue in CNO-injected mice (right). (I) Example of wet side of cage of CNO-injected mice. Mean±SEM. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

Secondly, we tested palatable food intake. Both palatable foods (lard and sugar cube) had a less solid structure compared to chow. When we presented the mice with sugar cubes in a plastic petri dish, there was no effect of LH GABA activation on sugar cube weight change (fig. 1C). We presumed this lack of effect was due to gnawing on both the petri dish and water bottle sippers, which was heard and observed (CNO injected mice had wet cages) throughout the test. We then repeated this test, but placed the sugar cubes on the floor of the cage. In this setting, sugar cube weight change was similarly not affected by CNO (main effect of Injection $F(1,5)=4.548$, p=0.086) (fig. 1D). It seemed that sugar cube weight change increased in the third hour post-CNO injection. However, we suspect this increase was the consequence of gnawing on the water bottle sippers, which resulted in wet cages and in slightly dissolved sugar cubes, making the measurements of sugar cube weight change less reliable. In 3/6 CNO injected mice, we found wet cages and wet sugar cubes after the third hour, whereas in the previous hours only 2/6 mice had wet cages and sugar cubes (fig. 1H depicts a wet cage during testing of sugar cubes). When presented with lard on a suspended spoon, over the period of 7h LH GABA activation decreased lard weight change (main effect of Injection $F(1,5)=7.639$, p=0.039) (fig. 1E). Here again, CNO injected mice had wet cages due to gnawing on water bottle sippers, but this did not affect lard measurements since lard was suspended.

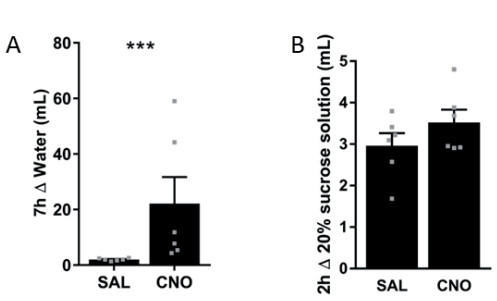

Figure 2. Consumption of liquids upon LH GABA stimulation.
(A) Home cage water intake. (B) Home cage 20% (w/c) sucrose solution intake. Individuals points are plotted as grey squares. Mean±SEM.

Thirdly, we tested the simultaneous presentation of chow, lard and sugar cubes. After 7h, CNO injected mice showed increased chow intake (main effect of Injection $F(1,5)=10.00$, p=0.025), but decreased lard intake (main effect of Injection $F(1,5)=22.01$, p=0.0054) and did not alter sugar cube intake (fig. 1F).

Since many studies tested intake of solutions, we tested intake of both a calorie (20% sugar solution) and a non-calorie (water) containing liquid. The weight change of a water bottle was tested in the home cage, which over 7h was increased in all CNO injected animals ($t(6)=4.891$, p=0.0027 (fig. 2A). Similar to water, the weight change of 20% sugar solution was increased in 5/6 CNO injected animals, but this did not reach statistical

significance $t(5)=1.705$, p=0.149 (fig. 2B). During testing, cage sides and saw dust directly under the liquid bottle became wet as a result of LH GABA induced gnawing on the bottle sippers, but not in the vehicle condition. However, we were unable to directly quantify the amount of water/sucrose solution that was spilled and not consumed. Therefore, the weight change of water/sucrose solution in vehicle treated mice reflected actual consumption, but in CNO treated mice this only partly reflects consumption, since cages became wet due to spillage.

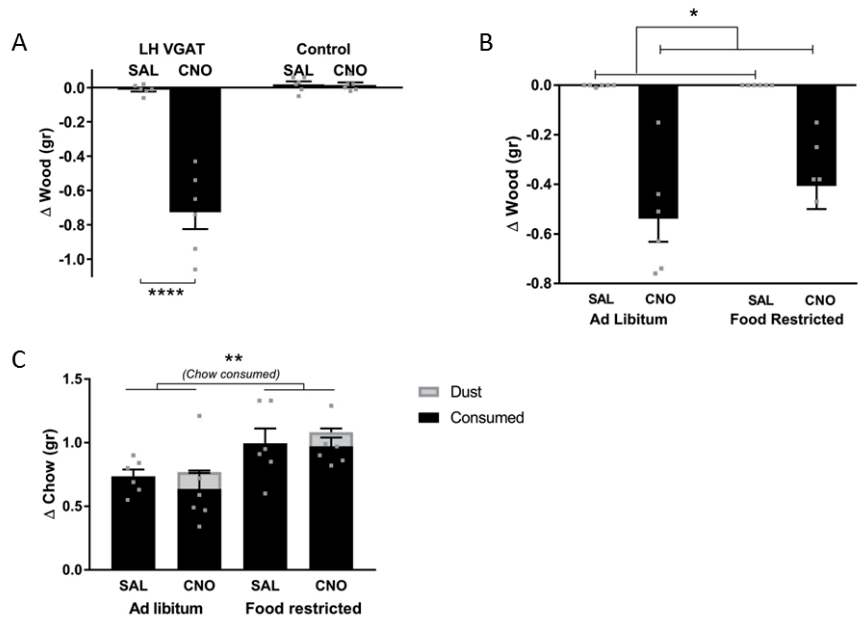

Figure 3. Gnawing behaviour upon LH GABA stimulation. (A) Gnawing on a wood block. (B,C) Simultaneous presentation of a wood block and chow in ad libitum and food restricted mice: (B) wood weight change and (C) chow intake. Individual points are plotted as grey squares (in C, dots represent chow consumed). Mean±SEM. * p<0.05, ** p<0.01.

Throughout testing, we observed mice gnawing on objects such as plastic petri dishes, metal wired cage tops, water bottle sippers and tissues (example of torn tissues and wet cage shown in fig. 1G and 1H, respectively). To test whether this was non-specific (non-food directed) gnawing behaviour, we presented the animals with a block of wood as previously done by Navarro et al. (Navarro et al., 2016). Indeed, LH vGAT animals shredded the wood and decreased the weight of the wood block after CNO injections, which was not seen after saline injections or CNO injections in control mice (interaction effect LH VGAT vs Control and Injections $F(1,10)=42.96$, p=0.000064; LH VGAT Sal vs CNO p=0.000058) (fig. 3A).

Since we expected mice to direct their gnawing behaviour towards food when available, we presented mice with a wood block and chow simultaneously. We further expected animals to increase their willingness to chew on food compared to wood when food restricted. Therefore, we tested simultaneous presentation of wood block and chow in both 24h food restricted and ad libitum fed mice. Wood weight was decreased upon CNO injections compared to saline injections, but this was not affected by hunger (main effect of Injection $F(1,5)=37.126$, p=0.0017) (fig. 3B). Since we observed more chow dust in CNO-treated mice in previous experiments, we decided to more accurately separate chow intake from spilling by weighing pieces of chow and chow dust separately, making sure to take all pieces of wood and faeces out of the chow dust. The change in the weight of chow (ΔChow= gr chow given at beginning of task – gr remaining pieces of chow after task) and the weight of the chow dust was used to calculate the amount of chow actually ingested (chow consumed: ΔChow-chow dust). This revealed that the chow consumed, but not chow dust formed, was increased in food restricted mice and that this was not affected by CNO injections (interaction effect of Ad libitum vs Food restriction x Chow consumed vs Chow dust $F(1,5)=19.973$, p=0.007; post hoc Chow consumed: Ad libitum vs Food restriction $t(11)=-4.977$, p=0.0008; Chow dust: Ad libitum vs Food restriction $t(11)=0.913$, p=0.762). Finally, we directly compared the decrease in wood weight between the presentation of only a wood block (-0.72±0.10gr, fig. 3A) or a wood block together with chow (-0.54±0.09gr, fig. 3B). This revealed that the simultaneous presentation of chow did not reduce the extent of gnawing on a wood block ($t(5)=-1.260$, p=0.263). Thus, mice did not decrease gnawing on wood when food was present or when food restricted, nor did mice decrease the amount of gnawing on chow (i.e. forming of chow dust) when food restricted.

Discussion

Activation of LH GABA neurons induced acute and transient consumption of chow, but also induced gnawing on a wood block, tissue and even water bottle sippers. Gnawing on wood blocks was induced to a similar extent in food restricted and ad libitum fed mice and did not decrease with the simultaneous presentation of food, i.e. chow. Thus, LH GABA activation induced gnawing is not specifically targeted to food, which is consistent with findings by Navarro et al. (Navarro et al., 2016).

Like Navarro et al (Navarro et al., 2016), our data showed that LH GABA activation induced gnawing on stimuli with calories (chow) and those without (wood, tissue and water bottle). Our data further revealed that gnawing of calorie-containing foods (chow, sugar cubes, lard) was dependent on the consistency or the chewability of the foods when presented in a standard laboratory setting (i.e. wired cage tops and water bottles present). Consumption and gnawing was seen for solid foods (chow), whereas intake of porous (sugar cubes) or soft (lard) foods was unaffected or even decreased. When given the choice between chow, sugar cubes and lard, mice consumed more chow at the expense of lard, further supporting that chewability rather than palatability determined consumption after LH VGAT neuronal stimulation. However, Nieh et al (Nieh et al., 2015) used moist, and thus softer, chow pellets and showed increased time spent feeding upon optogenetic activation of LH GABA neurons. Perhaps increased intake of soft chow was due to the absence of more appealing stimuli to gnaw on in the arena that those animals were tested in. Nonetheless, our data show that LH GABA-induced gnawing is dependent on the experimental setting and available food and non-food stimuli. This finding has consequences for the interpretation of behavioural data after LH GABA stimulation. For instance, since large amounts of water (60mL) were released from water bottles upon LH GABA stimulation, without actual consumption, any experiments testing liquids in bottles may overestimate consumption.

Several limitations to this study exist. First, although viral expression was extensively localized in the LH, expression extended into the zona incerta (ZI). Stimulation of ZI GABA neurons has been reported to increase feeding, also of sweet and high-fat foods (Zhang and van den Pol, 2017). Since in our experiments we observed transiently increased intake of chow, but not of palatable sweet (sugar cubes) and high-fat (lard) foods, we suspect that activation of ZI GABA had no major effects on behavioural tasks, although we cannot entirely exclude an involvement of the ZI. Second, the group size used was low. However, the most significant findings of increased weight change of wood, transiently increased weight change of chow when presented on the cage floor, or decreased lard intake, were observed in all mice. Furthermore, all or 5/6 mice increased the weight change of water or sugar solution, respectively, but this did not reach statistical significance. Thus, even though we used a low number of mice, the behavioural output was consistent between animals.

In summary, activation of LH GABA neurons induced gnawing behaviour towards both food and non-food items in normal fed and hungry mice. For calorie containing foods, it seemed that the chewability of a food predicted consumption when presented with other stimuli that can induce gnawing, such as water bottle sippers. Furthermore, we show that gnawing on wood was not reduced by the simultaneous presentation of food or by food restriction. Therefore, studies activating LH GABA neurons must be interpreted with caution as effects may be overestimated or misinterpreted due to gnawing behaviour.

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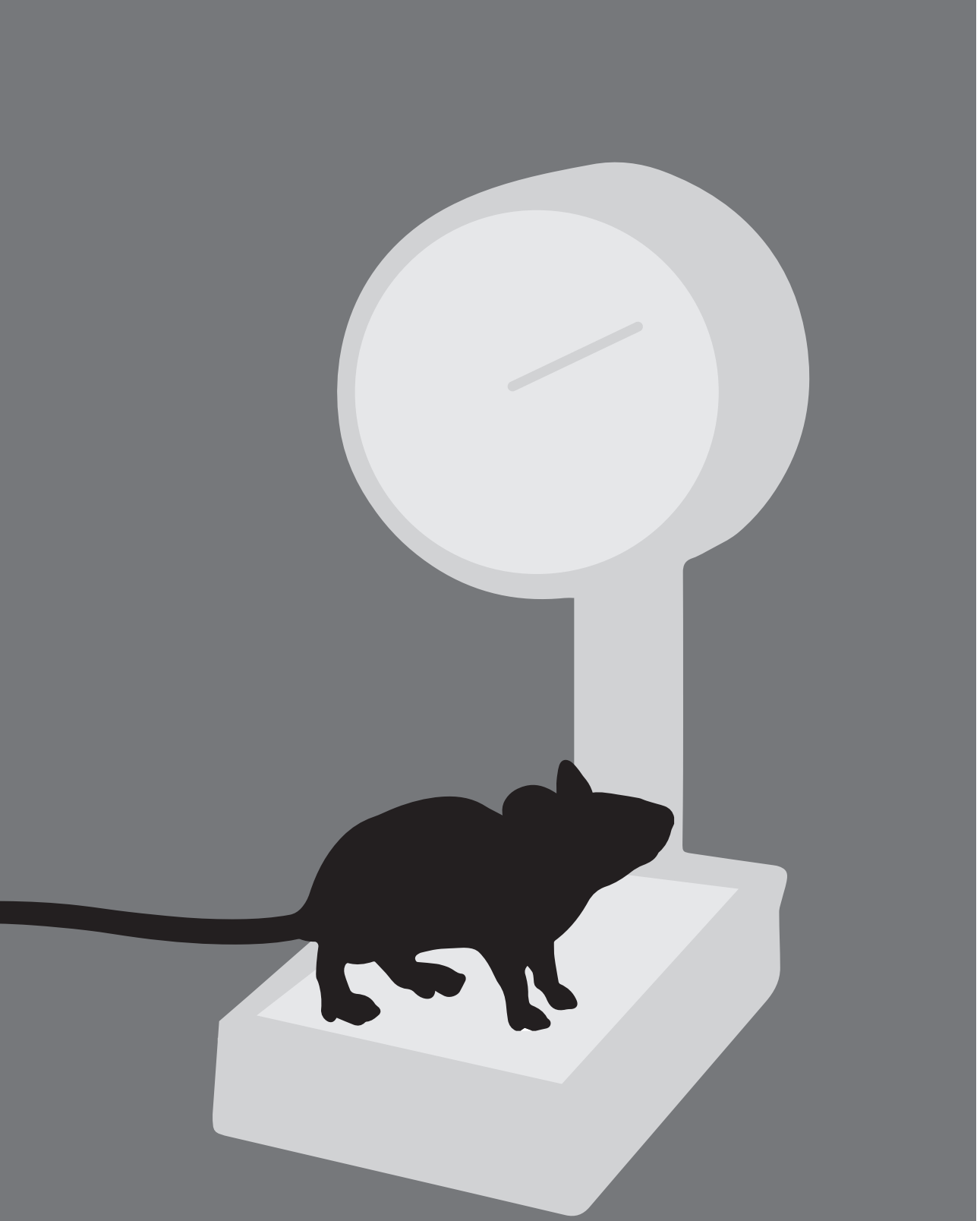

Chapter 6

Differential effects of short- and long-term stimulation of dorsomedial hypothalamic leptin receptor-expressing neuron activation on energy balance

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Abstract

Leptin and leptin receptor-expressing (LepR) neurons play an important role in energy homeostasis. The dorsomedial hypothalamus (DMH) contains a population of LepR neurons that modulate energy homeostasis. Previous studies have shown that intra-DMH leptin injections decreased chow intake and that decreased leptin signalling in the DMH is associated with enhanced weight gain on an obesogenic diet. However, whether DMH LepR neurons play a role in the feeding response to palatable foods remains unknown. Furthermore, repeated chemogenetic stimulation of DMH LepR in normal fed mice has been shown to induce body weight loss as a result of increased energy expenditure by both increased thermogenesis and locomotor activity. Whether this effect is also present in mice that are previously exposed to an obesogenic free-choice high-fat high-sucrose (fcHFHS) diet remains to be determined. We used chemogenetics to selectively activate DMH LepR neurons and determined the role of DMH LepR neurons in different aspects of body weight regulation: feeding, locomotion and thermogenesis. Neuronal activation of DMH LepR neurons increased locomotor activity and thermogenesis, but did not affect food intake. As a result, repeated DMH LepR stimulation decreased body weight in normal fed mice. However, in mice previously exposed to a fcHFHS diet, repeated DMH LepR stimulation did not enhance body weight loss despite increased thermogenesis. Our data show that DMH LepR neurons regulate energy expenditure and body weight, but activation of DMH LepR neurons is not sufficient to enhance body weight loss in mice previously exposed to an obesogenic diet.

Introduction

Body weight homeostasis is the result of balancing energy intake and energy expenditure. When becoming obese, this balance is lost as a result of the intake of excess calories. While many people try to lose weight, on the long term this is effective in only ~20% of individuals (Wing and Phelan, 2005). In humans, weight regain often follows weight loss as physiological counter regulatory mechanisms defend higher weight, for instance by increasing the salience of food cues (Ely et al., 2014; Kissileff et al., 2012; Rosenbaum et al., 2008) and by decreasing energy expenditure (Heilbronn et al., 2006; Soare et al., 2011). With an increasing incidence of obesity and no truly effective treatments, there is a need for a deeper understanding of the mechanisms underlying energy homeostasis.

Leptin is a hormone released by and in proportion to the amount of white adipocyte tissue (Frederich et al., 1995; Masuzaki et al., 1995) and is thereby a signal of the body's energy reserves. Leptin modulates many aspects of energy homeostasis as it is well-known for reducing food intake (Halaas et al., 1995) and it also mediates changes in locomotor activity (Ribeiro et al., 2011) and thermogenesis (Dodd et al., 2014; Enriori et al., 2011; Rezai-Zadeh et al., 2014). When during dieting fat mass is lost, circulating leptin levels decrease in both humans and rodents (Ahima et al., 1996; Maffei et al., 1995; Weigle et al., 1997; Wolfe et al., 2004). As part of the counter regulatory processes during dieting, decreased leptin is associated with reduced energy expenditure, for instance reduced thermogenesis (Fischer et al., 2016), and increased appetite during dieting (Hinkle et al., 2013; Kissileff et al., 2012).

Since dieting decreases thermogenesis, here we focused on a region where leptin receptors (LepR) are expressed and which is known for its role in thermogenesis: the dorsomedial hypothalamus (DMH). DMH LepR neurons have a role in body weight regulation. Leptin injections into the DMH decrease feeding and increase thermogenesis (Enriori et al., 2011) and chemogenetic activation of DMH LepR neurons decreases body weight as a result of increased thermogenesis and locomotion (Rezai-Zadeh et al., 2014). Furthermore, decreased leptin sensitivity in the DMH of normal weight rats predicts enhanced weight gain on a high caloric diet (de Git et al., 2018) and diet-induced obesity in rodents decreases leptin responsiveness in the DMH (de Git et al., 2018; Levin et al., 2004, 2003). Thus, DMH LepR signalling modulates energy homeostasis and decreased leptin signalling in the DMH is associated with obesity.

So far, DMH LepR effects on feeding in normal weight animals have been studied with regular chow only. Whether LepR neurons of the DMH play a role in the feeding response to palatable foods in obesogenic diets remains unknown. Furthermore, since DMH LepR

neurons decrease body weight by increasing energy expenditure, it is important to determine whether activating these neurons during dieting accelerates weight loss. We hypothesize that, when obese, activation of DMH LepR neurons will accelerate weight loss during dieting. If this is true, then these neurons may be a new therapeutic target to assist weight loss.

In the present study, the role of DMH LepR neurons in body weight homeostasis was analyzed using a chemogenetic approach. We determined behavioural effects of DMH LepR activation on the intake of both regular chow and palatable foods (sugar cubes and lard). Furthermore, we assessed energy expenditure by recording locomotor activity and body temperature. Finally, we analyzed body weight decrease upon repeated DMH LepR stimulation in both normal fed mice and those removed from an obesogenic diet, with which we induce a state of negative energy balance similar to dieting.

Materials & Methods

All experiments were approved by the Animal Experimentation Committee of the University Utrecht and were carried out in agreement with Dutch Laws (Herziene Wet op Dierproeven, Art 10.a2, 2014) and European regulations (Guideline 2010/63/EU).

Animals

In house bred, adult male homozygote LepRb-Cre transgenic mice (ObRb-Cre and Vgat-ires-cre from JAX, C57Bl/6J background) were used for experiments. Animals were housed individually in standard mouse cages (Type II L, 365x207x140mm, 530cm2, Tecniplast, Italy) and maintained in a temperature (21°C±2) and humidity (60-70%) controlled room on a reversed day/night (9:00 AM lights OFF, 9:00 PM lights ON) schedule. Ad libitum chow and water were always present unless stated elsewise.

Surgeries

At least 30min prior to anesthetization, mice were given carprofen (5mg/kg, subcutaneous (s.c.), Carporal, AST Farma BV). Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (75mg/kg, Narketan, Vetoquinol BV) and medetomidine (1mg/kg, SedaStart, AST Farma BV). Mice were given eye cream (CAF, CEVA Sante Animale BW) and placed in a stereotactic frame (Kopf Instruments, USA). An incision was made along the midline of the skull and additional analgesia was applied by spraying Xylocaine (lidocaine 100mg/ml, AstraZeneca BV) on the skull. Microinjections of AAV5-hSyn-DIO-hM3DGq-mCherry (0.3ul/side, 3.0*10^9gc/ul, UNC Vector Core, USA) or control virus, AAV-Ef1a-DIO-hChR2-eYFP (0.3ul/side, 3.0*10^9gc/ul, UNC Vector Core, USA) were performed bilaterally in the dorsomedial hypothalamus (-1.7 AP, +1.2 ML, -5.2 DV, 10° angle) at a rate of 0.1ul/min per side followed by a 10min waiting period before retracting the needles. Following surgery, mice were given atipamezole (2.5mg/kg, i.p., SedaStop, AST Farma BV) and saline for rehydration. The following 2 days, mice were given carprofen (5mg/kg, s.c.) and were allowed to recover for at least 1 week. To ensure viral expression, testing commenced 3 weeks after virus injection.

Drugs and food

Clozapine N-oxide (CNO 99%, AK Scientific, Inc., USA), was dissolved in 0.9% saline and injected i.p. at a dose of 1.0mg/kg. For all experiments, each animal received saline and CNO injections in a latin-square design with at least 1 day in between 2 consecutive injections except during repeated injection protocols.

Regular rodent chow (3,1kcal/gr, Standard Diet Service, United Kingdom), sugar cubes (4kcal/gr, Van Gilse, the Netherlands) and lard (9.1kcal/gr, Ossewit/Blanc de Boeuf, Belgium) were used in feeding studies. Mice were habituated to novel foods overnight at least 3 days prior to testing to prevent neophobia.

Experimental procedures

Feeding. To simplify the search of food pieces during measurements, all animals were habituated to a second standard mouse cage (Type II L, 365x207x140mm, 530cm2, Tecniplast, Italy), in which no bedding, but only 3 tissues (to retain comfort and environmental enrichment in the form of nest building) and a water bottle in the cage top were present at all times. All feeding tests were done in these cages, referred to as 'feeding cages' from hereon. When testing intake of chow, sugar cubes, lard or a combination of the three, mice were placed in their feeding cage directly after saline/CNO injections. Injections were done 30min prior to the onset of the dark phase. A pre-weighed amount of food was placed on the cage floor (for chow and sugar cube experiments) and/or on a suspended spoon (for lard experiments, Walking Dinner Amuse Spoon, Yong). Food intake was measured 1, 2, 3, 5 and 7h after injections, after which the mice were placed back in their home cage.

Locomotion. Locomotion was tested in an empty cage (Type III H, 425x266x185mm, 800cm2, Tecniplast, Italy) to which mice were habituated 2x1h prior to testing on separate days. The cages were surrounded by white cardboard to prevent interaction between mice. On test days, 30min prior to the locomotion test, mice were injected with either saline/CNO and placed in the behavioural testing room. At the start of the task, mice were placed in their own locomotion cage and horizontal movement was tracked using a camera placed above the cages that was coupled to a computer running Ethovision 7 (Noldus, Wageningen). Locomotion was tested 4-5h into the dark phase and tests lasted 1h.

Temperature. Temperature measurements started 4h into the dark phase. As proxy for body temperature we used the temperature of the eye, which we found to be the highest temperature spot detected on images produced by a thermosensitive camera. Moreover, we found eye temperature to increase the reproducibility of measurements, because temperature within the eye is more stable across different voxels compared to other regions that are used to detect temperature, such as brown adipose tissue. Prior to temperature measurements, food, water and tissues were removed from the home cage. To measure baseline temperature, 30min after removal a movie was made of mice in their homecage using a thermosensitive camera (FLIR T420, FLIR Systems, Inc., USA) and the accompanying software ResearchIR (version 4.40.6.24, 64-bit, FLIR Systems, Inc., USA). Directly after baseline temperature was recorded, mice were injected with saline or CNO and 30, 60 and 90min after injections additional thermal movies were made of each mouse. ResearchIR was used to determine body temperature by directing a 3x3 sized pixel towards the center of the eyes of mice standing on their hind legs and looking upwards. The temperature was noted from the area of the eye with the least standard deviation. For analysis, two movie shots were used and averaged. Temperature is displayed as a change in temperature from baseline (temperature - baseline temperature).

Repeated injections. For repeated injection protocols, mice were injected twice per day for three consecutive days. Injections were given at 8.30AM and at 3.30PM. Directly after injections, the mice, chow in cage top and water bottles were weighed to measure body weight, food intake and water intake. On the fourth day, mice, food and water were weighed at 8.30AM. Mice were allowed to recover for at least one week after repeated injections protocols.

HFHS: temperature, repeated injections. Previously, we found that a warm environment enhanced body weight gain in mice on a fcHFHS diet. So, to accelerate the impact of obesogenic diet on weight gain, mice were acclimated to a warm (28°C±2) room for 1 week and a cardboard shelter in the cage to decrease body heat lost. Then, animals were given free access to a free choice high-fat high-sucrose (fcHFHS) diet, which aside from regular chow and water, consisted of 10% (w/v) granulated sugar (4kcal/gr, Jumbo, Netherlands) solution and lard. After 2 weeks on the diet, room temperature was switched back to 21°C±2 and animals were kept on the fcHFHS for another week. Subsequently, lard and sugar were removed for the remainder of the experiment. 3 days after removal of fcHFHS diet, temperature measurements were performed as described above. The next day, the repeated injection protocol was performed as described above.

Immunohistochemistry

Animals were anesthetized with an overdose of sodium pentobarbital (Euthanimal, Alfasan BV, The Netherlands) and transcardially perfused with 1x phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS (PFA). Directly at the start of saline perfusion, subcutaneous and epididymal fat depots were removed and weighed. Brains were dissected and placed in PFA for 24h at 4°C, after which they were transferred to 30% sucrose for at least 48h at 4°C. Using a cryostat, the brains were then sectioned to 40um slices and stored in PBS with 0.01% sodium azide. For immunohistochemistry, slices were washed 3x10min in 1xPBS and then blocked for 1h in 1xPBS containing 10% normal goat serum (NGS) and 0,25% Triton-X100. Slices were then placed in 1xPBS containing primary antibody (Rabbit anti-dsRed 1:500, #632496, Clontech, Takara Bio USA Inc, USA) and 2% NGS overnight at 4°C. At room temperature, they were washed 3x10min in 1xPBS and placed in 1xPBS containing secondary antibody (Goat anti-Rabbit 569, 1:500, #ab175471, Abcam plc, UK) and 2% NGS for 2h. The slices were then washed in 1xPBS and mounted onto slides. Slides were dried and covered using Fluorsave (EMD Millipore Corporation, USA) and a coverslip. Images were collected on an epifluorescent microscope (Axio Scope A1, Zeiss, Germany).

Data Analysis and Statistics

Behavioural data was analyzed using Microsoft Excel and Graphpad Prism (version 7.04, Graphpad Software Inc., USA). Outliers or animals that ate <1gr chow over 7h (average: 1.9gr ±0.1, n=16) after injections were removed from analyses. Independent sample t-tests and two-way repeated-measures ANOVAs were used where applicable with post hoc Bonferroni adjusted tests. A significance criterion of $p<0.05$, two-tailed, was adopted in all statistical analyses.

Results

To assess behavioural effects of activating DMH LepR neurons, we injected 10 LepR-cre mice with AAV5-hSyn-DIO-hM3DGq-mCherry targeted at the DMH area and 6 LepR-cre mice with AAV-Ef1a-DIO-hChR2-eYFP which served as control mice. All mice showed bilateral hM3DGq expression in the DMH and were therefore included in further analyses (for example, figure 1A).

DMH LepR neuron activation decreased chow intake, but not intake of palatable foods

To test whether chemogenetically activating DMH LepR neurons affects intake of chow, sugar and fat, we injected mice with saline or CNO and measured intake of chow, sugar cubes and lard or a combination over 7h.

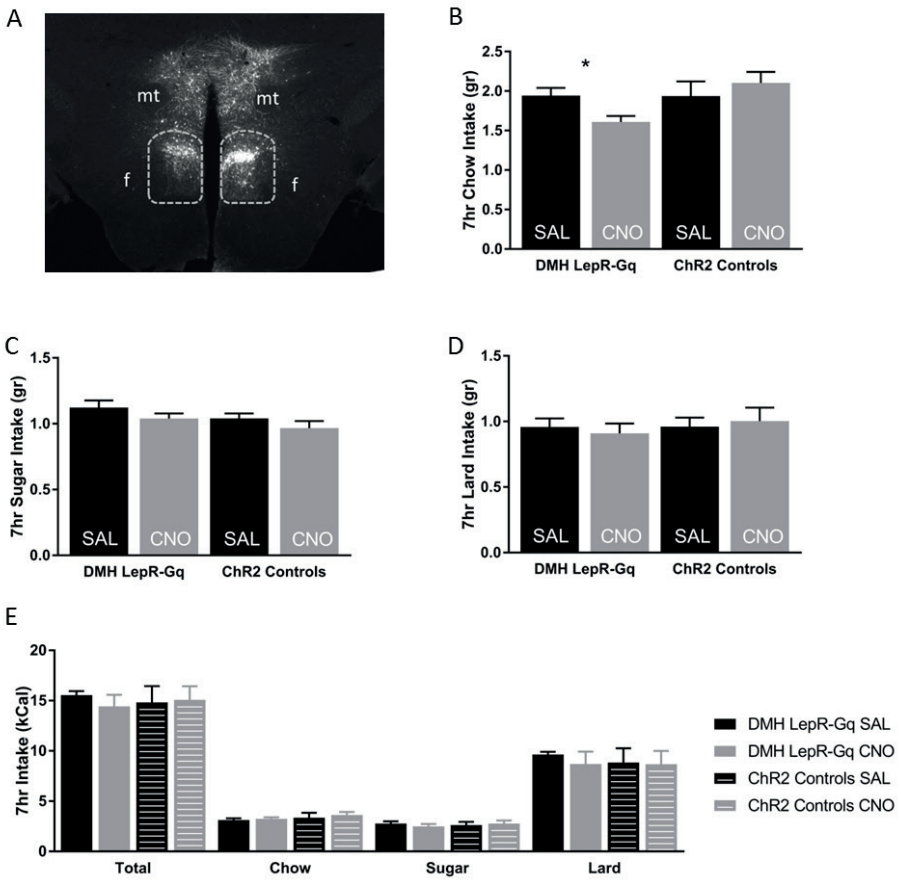

Figure 1. Food intake in DMH LepR-Gq and control mice. (A) Representative example of AAV5-DIO-hM3DGq targeted at the DMH in a LepR-cre mouse. The dashed lined shapes indicate the DMH. (B) CNO decreased chow intake in DMH LepR-Gq mice compared to saline (SAL), but not in control mice. (C) CNO had no effect on total sugar cube or (D) lard intake. (E) Total caloric consumption when given free choice between chow, sugar cubes and lard was unaffected as well as caloric consumption of the individual items. DMH LepR-Gq (n=10), Controls (n=6). Mean±SEM. * p<0.05.

A two-way repeated-measures ANOVA on chow data revealed an Interaction effect of DMH LepR-Gq vs ChR2 controls x Injection $F(1,13)=7.077$, p=0.020 (figure 1B). Further comparisons revealed decreased chow intake in DMH LepR-Gq, but not control, mice after CNO compared to saline injections ($t(13)=2.802$, p=0.030). Total sugar or lard intake was not affected by CNO injections in either DMH LepR-Gq or control mice (figure 1C, D). When chow, sugar and lard were presented simultaneously, total caloric intake and caloric intake of any type of food was not affected by CNO injections in DMH LepR-Gq or control mice (figure 1E).

Activating DMH LepR neurons increased locomotion and eye temperature

To test whether energy expenditure was affected by activation of DMH LepR neurons we assessed locomotor activity and body temperature using a thermosensitive camera. As proxy for body temperature, we took the warmest spot detectable with the thermosensitive camera which was the eye.

A repeated-measures ANOVA on cumulative locomotion revealed an interaction effect of Injection x Time $F(1,13)=8.902$, p=0.011 (figure 2A). Further contrasts revealed increased locomotion in DMH LepR-Gq, but not control, mice after CNO injections compared to saline $t(13)=6.162$, p<0.0001.

Eye temperature was measured in upright standing mice (for example, figure 2B). DMH LepR-Gq mice increased eye temperature after CNO injections compared to saline (main effect of Injection $F(1,9)=7.421$, p=0.024; (figure 2C). CNO did not affect eye temperature in control mice (data not shown). Comparing area under the curve of change in temperature after CNO injections in DMH LepR-Gq mice and control mice, revealed a significant increase in temperature in DMH LepR-Gq mice $t(14)=3.395$, p=0.004 (figure 2D).

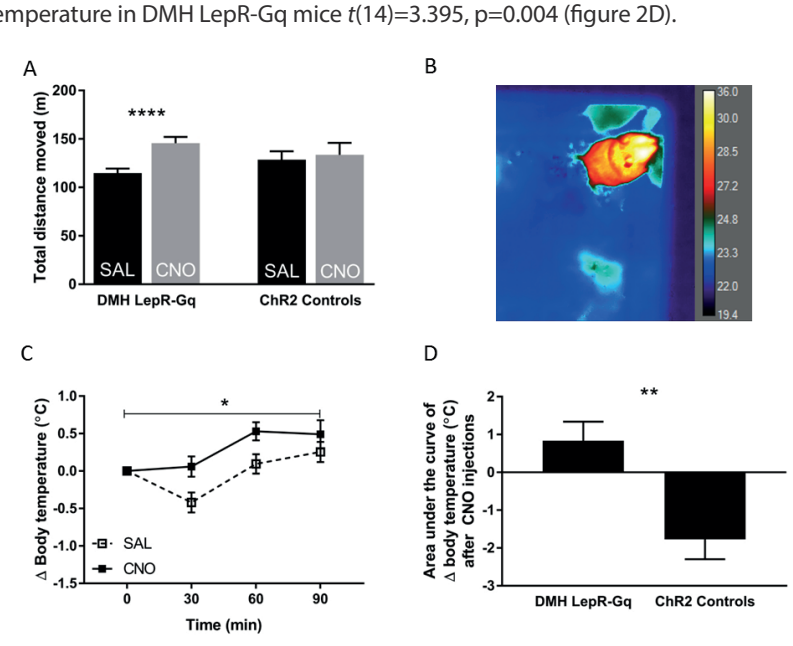

Figure 2. Energy expenditure in DMH LepR-Gq and control mice. (A) CNO increased locomotion in DMH LepR animals, but not in controls. (B) Example of movie frame from thermosensitive camera in which mouse is standing on hind legs with eyes facing upwards. (C) In DMH LepR-Gq mice CNO increased body temperature compared to saline. (D) Comparison of net area under the curve of CNO reveal an increase in body temperature in CNO injected DMH LepR-Gq mice compared to CNO injected controls. DMH LepR-Gq (n=10), Controls (n=6). Mean±SEM. * p<0.05, **** p<0.0001.

Repeated DMH LepR stimulation enhanced weight loss

We next addressed whether the effects seen on feeding, locomotion and temperature altered body weight with twice daily injections over 3 consecutive days.

A repeated-measures ANOVA on body weight loss over time upon repeated CNO injections revealed an interaction effect of DMH LepR-Gq vs Control mice x Time $F(3,42)=2.147$, p=0.029 (figure 3A). Further comparisons revealed that body weight was decreased in DMH LepR-Gq mice compared to control mice on days 2 (p=0.0058) and 3 (p=0.0008). Chow intake upon repeated CNO injections did not differ between the 2 groups (figure 3B). As a result, feeding efficiency (total chow intake/body weight change) was lower in DMH LepR-Gq compared to control mice, i.e. for each gram of chow eaten, DMH LepR-Gq mice lost more weight (figure 3C, $t(13)=2.649$, p=0.020).

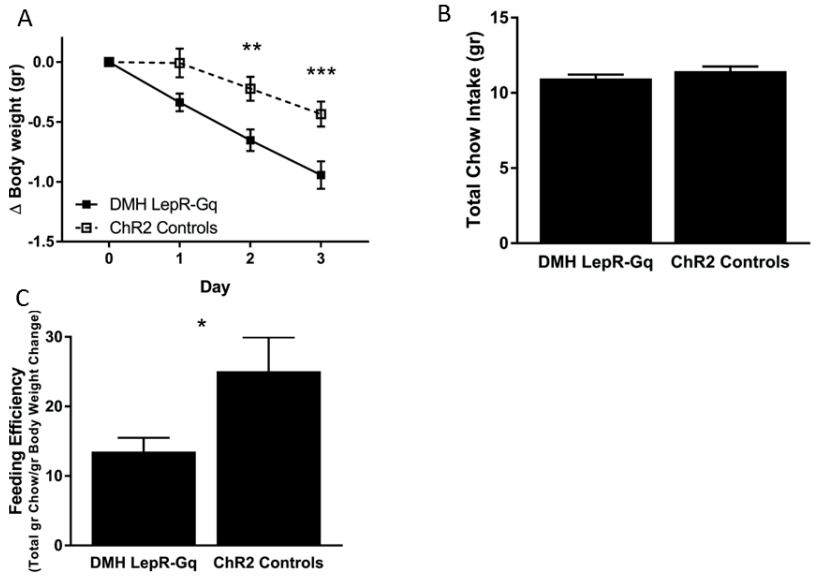

Figure 3. Twice daily injections of CNO for 3 consecutive days in DMH LepR-Gq and control mice. (A) Body weight loss was enhanced in DMH LepR-Gq compared to control mice. (B) Chow intake was unaffected by repeated CNO stimulation. (C) Feeding efficiency was lower in DMH LepR-Gq mice compared to controls. DMH LepR-Gq (n=10), Controls (n=6). Mean±SEM. * p<0.05, ** p<0.01.

DMH LepR-Gq stimulation increased eye temperature in obese mice

We then exposed mice to a free choice high-fat high-sucrose (fcHFHS) diet for 3 weeks. This diet increased body weight to a similar extent in all mice: LepR DMH-Gq mice, 121.1% ±2.2 and control mice, 121.6%±2.3 (figure 4A). 3 days after removal of the fcHFHS diet, CNO effects on body temperature were determined. A repeated-measures ANOVA on the change in body temperature revealed an interaction effect of DMH LepR-Gq vs Controls x Time $F(3,42)=2.893$, p=0.046 (figure 4B). Further comparisons revealed that eye temperature was increased in DMH LepR-Gq mice compared to control mice 90min after injections p=0.037.

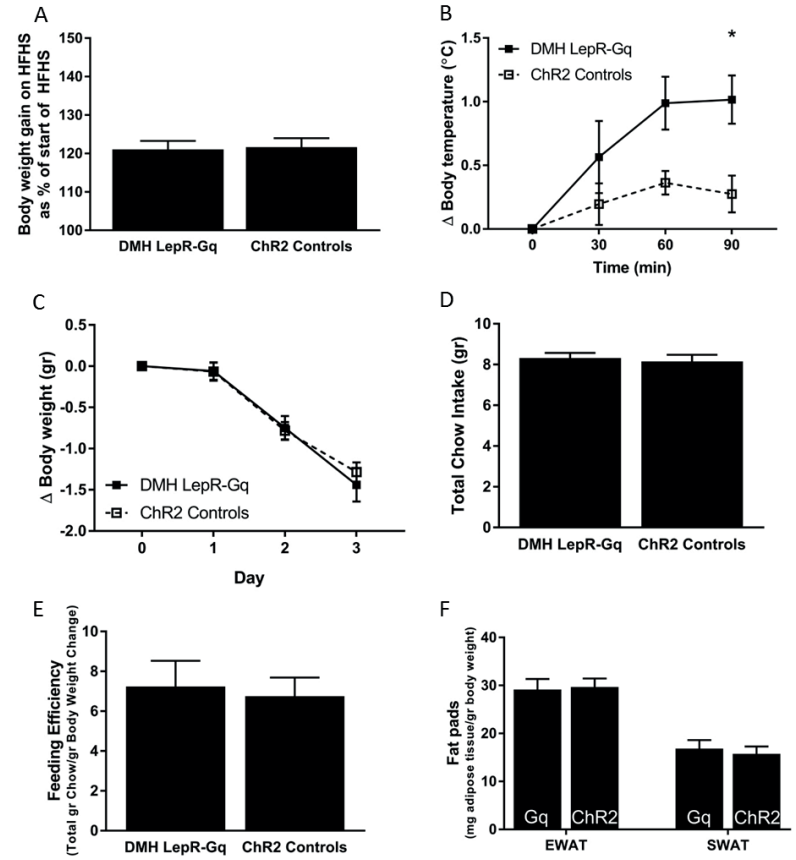

Figure 4. Effect of stimulating DMH LepR neurons after removal of a free choice high-fat high-sucrose diet (fcHFHS). (A) fcHFHS increased weight in all animals. (B) CNO increased eye temperature of DMH LepR-Gq compared to control mice. (C) Body weight loss, (D) food intake, (E) feeding efficiency and (D) amount of fat pads were similar in DMH LepR-Gq and control mice when repeatedly injected with CNO. EWAT=epididymal white adipose tissue; SWAT=subcutaneous white adipose tissue. DMH LepR-Gq (n=10), Controls (n=6). Mean±SEM. * p<0.05.

Repeated DMH LepR-Gq stimulation after fcHFHS diet removal failed to enhance weight loss

To test whether stimulation of DMH LepR-Gq enhanced weight loss during dieting, twice daily CNO injections were given starting 4 days after removal of the fcHFHS. Body weight loss over 3 days of repeated CNO injections was similar in DMH LepR-Gq and control mice (figure 4C). Similarly, food intake (figure 4D) and feeding efficiency (figure 4E) did not differ between the 2 groups. After 3 days of twice daily CNO injections, fat pads were removed and weighed. Repeated DMH LepR-Gq stimulation did not affect fat pad amount compared to control mice (figure 4F).

Discussion

Using chemogenetics we were able to further establish the role of DMH LepR neurons in energy homeostasis. Repeated chemogenetic stimulation of DMH LepR neurons in normal fed mice resulted in body weight loss as a result of increased locomotor activity and thermogenesis without affecting feeding, in line with a study by Rezai-Zadeh et al. (Rezai-Zadeh et al., 2014). In contrast, repeated DMH LepR stimulation in mice previously exposed to an obesogenic diet did not affect body weight, despite increased thermogenesis.

We observed a decrease in chow intake 7h after CNO injections when chow was placed on the cage floor, which is in agreement with decreased feeding seen upon intra-DMH leptin injections (Enriori et al., 2011). However, we saw no effect on feeding during repeated CNO injections, also not 7h after the first CNO injections (data not shown). This is consistent with Rezai-Zadeh et al (Rezai-Zadeh et al., 2014) who saw no effect on 24h feeding in a similar repeated injection scheme. The different outcome in feeding between single and repeated injections may be explained by the differences in experimental set up, i.e. during repeated injections chow was presented in the cage top, whereas with single injections chow was presented on the cage floor. Apparently, mice will eat more chow when it is presented on the floor (CNO injected ChR2 controls: 2.10±0.3gr with chow on floor; 1.50±0.1gr when in cage top). So, activation of DMH-LepR neurons decreases consumption of chow, but only when food is easily accessible. Furthermore, intake of palatable foods was unaffected by stimulation of DMH LepR neurons, suggesting that DMH LepR neurons do not play a role in the feeding response to obesogenic diets.

It is important to note that leptin was previously found to depolarize only a subset (38,2%) of DMH LepR neurons (Simonds et al., 2014). A group of leptin-activated DMH prolactin-releasing peptide neurons decreased feeding (Dodd et al., 2014), whereas a group of DMH GABA neurons that were found to be hyperpolarized by leptin increased feeding (Otgon-Uul et al., 2016). Here we used chemogenetics, with which all DMH LepR Gq-targeted neurons are stimulated. As such, we may have targeted a mixed population of neurons that are oppositely regulated by leptin and activating them all using chemogenetics may have masked effects on feeding.

We found, similar to a previous study (Rezai-Zadeh et al., 2014), that repeated DMH LepR stimulation enhanced weight loss in normal fed mice. Since in these animals food intake was not affected, weight loss was primarily the result of increased energy expenditure, which was reflected in an increase in both locomotion and thermogenesis. Similarly, in mice previously exposed to fcHFHS diet for 3 weeks, DMH LepR stimulation increased thermogenesis. This is in agreement with a previous study that showed that diet-induced obese mice remained sensitive to the thermogenic effects of intra-DMH leptin injections, despite increased baseline temperature (Enriori et al., 2011). Even though DMH LepR stimulation increased thermogenesis, our results show that repeated stimulation of DMH LepR neurons starting on the fourth day after fcHFHS removal was unable to enhance body weight loss. An explanation for this could be that we did not observe a decrease in body temperature after the fcHFHS diet was removed (on the fcHFHS diet 35.2±0.2°C; four days after fcHFHS removal 35.3±0.2°C), even though caloric restriction is associated with reduced thermogenesis (Fischer et al., 2016). Because body temperature was not reduced, this may have dampened the effect that DMH LepR stimulation induced thermogenesis may have had on body weight loss. Perhaps body temperature is more prominently reduced after longer periods of weight loss, at which DMH LepR stimulation could enhance body weight loss. Thus, further studies will be necessary to determine whether DMH LepR neuron activation could enhance weight loss during longer periods of dieting.

Together, our data show that DMH LepR stimulation increased body temperature, increased locomotor activity and promoted weight loss in normal fed mice. However, when dieting was mimicked in mice by removing a fcHFHS diet, DMH LepR stimulation was unable to enhance weight loss, despite increased thermogenesis. The DMH is a region in which leptin resistance exists naturally in some animals (de Git et al., 2018) and occurs as a result of diet-induced obesity (de Git et al., 2018; Levin et al., 2004, 2003). It seems that reduced signalling of DMH LepR neurons contributes to inducing and maintaining obesity, suggesting that increasing DMH LepR signalling may be a treatment option for obesity. However, our data show that stimulating DMH LepR neuron activity during dieting does not enhance body weight loss after obesity, challenging the idea that DMH LepR neurons might be a feasible target for weight loss therapies.

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Appendix LH LepR and VTA LepR neurons modulate different aspects of motivational behaviour

Background

In this thesis, we determined whether stimulation of leptin receptor-expressing (LepR) neurons in the ventral tegmental area (VTA) or lateral hypothalamus (LH) modulated motivational behaviour. To test motivational behaviour, we trained and tested mice in an operant task using the progressive ratio (PR) schedule (Richardson and Roberts, 1996). The amount of active lever presses (ALP) made were used as a measure of motivation. In chapter 2, we showed chemogenetic activation of VTA LepR expressing neurons decreased motivation in food restricted mice. In chapter 3, we found that LH LepR neurons project to the VTA and that activation of LH LepR neurons increased motivation in ad libitum fed mice.

A recent study that tested motivation in rats using the PR task, analysed the individuals ALP patterns and found that transient increases in DA levels enhanced the initiation of an action, i.e. an ALP, ("initiation vigor") and enhanced the number of repeated actions made once a behaviour was initiated (increased number of ALP in a bout; "effort exerted") (Ko and Wanat, 2016). Here, we sought to identify which aspects of motivation were affected by chemogenetic activation of VTA LepR or LH LepR neurons.

Methods

Using R (R Core Team, 2018), we assessed the effect of neuronal activation of VTA LepR or LH LepR on ALP patterns during the progressive ratio task. We assessed the following parameters:

- the total number of ALP,
- initiation vigor by assessing:
    - the number of bouts (based on a study by Ko and Wanat (Ko and Wanat, 2016), ALP were divided into separate bouts, which were defined as one or
    - more ALP at least 2sec separated from adjacent bouts), the average initiation time of ALP after a reward was received (time point of first ALP after reward – time of reappearance of the levers; at the moment a ratio is completed, the levers retract, 5sec later a pump is activated for 2sec to deliver the sucrose reward and 10sec after the levers reappear)
- the effort exerted by assessing the average number of ALP per bout (total number ALP/total number bouts),
- the average interval between two consecutive rewards, and
- cumulative time of inactivity (inactivity was defined as no ALP for >2sec).

Results & Discussion

Our analysis of lever press activity revealed that VTA LepR neuronal activation in food restricted mice decreased the number of ALP (fig. 1A), which was associated with a decreased number of ALP per bout (fig. 1C). Thus, VTA LepR neuronal activation decreased the number of ALP made once lever pressing was initiated, i.e. modulated the exerted effort.

Furthermore, we found that LH LepR neuronal activation in ad libitum fed mice increased the number of ALP (fig. 2A), which was driven by a decrease in initiation time (fig. 2D) and inactive time (fig. 2F). There was also a trend towards an increased number of bouts (fig. 2B). Together, this shows that LH LepR neuronal activation enhanced the initiation of reward-seeking actions and thus, modulated the initiation vigor. Since chemogenetic activation of VTA DA neurons in rats enhanced initiation vigor (Boekhoudt et al., 2018), this supports the idea that LH LepR neurons enhanced motivation by increasing VTA DA neuron activity (Leinninger et al., 2009).

Together, these results suggest that LepR neurons modulate different aspects of motivational behaviour via VTA DA projecting LepR neurons originating in the VTA and LH.

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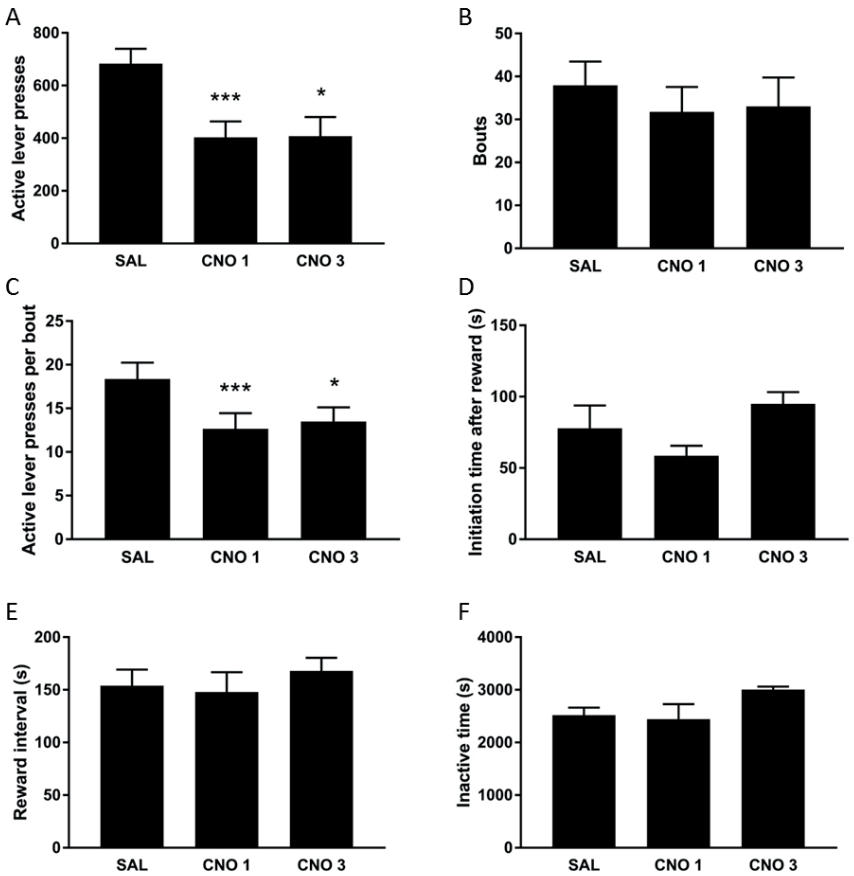


Figure 1. Active lever press pattern after chemogenetic activation of VTA LepR neurons in food restricted mice. (A) Active lever presses (ALP). (B) Number of bouts. (C) Average number of ALP per bout. (D) Average initiation time of ALP after a reward was received. (E) Average interval between two consecutive rewards. (F) Cumulative inactive time. Mean±SEM. One-way repeated measures ANOVA. * p<0.05, *** p<0.001.

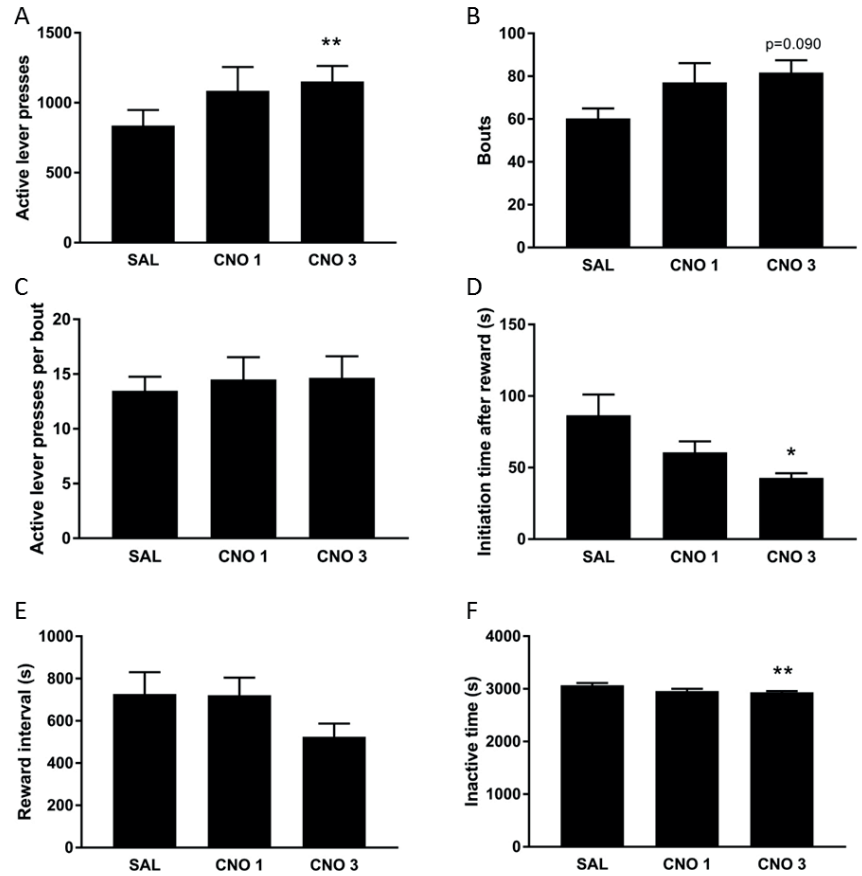
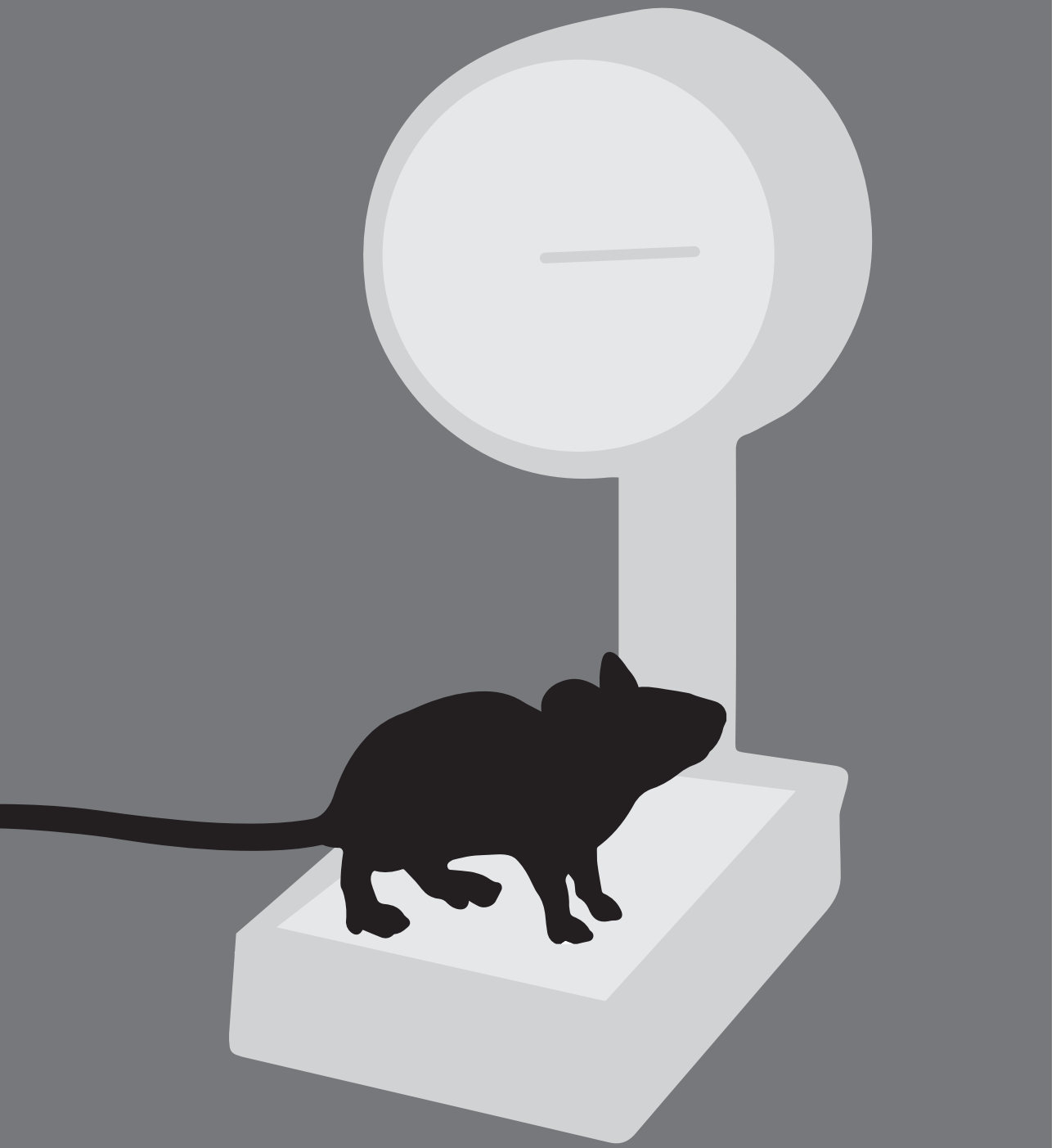


Figure 2. Active lever press pattern after chemogenetic activation of LH LepR neurons in ad libitum fed mice. (A) Active lever presses (ALP). (B) Number of bouts. (C) Average number of ALP per bout. (D) Average initiation time of ALP after a reward was received. (E) Average interval between two consecutive rewards. (F) Cumulative inactive time. Mean±SEM. One-way repeated measures ANOVA. * p<0.05, ** p<0.01.



Summary of findings & Discussion

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Key highlights of this thesis:

1. Leptin sensitivity is variable in mice and correlates with body weight gain on an obesogenic diet.
2. Midbrain LepR subpopulations have different behavioural effects: LepR neurons of the VTA regulate motivation and feeding and LepR neurons of the SN locomotion.
3. LH LepR neurons directly modulate motivational behaviour for food reward.
4. LH LepR neurons more specifically regulate body weight than LH GABA neurons and promote a negative energy balance by increasing energy expenditure.
5. Gnawing on food induced by LH GABA activation is dependent on the consistency of the food presented.
6. DMH LepR stimulation decreases body weight by increasing energy expenditure in normal weight mice, but not in mice previously exposed to an obesogenic diet.

Summary of findings

In western society, maintaining a healthy body weight can be a difficult task, as evidenced by the vast amount of people that are overweight or obese. Body weight is regulated by a complex system. One hormone important in this system is leptin, which is known for decreasing food intake and increasing energy expenditure (1–3). However, studies have shown that in rats the feeding response to leptin is variable, i.e. some decreased feeding, while others increased feeding after leptin administration (4, 5). This feeding response to leptin ("leptin sensitivity") has been shown to predict weight gain on an obesogenic diet. These results suggest that leptin sensitivity may in part explain differences in susceptibility to obesity and that leptin resistance may exist before becoming obese. In this thesis, we determined whether this relationship also exists in mice (***chapter 1***), which, if so, increases the likelihood that this phenomenon is generalizable to other species and perhaps even to humans. Furthermore, besides the arcuate nucleus (ARC), it remains largely unknown which brain regions expressing the leptin receptor (LepR) modulate which aspects of energy homeostasis. This thesis aimed to further understand how different LepR neuronal populations contribute to the regulation of body weight using chemogenetics. We determined the behavioural effects of chemogenetic activation of LepR neurons in the ventral tegmental area (VTA, ***chapter 2***), substantia nigra (SN, ***chapter 2***), lateral hypothalamus (LH, ***chapter 3, 4***) and dorsomedial hypothalamus (DMH, ***chapter 6***).

Chapter 1: Highly variable sensitivity to anorexic effect of leptin in mice

In ***chapter 1***, we evaluated the feeding response to leptin injections in mice, the extent of which is referred to as leptin sensitivity. We found, similar to studies in rats (4, 5), that leptin sensitivity is highly variable between mice. Based on the individual leptin sensitivity, mice were grouped into leptin sensitive (LS) mice that decreased feeding after leptin compared to vehicle injections or leptin resistant (LR) mice that increased feeding after leptin injection. We then investigated whether individual leptin sensitivity on a chow diet predicted weight gain on an obesogenic free choice high-fat high-sucrose (fcHFHS) diet. Although there was no significant difference in weight gain between LS and LR mice on a group level, we found that leptin sensitivity correlated with subsequent weight gain on a fcHFHS diet.

Previous studies showed that the extent of motivation for food predicted subsequent weight gain (6) and that leptin decreases motivation (7, 8). Therefore, in chapter 1, we also studied whether the response to leptin on motivation 1) correlated with leptin sensitivity and 2) correlated with subsequent weight gain on a fcHFHS diet. We found that the effect of leptin after 6h food restriction was variable and that this variable response correlated with the feeding response to leptin.

Chapter 2: Leptin receptor-expressing neurons in the substantia nigra regulate locomotion, and in the ventral tegmental area they regulate motivation and feeding

In ***chapter 2***, we determined whether chemogenetic activation of VTA LepR or SN LepR influenced different behaviours related to body weight regulation. Activation of VTA LepR neurons decreased motivation and feeding. SN LepR activation decreased locomotion.

Chapter 3: Do lateral hypothalamic leptin receptor-expressing neurons modulate motivational behaviour?

Since literature provided evidence that VTA DA neurons that express LepR do not modulate motivation (9), in ***chapter 3*** we addressed whether a prominent group of VTA projecting LepR neurons originating in the LH directly modulate motivation. Permanent silencing of LH LepR neurons did not affect baseline motivation, but decreased the amount of mice that were responsive to the decreasing effect of leptin on motivational behaviour. Chemogenetically activating LH LepR neurons increased locomotion and motivation. Thus, our results provide evidence that LH LepR neurons directly modulate motivational behaviour for food reward.

Chapter 4: Effects of GABA and leptin receptor-expressing neurons in the lateral hypothalamus on energy homeostasis

LH GABA neurons are orexigenic and LH LepR neurons are a subpopoulation of these GABAergic neurons. However, leptin is known to be anorexigenic and to promote a negative energy balance. In ***chapter 4***, we compared behavioural effects of chemogenetically activating LH GABA and LH LepR neurons. We found that activation of LH LepR neurons promoted a negative energy balance by increasing energy expenditure via both thermogenesis and locomotion. LH GABA neuron activation similarly resulted in a negative energy balance by increasing thermogenesis, despite decreased locomotion and transiently increased feeding. Thus, LH LepR neurons are a subgroup of LH GABA neurons that more specifically regulate body weight.

Chapter 5: Gnawing induced by LH GABA activation differently affects consumption dependent on experimental settings

In chapter 4, we noticed that activation of LH GABA neurons induced gnawing on different objects. ***Chapter 5*** describes which objects or foods are gnawed on depending on the experimental setting. We showed that the gnawing of food items in the presence of other gnawable objects, such as water bottle sippers, depended on the chewability of the food, i.e. solid food (chow) was gnawed on while porous (sugar cubes) or soft (lard) foods were not. We further showed that food restricting animals did not affect the extent of gnawing on food or non-food items.

Chapter 6: Differential effects of short- and long-term stimulation of dorsomedial hypothalamic leptin receptor-expressing neuron activation on energy balance

Chapter 6 addresses the weight loss enhancing effects of DMH LepR neurons. Activation of DMH LepR neurons in normal fed mice increased weight loss as a result of increased energy expenditure (both thermogenesis and locomotion), without affecting food intake. We then subjected mice to a fcHFHS diet and showed that after removing the diet, i.e. mimicking the start of dieting by restricting palatable foods, DMH LepR activation did not enhance weight loss. Thus, the latter results challenge the idea that DMH LepR neurons could be an interesting target for weight loss therapies.

		Motivation	Feeding	Locomotion	Temperature	Body weight
VTA	Ad Lib	=	=	=	·	·
	FR	↓	↓	=	·	·
SN	Ad Lib	=	=	=	·	·
	FR	=	=	↓	·	·
LH	Ad Lib	↑	=	↑	↑	↓
	FR	=	=	↑	·	·
DMH	Ad Lib	·	=	↑	↑	↓
	FR	·	·	·	·	·

Discussion

Leptin action multifaceted

Leptin is known for decreasing body weight, most prominently by decreasing food intake (1, 2). Leptin crosses the blood-brain-barrier after which it binds to LepR to exert behavioural effects. Numerous LepR neuronal populations exist, with particularly high expression in the hypothalamus, midbrain and brainstem (10). Especially the hypothalamus has been shown to be crucial for leptin action (11). The study of leptin in the hypothalamic ARC has received by far the most attention in leptin research, perhaps because the two LepR neuronal subpopulations express well-characterized neuropeptides: the anorexigenic proopiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) neurons and the orexigenic neuropeptide Y (NPY)/agouti-related peptide (AGRP) neurons. Leptin reduces

feeding by inhibiting NPY/AgRP neurons and activating POMC/CART neurons (12, 13). Although the ARC is recognized as an important site for leptin action, leptin in the ARC does not cover all aspects of central leptin signalling. Deletion of LepR from POMC/CART or AgRP/NPY in the ARC results in obesity that is less severe than that seen in *db/db* (14, 15). Similarly, restoration of leptin signalling in the ARC of *db/db* mice only modestly decreases body weight and food intake (16). Since ARC LepR signalling does not explain all aspects of leptin signalling, it is important that we study other LepR-expressing regions to increase our knowledge of the multifaceted way that leptin regulates body weight.

The hypothalamus, an essential brain area for leptin action, contains many other nuclei besides the ARC that express LepR, such as the LH and the DMH (10). We observed that activation of LH LepR or DMH LepR resulted in body weight loss (***chapter 4, 6***). Body weight loss was the result of increased energy expenditure, in the form of locomotion and thermogenesis. Also many extra-hypothalamic nuclei express LepR, such as the VTA and SN, which are located in the midbrain (10). One of the questions of this thesis was to compare the behavioural effects of midbrain LepR neuronal populations in the VTA and SN. We found that stimulating these midbrain LepR populations affected different aspects of weight regulation, namely activation of VTA LepR neurons modulated feeding and motivational behaviour while activation of SN LepR neurons modulated locomotion (***chapter 2***).

Something that becomes apparent from not only literature, but also our behavioural experiments, is that leptin modulates a singular behaviour via action at many different brain nuclei. For instance, local leptin infusions into the ARC, but also the LH, DMH and the VTA result in decreased feeding (13, 17–20). The results of this thesis further showed that this is also true for motivational behaviour, locomotion and thermogenesis: LepR neurons at both the level of the LH (***chapter 3***) and the VTA (***chapter 2***) modulated motivation, LepR neurons in the SN (***chapter 2***), LH (***chapter 3, 4***) and DMH (***chapter 6***) altered locomotor activity and LepR neurons in the LH (***chapter 4***) and DMH (***chapter 6***) enhanced thermogenesis. Not only do multiple LepR-expressing neuronal populations of different brain regions modulate the same behaviour, LepR neurons that regulate a particular behaviour also project to another LepR containing brain region that affects the same behaviour. For example, although with contrasting effects on motivation, both LH and VTA LepR neurons modulate motivation and LH LepR project to the VTA (***chapter 2, 3***). Thus, leptin manipulates the same behaviour via many different brain regions.

Why does leptin act via so many different neuronal populations? Body weight regulation is controlled by both physiological needs and the external environment. For instance, an animal must decide whether the desire for food outweighs both the energy requirements and predatory threats associated with the gathering of food. As such, different signals that convey information about hunger, energy reserves, stress, environmental cues etc. must be integrated before an informed decision can be made. LepR are expressed in many brain areas, so that leptin can modulate signalling of different kinds of information, such as energy balance but also stress. This is necessary, because when adiposity levels are low, food must be gathered even in the face of stress or threats in order to prevent death. However, when adiposity levels are sufficient, an animal would be better off not to hunt for food when threats exist. Thus, leptin adjusts signalling of information and behaviours dependent on the amount of bodily energy reserves with the aim of maintaining body fat at levels required for survival.

Leptin treatment

When leptin was discovered, this was accompanied by excitement of the possibility that it could cure human obesity, since leptin administration decreased feeding and leptin deficient mice that were hyperphagic and obese quickly normalized feeding and body weight after leptin administration (1, 21–23). This excitement was, however, quickly dampened by the discovery that obesity is associated with increased leptin levels and reduced leptin responsiveness, "leptin resistance" (24–28).

Although leptin deficient humans highly benefit from leptin treatment, leptin treatment in both lean and obese humans has only marginal effects or even no effect on body weight (29–31). However, differences in leptin responsivity may have compromised the effect size of leptin treatment. Studies have shown that the feeding and thermogenic response to leptin is highly variable between rats but stable within a rat; leptin sensitive animals decrease food intake and increase thermogenesis after leptin injections compared to vehicle injections, while leptin resistant animals increase feeding and decrease thermogenesis (4, 5). In agreement with this, in chapter 1, we showed that the feeding response to leptin is also variable in mice. These results suggest that this phenomenon may also be generalizable to other species, and perhaps even to humans. If so, the differences in the response to leptin may have compromised the effects of leptin as a weight loss treatment, since leptin would only be effective in a subgroup of people, i.e. those that are sensitive to the effects of leptin. As such, the weight loss effects of leptin may be larger in leptin sensitive individuals than effects reported by previous studies and leptin may prove to be beneficial as weight loss therapy in this subgroup of individuals. However,

future studies will need to show whether, in humans, this variable sensitivity to leptin is also present.

Even though leptin did not turn out to be a general treatment against obesity, studying mechanisms deployed by leptin continues to shed light on the neurobiological underpinnings of body weight regulation and may lead to the discovery of new drug targets that aid in weight loss or new prevention strategies.

Possible contribution of leptin research to prevention strategies

A major focus point in light of the obesity pandemic is the prevention of weight gain and the promotion of a healthy lifestyle. Both in humans and rodents, there is a large variability in the susceptibility to obesity when exposed to the same environment (32, 33). Studies focussing on what determines how susceptible one is for weight gain will help identify obesity prone individuals and which strategies are beneficial for these individuals.

Leptin research has provided us with insights that contribute to the susceptibility of obesity. The feeding (and thermogenic) response to leptin administration is variable in both rats and mice (1, 2, ***chapter 1***). This variable response to leptin administration predicted subsequent weight gain on an obesogenic diet (1, 2, ***chapter 1***). Interestingly, studies have shown that rodents that are prone to obesity have intact leptin transport across the blood-brain-barrier, but LepR mRNA expression and LepR signalling are reduced in, amongst others, ARC, DMH and ventromedial hypothalamus (VMH) compared to obesity resistant rats (4, 32–34). These studies suggest that cellular leptin resistance may contribute to the development of obesity. As such, deciphering the cause of cellular leptin resistance may enable the restoration of leptin signalling in obesity prone individuals, which may prevent the development of obesity.
Rather interestingly, the sensitivity to leptin can be altered by the act of physical activity. In rats, the act of wheel running, but not per se the extent of it, induced weight loss and increased leptin sensitivity (35–37). Even acute exercise, no more than two 10min sessions of swimming increased LepR signalling in the hypothalamus, without affecting leptin levels themselves (36). Thus, rodents less sensitive to leptin may be able to enhance the response to leptin by physical activity.

To date, it is unknown whether variable leptin sensitivity in humans exists or whether it explains differences in the susceptibility to obesity. However, if this is the case, then leptin sensitivity may prove to be a biomarker with which we can identify populations at risk for obesity. Prevention strategies could then be adjusted to the needs of these populations, for instance by promoting physical activity to increase the sensitivity to leptin in individuals that are less sensitive. However, this would mean administering leptin in humans and measuring the feeding or thermogenic response, which would be a laborious feat to undertake. Perhaps future research will find genetic components that explain the differences in leptin sensitivity, so that genetic analyses instead of a behavioural test can identify the individuals at risk for obesity based on their leptin sensitivity.

Increasing dieting success by mimicking effects of leptin

Although prevention is of utmost importance, there is a large proportion of humans already suffering from obesity. Therefore, another focus point is the treatment of obesity. The current treatment of obesity focuses on weight reduction, which is most successfully achieved by bariatric surgery. Bariatric surgery improves the health and quality of life, but is associated with risks and patients must be willing to make significant lifestyle changes. Moreover, these surgeries are only considered for people that made serious non-surgical weight loss attempts. However, the success rates of dieting are low (~20%) and maintaining weight loss has been shown to be difficult (38–40).

In theory, dieting seems rather simple: one must reduce caloric consumption and increase energy expenditure. A compromising factor during dieting is that weight loss causes a state of negative energy balance. In humans, this is associated with increased hunger, food craving and neuronal activity to food cues in brain reward regions, but also with reduced energy expenditure (41–43). Together, these adaptations promote weight regain and, thus, counteract weight loss. Rather than viewing recovery of lost weight as a failure of dieting, this phenomenon should be viewed as an expected response to dieting which can be pharmacologically treated. In that regard, the success rate of dieting could be increased by targeting weight-reduced associated adaptations.

Although leptin is mostly associated with obesity, effects of the loss of leptin during fasting are gaining attention. Leptin levels drop quickly with food restriction and lowered leptin levels in dieting individuals is often referred to as a leptin deplete state (27, 44–46). The subsequent physiological adaptations, such as food becoming more rewarding or reduction of energy expenditure, have partially been restored by leptin administration in weight-reduced humans (47–51). Thus, effects associated with dieting are in part the result of a depletion of leptin. As such, mimicking leptin action to restore the weight loss counter regulatory behaviours may contribute to the sustained effects of dieting. Determining where in the brain leptin acts to modulate, for instance, food reward and energy expenditure is therefore an important step in identifying targets that could contribute to weight loss.

Leptin modulates motivational behaviour via both the LH and the VTA

One behavioural response to food restriction shown to be decreased by leptin is the increased rewarding effect of food in both humans and rodents (7, 8, 47, 48, 52, 53). To test the willingness to work for a reward ("motivation for food"), we trained and tested mice in an operant task using the progressive ratio (PR) schedule (54). The amount of active lever presses (ALP) made were used as a measure of motivation.

The mesolimbic DA system of the brain is associated with motivational behaviour. Enhanced motivational behaviour is related to an increase in DA transmission in the nucleus accumbens (NAc) (55). A recent study that tested motivation in rats using the PR task, evaluated the pattern of ALP and found that enhanced DA levels decreased the latency to initiate an action, i.e. ALP, (referred to as "initiation vigor") and enhanced the number of repeated actions made once a behaviour is initiated (increased number of ALP within a bout of ALP; "effort exerted") (56). Interestingly, our results showed that both VTA LepR and LH LepR modulated motivational behaviour, but that they modulated different aspects of this behaviour.

Activation of VTA LepR neurons decreased motivation in food restricted mice (***chapter 2***). When specifically looking into the pattern of ALP, we found that decreased responding in the PR task was associated with a decreased number of ALP per bout, i.e. decreased effort exerted (appendix).

The decrease in motivation after activation of VTA LepR neurons was a surprising finding. Most VTA LepR are DAergic (***chaper 2***), increased midbrain DA activity is associated with enhanced motivation (57–59) and chemogenetic activation of VTA DA neurons increases motivation, which was the result of enhanced initiation vigor (60). As such, we would have expected an increase in motivation and enhanced initiation vigor. Instead, our results clearly show that activation of VTA LepR neurons decreased motivational behaviour and modulated effort exerted and not initiation vigor. Moreover, the decrease in motivation was only observed in food restricted mice, which have lowered leptin levels and increased VTA DA neuronal activity (61, 62), making it unlikely that stimulation of VTA DA-LepR neurons contributed to the decrease in motivation. Thus, although VTA DA-LepR neurons were originally proposed to mediate the effects of leptin on motivation, our findings challenge this view. This view is in line with other studies that showed that LepR are scarcely expressed in the mesolimbic DA pathway important for motivational behaviour and instead majorly project to the extended amygdala, which is important in fear behaviour (9, 63). These findings were supported by studies showing that intra-VTA leptin injections did not affect motivation, but instead decreased anxiety-like behaviour (64, 65). Together, these results suggest that VTA DA-LepR neurons do not modulate motivation.

Another chemically defined group of neurons within the VTA are VTA GABA neurons, which are known to provide local inhibition of DA neurons and modulate reward behaviour (66). One interpretation of our results is therefore that decreased motivation is the result of chemogenetic activation of VTA GABA-LepR neurons that reduce VTA DA activity. Our results would then further imply that VTA GABA-LepR neurons reduce the neuronal activity of VTA DA neurons that modulate the amount of effort exerted.

In contrast to VTA LepR activation, activation of LH LepR neurons increased motivation (***chapter 3***). This was most likely the result of LH LepR mediated modulation of the VTA, a phenomenon already shown by others (18). Of interest, LH LepR neuron stimulation in ad libitum fed mice increased locomotion and enhanced the number of ALP, but not inactive lever presses (ILP). Increased locomotor activity can enhance general behaviour initiation and may have contributed to the enhanced motivation. However, since only ALP, but not ILP (which is never reinforced by a reward), was increased, this showed that the behaviour was goal-directed and related to the motivation for food reward.

By analysing the ALP pattern, we showed that the increase in motivation was the result of decreased initiation time of ALP after a reward was earned and an increase in the number of ALP bouts, but the amount of ALP per bout remained the same (***appendix***). Thus, LH LepR activation enhanced the initiation of reward-seeking events in mice, i.e. initiation vigor. Recently, VTA DA neuronal activation was shown to increase initiation vigor, but not effort exerted (67), which provides support that LH LepR activation enhanced motivation through increased VTA DA activity. It must be noted that motivational behaviour was only enhanced in mice injected with high viral titres and hence higher expression of the DREADD receptor (***chapter 3***). When titres were low, as with LH LepR Gq-biased experiments, motivation was not affected, but locomotor activity was still enhanced by LH LepR stimulation. This suggests that the activation of a limited number of LH LepR neurons affects locomotor activity and that higher numbers of activated LH LepR neurons are required to affect motivational behaviour.

Together, these results suggest that leptin (indirectly) modulates different aspects of motivational behaviour via VTA DA projecting LepR neurons originating in the VTA and LH. It would be interesting to determine the exact projections and mechanisms with which leptin modulates motivation in order to find therapeutic targets to combat the increase in food motivation observed during dieting.

The relevance of finding targets to decrease motivation for food becomes particularly evident in patients treated with antipsychotics. Many antipsychotics, such as haloperidol and olanzapine, are antagonists for the dopamine receptor type 2 (D2R) and are associated with weight gain (68, 69). This has been suggested to be, in part, linked to leptin (signalling) (70–73). Mice deficient for D2R in the midbrain show enhanced motivation to work for food (74) and D2R are necessary for the anorexic effect of peripheral leptin injections (75–78), showing that leptin mediates behaviours via D2R signalling. Further research into the relationship of leptin and D2R on both molecular and behavioural levels could possibly lead to the development specialized treatment for psychosis that does not affect food motivation.

LH and DMH LepR neurons increase energy expenditure

Another interesting physiological response to dieting is the decrease in energy expenditure (43, 51). By counteracting the decrease in energy expenditure, dieting could be made more successful. We found that stimulation of both LH LepR and DMH LepR stimulated weight loss, by increased energy expenditure in the form of locomotion and thermogenesis (***chapter 4, 6***).

The LH has previously been studied as a potential target for weight loss therapies in humans. LH lesions in humans induced a transient decrease in feeding and body weight and deep brain stimulation in both humans and rats led to long lasting decreases in feeding and body weight (79–82). Our results suggest that both LH GABA and LH LepR may have contributed to these effects. We showed that stimulation of LH LepR neurons, which are all GABAergic, more specifically promoted weight loss than LH GABA neurons (***chapter 4***). The most apparent discrepancy between the two populations was that LH GABA stimulation decreased locomotion, while LH LepR stimulation increased locomotion, and that LH GABA stimulation induced a transient increase in feeding. Stimulation of LH LepR neurons did not alter food intake, but motivational behaviour was enhanced (***chapter 3***). The majority (>60%; 76–78) of LH LepR neurons co-express Galanin of Neurotensin and both of these LH LepR neuronal populations have been associated with motivational behaviour (8, 86). It therefore seems unlikely that a LepR neuronal population will be found that increases energy expenditure but does not affect food motivation. Thus, LH LepR neurons are an unlikely target for future therapies, since enhancing motivation could have undesirable effects. However, insights like these will help pinpoint neuronal populations of the LH that as a group do promote weight loss without affecting food motivation.

Like the LH, the DMH contains LepR neurons that promote weight loss. Stimulation of DMH LepR neurons reduce weight by inducing energy expenditure via thermogenesis and locomotor activity (77). We found that this phenomenon was only observed in normal fed mice, but not in mice previously exposed to an obesogenic diet (***chapter 6***). However, since we assessed the weight-loss effects at only one time point after removal of the diet, it remains possible that DMH LepR activation could enhance body weight loss at other time points. Further studies will be necessary to determine whether DMH LepR neuron activation could enhance weight loss during dieting.

To conclude, the results in this thesis show that both LH and VTA LepR neurons modulate motivation and that both LH and DMH LepR neurons increase energy expenditure. These findings contribute to a deeper understanding of which neurons modulate which aspects of energy expenditure and could eventually lead to interesting new drug targets.

Using chemogenetics to study leptin and future directions

Chemogenetics has proved to be a valuable tool for behavioural neuroscience. It allows us to investigate behavioural effects of manipulating neuronal activity of neurons in freely moving animals and in a relatively non-invasive manner. Combined with Cre-recombinase, such as in LepR-cre mice that were used in this thesis, chemogenetics enables manipulation of specific subsets of neurons. Furthermore, since the effect of neuronal modulation is transient, chemogenetics is a tool that allows for within subject comparisons, which increases the power of the study and decreases the number of animals needed. Since chemogenetics only transiently alters neuronal activity, effects on downstream circuits will be absent or less prominent than the effects of permanent strategies, such as lesions. Thus, chemogenetic altering of neuronal activity occurs in an otherwise intact brain. Lastly, chemogenetics is also interesting for the human situation, since it could become a gene therapy that may be used in the clinical setting. This implies that positive results from fundamental rodent studies could have direct therapeutic potential in humans. In non-human primates, chemogenetics has already been successfully used (88, 89). However, additional studies focusing on safety, efficacy, but also adequate targeting of specific cell types or neuronal projections are necessary to allow a clinical application of chemogenetics.

One potential drawback of chemogenetics is the use of the designer drug, clozapine-N-oxide (CNO), which is used to manipulate neuronal activity. CNO was found to have only limited access to the brain and that instead clozapine, to which CNO is readily metabolized in humans, non-human primates and rodents, is the in vivo DREADD

agonist (90–94). Clozapine is a drug with many endogenous targets and binds to DREADDs with high affinity (92, 95). Therefore, proper controls are detrimental when using chemogenetics, because CNO derived clozapine may lead to off target effects. With the use of control mice (***chapter 2-6***) that received viral injections expressing something other than hM3DGq, we were able to show that CNO by itself did not induce behavioural effects in our experiments. This drawback of chemogenetics is being resolved by the development of new designer drugs, such as compound 21, which does not convert to clozapine (96).

While chemogenetics has many benefits, aspects of leptin signalling make using this technique rather complicated. For instance, LepR neurons within the arcuate nucleus, but also within the LH and DMH studied in this thesis, differently respond to leptin. In the LH, only 34% of neurons are depolarized by leptin and, in the DMH, 38% of neurons are depolarized by leptin (18, 97). Furthermore, in the midbrain, it is known that VTA DA neurons are hyperpolarized by leptin (76, 98–100). Based on our results in ***chapter 2***, we proposed that midbrain GABA neurons are depolarized by leptin, which is supported by unpublished electrophysiological work from our lab (Omrani, unpublished). Finally, the response of SN DA and SN GABA to leptin remains unknown, but could be similar to the response of VTA DA and VTA GABA neurons. Thus, LH LepR, DMH LepR, VTA LepR and perhaps even SN LepR, all contain a mixed population of LepR neurons that differently alter their neuronal activity in response to leptin. With the use of the hM3DGq-excitatory DREADD receptor in the LepR-cre mouse, we enhance excitability of all Gq-targeted neurons in a specific area. As such, by targeting this mixed population that are oppositely regulated by leptin, we 1) do not directly assess the effect of leptin in this region on behaviour and 2) may have even masked behavioural effects as a result. The latter is evidenced by the fact that both leptin injections into the DMH and LH decrease feeding (18, 19), yet chemogenetic activation of DMH LepR or LH LepR did not (chapter 4, 6). Thus, it seems that leptin-induced feeding effects in the DMH and LH are the result of the intricate activation and inhibition of LepR neurons in these regions.

For future experiments, it will be interesting to target specific subtypes of LepR neurons. VTA GABA-LepR and VTA DA-LepR neurons could be targeted by using viral vectors with promotors specific for that cell type. A more difficult task will be to target LepR neurons that have identical chemical markers but are oppositely regulated by leptin. New techniques are constantly being developed which enable access to activated neurons. For instance, immediate early gene promoters have been used to drive Cre-expression in a technique referred to as targeted recombination in active populations (TRAP) (101). If this could be adjusted to genes that are upregulated after leptin-induced depolarization, it would be possible to selectively target leptin-depolarized neurons in a specific brain region.

Another aspect that is not addressed by chemogenetically activating LepR neurons in a specific brain area, is how this affects downstream targets. For instance, with respect to LH LepR activation and the resultant increase in motivation (chapter 3), it would be interesting to study: 1) whether the LH LepR -> VTA projection modulated motivation? and 2) whether the increase in motivation was the result of increased VTA DA activity?

The former can be addressed by chemogenetically targeting LH -> VTA projections. Projection specificity can be achieved by locally infusing CNO into a downstream area of interest. However, the non-invasive nature of DREADD would be compromised as a result, since a cannula would have to be implanted into the brain. Another possibility to reach projection and cell-type specificity is the combination of the Cre- and flipase(Flp)-recombinase (102). For instance, a Cre-dependent retrograde virus expressing Flp could be injected into the VTA of LepR-cre mice, which would result in the expression of Flp in VTA projecting LepR neurons. When a Flp-dependent DREADD is then injected into the LH, only VTA projecting LH LepR neurons will express DREADD. The second question could be answered, for instance, by combining chemogenetics and fiber photometry, which allows for (repeated) measurement of neuronal activity using calcium indicators. By measuring neuronal activity of VTA DA neurons, the effect of chemogenetically activating LH LepR neurons on VTA DA neurons can be measured. Together, this would enable the researcher to simultaneously examine the effects chemogenetic manipulation has on downstream targets; in this case how LH LepR activation affects VTA DA neuronal activity.

To conclude, in our experiments we targeted a mixed population of LepR neurons in the LH, DMH, VTA and perhaps even SN that differently alter neuronal activity in response to leptin. As such, our results do not mimic the effect leptin would have in a specific region. Moreover, modulation of neuronal activity with chemogenetics remains artificial and does not necessarily mean that a similar change in neuronal firing would be seen in a physiological setting. Therefore, studies using chemogenetics should be complemented by more physiologically relevant approaches to ensure correctness of the conclusions drawn from the experiments. However, chemogenetically activating LepR neurons in specific regions has enhanced our knowledge on the different behaviours these neurons are capable of modulating. In order to get a complete picture of the role of LepR neurons in behaviour, future studies will have to determine the roles of more specific subgroups of LepR neurons,

by targeting specific projections or specifically those neurons that are depolarized or hyperpolarized by leptin. Since leptin is known to hyperpolarize neurons such as VTA DA neurons, chemogenetic inhibition of LepR neurons will also provide valuable information. Dissecting the various behaviours modulated by different LepR neurons will contribute to a deeper understanding of body weight regulation and may lead to potentially useful therapeutic targets for the treatment of obesity.

Concluding remarks

Obesity is a growing pandemic concerning much of the human civilization. However, treatment options for obesity remain minimal, with bariatric surgery being the most beneficial at the moment, which is associated with risks and demands significant lifestyle changes. Fundamental research can open new paths that will lead to a larger array of treatment options. Insights into the underlying mechanisms of body weight regulation have already led to the discovery of new weight loss therapies for specific diseases, such as LepR deficiency. For instance, in LepR deficient patients weight loss can be achieved by an agonist of a peptide that is downstream of the LepR (103) and metreleptin, a leptin analog, has been approved in Europe to treat lipodystrophy, the abnormal distribution of fat (104, 105). Another interesting line of research is that rat studies have shown that an orally administered pill can produce a luminal coating of the gastrointestinal track that mimics bariatric surgery by preventing nutrient absorption (106). In this thesis we shed more light on the neuronal control of body weight regulation by LepR neurons by using chemogenetics and behavioural tasks that are easily translatable to the human situation. With this we hope to contribute to exciting new discoveries that will aid in the reduction of the obesity pandemic.

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Addendum

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Nederlandse samenvatting

Obesitas en overgewicht zijn wereldwijd nog steeds een groeiend probleem. In 2017 had 48.7% van de Nederlanders van 18 jaar en ouder overgewicht, 13.9% had obesitas. Dit proefschrift onderzoekt de rol van leptine signalering in het brein in de regulatie van lichaamsgewicht. Een constant lichaamsgewicht is simpel gezegd het gevolg van een balans tussen voedselinname en energieverbruik, waarbij temperatuurhuishouding en fysieke beweging betrokken zijn.

Leptine is een hormoon dat wordt afgescheiden door vetweefsel. De hoeveelheid leptine dat wordt afgescheiden is nagenoeg evenredig met de hoeveelheid vetmassa van een individu; met andere woorden, is er meer vetmassa, dan wordt er meer leptine afgescheiden. Leptine wordt naar de hersenen vervoerd, waar het bindt aan leptine receptoren (LepR), die in zeer veel hersengebieden voorkomen. Het meest bekende effect van leptine is een verlaging in voedselinname, maar leptine staat ook bekend om een verhoging van het energieverbruik. Zo stimuleert leptine gewichtsverlies. Daarnaast is bekend dat leptine ook nog een verlagende werking heeft op de motivatie om te willen werken voor voedselbeloning. In dit proefschrift onderzochten wij hoe leptine en leptine receptor-expresserende neuronen (LepR-neuronen) in verschillende hersengebieden van de muis betrokken zijn bij de regulatie van lichaamsgewicht.

In ***hoofdstuk 1*** onderzochten wij hoe de voedselinname van muizen veranderde na leptine injecties en vervolgens hoeveel zij in gewicht toenamen op een vet- en suikerrijk dieet. Wij vonden, net als eerdere experimenten in ratten, dat er 2 type muizen zijn, waarbij 1 groep voedselinname verlaagt en de andere juist voedselinname verhoogt. Ook zagen wij dat er een correlatie was tussen de voedselinname na leptine injecties en gewichtstoename op het calorierijke dieet: muizen die meer aten na leptine injecties, kwamen meer aan op een calorierijkdieet en andersom. Net als voedselinname, zagen wij dat sommige muizen hun motivatie verlaagden na leptine injecties, terwijl anderen juist hun motivatie verhoogden. Ook zagen wij dat de muizen die hun voedselinname verlaagden na leptine, ook hun motivationeelgedrag voor voedsel verlaagden. Samengevat laten wij in hoofdstuk 1 zien dat de verandering in voedselinname na leptine injecties correleert met zowel de verandering in motivationeelgedrag na leptine en gewichtstoename op een calorierijk diet. Dit verschil in gevoeligheid voor leptine zou mogelijk kunnen verklaren waarom sommige muizen dik worden en anderen niet, terwijl ze in dezelfde omgeving leven en hetzelfde eten voorgeschotelt krijgen. Gezien dit fenomeen in zowel ratten als muizen bestaat, is de kans aanwezig dat dit in meerdere soorten bestaat en wellicht zelfs in de mens. Als dit zo is, dan zou dit tevens een verklaring kunnen zijn waarom sommige mensen gevoeliger zijn dan andere voor gewichtstoename en zelfs obesitas.

In de resterende hoofdstukken van dit proefschrift gebruiken wij een techniek dat chemogenetica heet. Dit is een techniek waarbij je door middel van een virus een receptor tot expressie brengt in een bepaald hersengebied. In ons geval werd deze receptor specifiek in LepR-neuronen van een hersengebied tot expressie gebracht. Als deze receptor tot expressie is gebracht in hersencellen, kunnen wij door een gemodificeerd geneesmiddel “clozapine-N-oxide” (CNO) in te spuiten deze neuronen activeren of inactiveren. Met behulp van deze techniek kunnen wij het gedrag van muizen observeren na het activeren van LepR-neuronen in verschillende hersengebieden.

Hoofdstuk 2 behandelt het beloningscentrum, waarin twee hersengebieden liggen die LepR-neuronen bevatten: het ventraal tegmentaal gebied (afgekort tot VTA) en de substantia nigra (SN). De VTA staat vooral bekend om zijn betrokkenheid bij motivationeel gedrag en de SN is het meest bekend van de ziekte Parkinson, waarbij bewegingsstoornissen ontstaan door een vermindering van dopamine producerende neuronen in dit gebied. Voorheen waren de meeste experimenten naar LepR-neuronen in het beloningscentrum gericht op alleen de VTA of het gehele beloningscentrum. In hoofdstuk 2 wilden wij daarom een onderscheid maken tussen de gedragseffecten na het activeren van LepR-neuronen in de VTA en die in de SN. Wij vonden dat het activeren van VTA LepR-neuronen motivationeel gedrag voor voedselbeloning en voedselinname onderdrukt, maar dat dit niet locomotie beïnvloedt. Activeren van SN LepR-neuronen onderdrukt locomotie, maar had geen effect op voedselinname of motivationalgedrag voor voedselbeloning. Zodoende laten wij zien dat de twee LepR-populaties andere gedragingen beïnvloeden.

In ***hoofdstuk 3*** onderzochten wij of LepR-neuronen van de laterale hypothalamus (LH) motivationeel gedrag beïnvloeden. De LH staat bekend om zijn rol in voedselinname, omdat LH lesies ervoor zorgen dat dieren stoppen met eten. De LH speelt ook een rol in motivationeel gedrag en LH LepR-neuronen projecteren naar de VTA, een gebied dat belangrijk is voor motivationeel gedrag. Wij hebben eerst gekeken naar het effect van leptine injecties op motivationeel gedrag in twee groepen: in de ene groep muizen waren de LH LepR-neuronen uitgeschakeld en in de andere groep waren de LH LepR-neuronen intact. In de groep waarbij LH LepR-neuronen intact waren, zagen wij dat leptine injecties motivationeel gedrag verlaagden. In de groep waarbij de LH LepR-neuronen waren uitgeschakeld, zagen we dat dit effect in mindere mate te zien was. Daarnaast hebben wij in een aparte groep chemogenetica toegepast, waarbij wij LH LepR-neuronen konden activeren. Opnieuw zagen we dat LH LepR-neuronen betrokken zijn bij motivationeel gedrag, waarbij het gedrag na activeren werd verhoogd. Zodoende lieten onze resultaten van hoofdstuk 3 zien dat, net als VTA LepR-neuronen, LH LepR-neuronen motivationeel gedrag voor voedselbeloning kunnen beïnvloeden.

LH LepR-neuronen zijn neuronen die een inhibitoire neurotransmitter, GABA, tot expressie brengen. LH GABA-neuronen zijn een grote groep neuronen binnen de LH die aspecten van energiehuishouding beïnvloedt. LH LepR-neuronen zijn een subgroep van LH GABA-neuronen die LepR tot expressie brengen. In ***hoofdstuk 4***, vergelijken wij de gedragseffecten van het activeren van LH GABA-neuronen en LH LepR-neuronen. We zagen dat activatie van LH GABA-neuronen leidde tot gewichtsafname, als gevolg van verhoogde lichaamstemperatuur, ondanks dat locomotieactiviteit verlaagd was en voedselinname tijdelijk verhoogd was. Activatie van LH LepR-neuronen leidde eveneens tot gewichtsafname, als gevolg van verhoogde lichaamstemperatuur en verhoogde locomotieactiviteit, zonder voedselinname te beïnvloeden. Hieruit concludeerden wij dat LH LepR-neuronen gerichter de mechanismes beïnvloeden die te maken hebben met gewichtsafname vergeleken met LH GABA-neuronen.

Naast de bovengenoemde resultaten vonden wij in hoofdstuk 4 ook dat het activeren van LH GABA-neuronen, maar niet LH LepR-neuronen, in muizen leidde tot knaaggedrag. In ***hoofdstuk 5***, wilden wij dit knaaggedrag verder onderzoeken. De resultaten van hoofdstuk 5 lieten zien dat het activeren van LH GABA-neuronen tot knagen leidde van harde (muizenbrokjes, houtblokken en waterflesmondjes), maar niet van zachte(re) (suikerblokjes en ossewitvet) voedsels of objecten en dat dit knaaggedrag niet afneemt als een dier hongerig is. Andere onderzoekers die LH GABA-neuronen activeren en knaaggedrag observeren, moeten rekening houden met het feit dat niet alles wordt geconsumeerd en dus dat er goed moet worden gemeten wat daadwerkelijk wordt gegeten/gedronken en wat wordt verspild.

In ***hoofdstuk 6*** onderzochten wij de gedragseffecten van LepR-neuronen in de dorsomediale hypothalamus (DMH). We weten dat als DMH LepR-neuronen minder functioneren, dat dieren zwaarder worden op een calorierijk dieet. Wij vonden echter dat activatie van DMH LepR-neuronen geen invloed had op voedselinname. Vervolgens onderzochten wij de effecten op energieverbruik. Net als vorig onderzoek lieten verdere resultaten zien dat het activeren van DMH LepR-neuronen leidde tot gewichtsafname, als gevolg van verhoogde lichaamstemperatuur en locomotie. Om te zien of DMH LepR-neuronen een mogelijk doelwit kunnen zijn voor medicatie gericht op gewichtsverlies, onderzochten wij ook of gewichtsafname door DMH LepR-neuron activatie plaatsvond nadat muizen dik waren geworden op een calorierijk dieet. Dit was echter niet het geval, ondanks dat lichaamstemperatuur nog steeds verhoogde na activatie van DMH LepR-neuronen.

Obesitas is een groeiend probleem en er zijn maar weinig effectieve behandelingen beschikbaar. Fundamenteel onderzoek naar de regulatie van lichaamsgewicht opent nieuwe wegen naar een uitgebreider aanbod behandelingen. Dit proefschrift levert verdere inzichten in de rol van LepR-neuronen in verschillende hersengebieden van de muis in de regulatie van lichaamsgewicht. Met deze kennis hopen wij bij te dragen aan het zetten van stappen in de doorontwikkeling van behandelingen die gewichtsverlies stimuleren, waardoor deze op termijn gerichter kunnen worden ingezet.

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Véronne de Vrind, Jacqueline Scuvée-Moreaua, Guillaume Drion, Cyrine Hmaied, Fabian Philippart, Dominique Engel, Vincent Seutin. (2016) Interactions between calcium channels and SK channels in midbrain dopamine neurons and their impact on pacemaker regularity: Contrasting roles of N- and L-type channels. E*uropean Journal of Pharmacology* 788: 274-279; doi: 10.1016/j.ejphar.2016.06.046.

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