Ghrelin in feeding: new insights into its role and the neurocircuits involved

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À mes parents Christine & Guy
et
à Gurdeep

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To my parents Christine & Guy
and
to Gurdeep

Abstract

Appetite, originally evolved to ensure we consume enough of diverse nutrients to survive famines, has lost its survival advantage in our modern society, where food is plentiful. The hedonic aspect of appetite can indeed induce over-consumption of food, a major cause for the obesity pandemic. In this context, ghrelin, the only hormone known to promote feeding, is of particular interest because studying it helps us understand how food consumption is regulated and provides potential targets for the treatment of obesity. With the aim to further our understanding of the effects of ghrelin within the brain, we sought to investigate, first, the valence/emotion signal carried by ghrelin signalling in the brain and, second, novel central regions that mediate ghrelin's feeding effects.

Firstly, using simple behavioural tests measuring preference/avoidance in rats and mice, we demonstrate that ghrelin injection into the brain carries a negative valence signal, which leads to the animals avoiding situations paired with this injection. Secondly, our results show the hypothalamic supramammillary nucleus (SuM) to be a brain area activated by peripheral ghrelin injection as well as by anticipation of chow and palatable food, two physiological states associated with elevated ghrelin blood levels. Moreover, ghrelin delivery directly into the SuM could drive a feeding response. Thirdly, we found the lateral parabrachial nucleus (lPBN) of the brainstem, an area rich in ghrelin receptor (GHSR), to be a novel target for the effects of ghrelin on food intake and dietary choice, whereby intra-lPBN ghrelin injection increased consumption of both standard chow and high-fat diet when presented separately and induced an increase in only chow intake when the rats were offered a choice diet consisting of chow, lard and sucrose. This ghrelin treatment did not alter food motivation or reward as tested by sucrose-induced operant responding and conditioned place preference for chocolate, respectively. Fourthly, using *Ghsr-IRES-Cre* mice and a Cre-inducible viral vector, we provide evidence that the GHSR-expressing cells of the lPBN are necessary for the development of diet-induced body weight gain via a role in the regulation of energy intake (as opposed to energy expenditure) and dietary choice (notably sucrose intake). The lPBN GHSR-expressing cells were identified as a distinct population from the well-described anorexigenic lPBN cells containing the calcitonin gene-related peptide.

In summary, the work presented in this thesis determines the reinforcing properties of central ghrelin administration as being negative and identifies the SuM and lPBN as novel brain targets for ghrelin's effects on feeding. Furthermore, the GHSR-expressing cells of the lPBN are introduced as a neuronal population of importance in feeding and body weight control, thus providing a novel potential target for pharmacological therapies against obesity and other eating disorders.

Keywords: ghrelin, feeding, supramammillary nucleus, parabrachial nucleus

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Sammanfattning på svenska

Aptit utvecklades ursprungligen för att säkerställa att vi konsumerar tillräckligt av olika näringsämnen för att överleva. I vårt moderna samhälle där mat alltid finns tillgängligt skapar istället aptit problem, där god aptit kan framkalla överkonsumtion av mat, vilket är en viktig komponent i den fetmapandemin vi ser idag. I det här sammanhanget är hormonet ghrelin av särskilt intresse eftersom det är det enda kända hormon som ökar födointag. Ghrelinets verkningsmekanismer kan hjälpa oss att förstå hur konsumtion av mat regleras och ge ökad kunskap för att hitta nya behandlingsmetoder mot fetma. I syfte att öka vår förståelse för effekterna av ghrelin i hjärnan försökte vi i arbetet med den här avhandlingen dels undersöka om ghrelinsignalering i hjärnan kan ge upphov till positiva eller negativa känslor kopplade till hunger, och dels identifiera nya viktiga regioner i hjärnan som förmedlar ghrelinets effekt på födointag.

Med hjälp av enkla beteendestest som mäter preferens/undvikande hos råttor visar vi att ghrelin som administreras till hjärnan ger negativa känslor, eftersom råttor undviker situationer som de förknippar med en sådan administration. I en separat studie visar vi att ett specifikt område i hjärnan som heter supramammillär kärnan (SuM) aktiveras vid perifer administration av ghrelin. Det här området aktiveras normalt också vid förväntan innan en måltid, ett fysiologiskt tillstånd som är förknippat med förhöjda ghrelinnivåer i blodet. Vi fann även att administration av ghrelin direkt till SuM ger ökat födointag. Ytterligare ett område i hjärnan som vi visade vara viktig för ghrelinets effekt på födointag och matval är laterala parabrachial kärnan (lPBN) i hjärnstammen, ett område som är rikt på ghrelinreceptorer. Vi fann att administrering av ghrelin till lPBN ökade konsumtionen av dels standardfoder och dels fettrikt foder i två separata experiment. När vi studerade matval kunde vi framförallt se en ökning av intaget av standardfoder när råttorna erbjöds att fritt välja mellan standardfoder eller kaloririka alternativ som rent ister och rent socker. I beteendetester som mäter motivation (operant betingning) och belöning (konditionerad platspreferens) kunde vi se att administration av ghrelin till lPBN dock inte förändrade varken motivationen att äta mat eller graden av belöning från mat. Med molekylära verktyg som cre-inducerbara viralvektor och genmodifierade möss som utrycker cre endast i neuron som har ghrelinreceptorer kunde vi visa att de neuron i lPBN som utrycker

ghrelinreceptorer är nödvändiga vid utvecklingen av dietinducerad kroppsviktökning. Vi kunde även visa att dessa neuron är viktiga vid reglering av energiintag snarare än energiförbrukning, samt att de är viktiga vid matval. De neuron i lPBN som utrycker ghrelinreceptorer visade sig vara en ny grupp celler som skiljer sig från en grupp tidigare välbeskrivna anorexigena neuron i lPBN.

Sammanfattningsvis visar denna avhandling dels att centralt verkande ghrelin ger negativa känslor, och dels att SuM och lPBN är viktiga områden i hjärnan där ghrelin reglerar födointag. Vidare visar den här avhandlingen att de neuron i lPBN som utrycker ghrelinreceptorer är av betydelse för födointag och kroppsviktsreglering, och därmed ett potentiellt mål för farmakologiska behandlingar mot fetma och andra ätstörningar.

Résumé en français

L'appétit, qui à l'origine nous assure de consommer suffisamment de nutriments divers pour surmonter des périodes de famine, a perdu son avantage de survie dans notre société moderne, où la nourriture est très abondante. L'aspect hédonique de l'appétit peut en effet induire une surconsommation alimentaire, cause majeure de la pandémie d'obésité. Dans ce contexte, la ghréline qui est la seule hormone connue pour favoriser la consommation de nourriture, est particulièrement intéressante car l'étudier nous aide à comprendre comment la consommation alimentaire est régulée et fournit des cibles potentielles pour le traitement de l'obésité. Dans le but d'approfondir notre compréhension des effets de la ghréline dans le cerveau, nous avons cherché à déterminer le signal de valence ou émotion produit par la ghréline dans cet organe ainsi qu'examiner de nouvelles régions cérébrales pouvant intervenir dans les effets de la ghréline sur l'alimentation.

Premièrement, à l'aide de tests comportementaux simples mesurant la préférence et l'évitement chez le rat et la souris, nous démontrons que l'injection de ghréline dans le cerveau porte un signal de valence négatif qui conduit les animaux à éviter un endroit ou un goût associé à cette injection. Deuxièmement, nos résultats montrent que le noyau supramammillaire hypothalamique (SuM) est une zone cérébrale activée par l'injection périphérique de ghréline ainsi que par l'anticipation de nourriture associée à des niveaux élevés de ghréline dans le sang. De plus, nous montrons que l'administration de ghréline directement dans le SuM génère une prise alimentaire. Troisièmement, nous avons constaté que le noyau parabrachial latéral (lPBN) du tronc cérébral, une zone riche en récepteurs de la ghréline (GHSR), est une nouvelle cible pour les effets de la ghréline sur l'apport alimentaire et le choix de nourriture. En effet, nous montrons que l'injection intra-lPBN de ghréline augmente la consommation de nourriture standard et de celle à forte teneur en matières grasses lorsqu'elles sont présentées séparément. En revanche, lorsque les rats se voient offrir un régime de choix composé de nourriture standard, de saindoux pur et de sucre pur, ce même traitement induit uniquement une augmentation de la consommation de nourriture standard. Lors de la performance de tests comportementaux mesurant la motivation pour (réponse opérante pour du sucre) et la récompense associée à de la

nourriture appétissante (préférence de place conditionnée pour du chocolat), l'injection de ghréline directement dans le lPBN n'affecte pas ces comportements. Quatrièmement, en utilisant des souris génétiquement modifiées qui contiennent l'enzyme Cre uniquement dans les cellules exprimant le GHSR ainsi qu'un vecteur viral inductible par Cre, nous montrons que les neurones exprimant le GHSR dans le lPBN sont nécessaires au développement de gain de poids corporel induit par l'alimentation. Plus particulièrement, ces neurones ont un rôle dans la régulation de la prise alimentaire (et non de la dépense énergétique) et des choix alimentaires (notamment la consommation de sucre). Ces cellules du lPBN exprimant le GHSR ont été identifiées comme une population distincte des neurones anorexigéniques bien connus dans le lPBN.

Pour conclure, les travaux présentés dans cette thèse déterminent les propriétés de renforcement de l'administration de ghréline dans le cerveau comme étant négatives et identifient le SuM et le lPBN comme de nouvelles cibles cérébrales pour les effets de la ghréline sur l'alimentation. De plus, les cellules du lPBN qui expriment le GHSR sont introduites comme étant une population neuronale importante dans le contrôle de l'alimentation et du poids corporel, fournissant ainsi une nouvelle cible potentielle pour les thérapies pharmacologiques contre l'obésité et autres troubles de l'alimentation.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Central administration of ghrelin induces conditioned avoidance in rodents**
Schéle E, Cook C, Le May M, Bake T, Luckman SM, Dickson SL
European Neuropsychopharmacology, 2017; 27: 809-815.

II. **Activation of the rat hypothalamic supramammillary nucleus by food anticipation, food restriction or ghrelin administration**
Le May MV*, Hume C*, Sabatier N, Schéle E, Bake T, Bergström U, Menzies J, Dickson SL
Journal of Neuroendocrinology, 2019; e12676.
*Le May MV and Hume C contributed equally to this work.

III. **Ghrelin receptor stimulation of the lateral parabrachial nucleus in rats increases food intake but not food motivation**
Bake T, Le May MV, Edvardsson CE, Vogel H, Bergström U, Albers MN, Skibicka KP, Farkas I, Liposits Z, Dickson SL
Submitted

IV. **Silencing the GHSR neurones of the lateral parabrachial nucleus in mice protects against diet-induced weight gain and alters food choice**
Le May MV, Peris-Sampedro F, Stoltenborg I, Adan RAH, Dickson SL
Manuscript

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Abbreviations

AAV	Adeno-associated viral vector
aCSF	Artificial cerebrospinal fluid
AgRP	Agouti-gene related peptide
α-MSH	Alpha-melanocyte stimulating hormone
ARC	Arcuate nucleus of the hypothalamus
BNST	Bed nucleus of the stria terminalis
CCK	Cholecystokinin
CeA	Central nucleus of the amygdala
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CPP/CPA	Conditioned place preference/avoidance
CFP/CFA	Conditioned flavour preference/avoidance
DMH	Dorsomedial nucleus of the hypothalamus
EGFP	Enhanced green fluorescent protein
GABA	Gamma amino butyric acid
GH	Growth hormone
GHSR	Growth hormone secretagogue receptor (ghrelin receptor)
GI	Gastrointestinal

GLP-1	Glucagon-like peptide 1
HFHS	High-fat high-sugar
GOAT	Ghrelin-O-acyl-transferase
I.c.v.	Intracerebroventricular
I.p.	Intraperitoneal
LatH	Lateral hypothalamus
LDTg	Laterodorsal tegmental area
MC4R	Melanocortin-4 receptor
NAcc	Nucleus accumbens
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
PBN	Parabrachial nucleus (lPBN is the lateral PBN)
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus of the hypothalamus
PYY	Peptide tyrosine tyrosine
S.c.	Subcutaneous
SCM	Sweetened condensed milk
SuM	Supramammillary nucleus
TetoxLC	Tetanus toxin light chain
VMH	Ventromedial nucleus of the hypothalamus
VTA	Ventral tegmental area

Introduction

In many countries, it is common to wish each other a good meal as we start eating. In French, we say “bon appétit!” which translates as wishing someone to have a good appetite... but what is really behind this term “appetite”?

Appetite describes the natural desire to eat food, including specific food components. It is an umbrella term that covers “hunger” (indicative of the need to eat in situations of energy deficit) as well as the wish to eat specific foods because we find them palatable or desirable. Thus, hunger emanates from our metabolic need for calories and nutrients present in foods, whereas appetite also includes eating that surpasses metabolic need. From an evolutionary perspective, hunger is key for survival as it drives us to seek out foods in our environment and maintain our energy balance. The desire to eat foods we find palatable/rewarding also provides an evolutionary advantage because it helps us to seek out and consume foods of diverse nutritional composition and to over-eat, such that we gain sufficient energy stores to prepare for a future famine. However, in our modern society where food is plentiful, the hedonic aspect of appetite can drive over-consumption of foods, a major contributor to the obesity pandemic.

The neuronal circuits engaged in appetite comprise extensive pathways found throughout the brain, including those that detect energy deficit as well as hunger and satiety signals coming from the gastrointestinal tract and other peripheral tissues, those that predict and process the energy and reward value of foods and those that drive behaviours adapted to the nutritional status. The discovery, in 1994 by the group of Jeffrey Friedman, that there exists an endocrine signal derived from adipose tissues (leptin) that acts within the brain to control body weight homeostasis (Zhang et al., 1994), opened a window to unravel the specific neuronal pathways in the brain that control food intake and food-linked behaviours as well as those that regulate energy expenditure.

Many circulating anorexigenic hormones other than leptin also target pathways controlling feeding behaviours and energy balance. One example

is the pancreatic hormone, insulin, the first endocrine signal attributed a role in the control of body weight by the brain (Woods et al., 1974); it enters the brain and acts on the same "appetite-regulating" neurones as leptin in the hypothalamus (Porte et al., 2002). On the other hand, many gastrointestinal hormones reduce food intake by targeting neurones in the brainstem via vagal afferents. For example, cholecystokinin (CCK) is known to induce meal termination (Gibbs et al., 1973; Kissileff et al., 1981) while glucagon-like peptide-1 (GLP-1) and peptide YY (3-36) (PYY_{3-36}) have long-term anorectic effects reducing body weight (Zander et al., 2002; Batterham et al., 2002 ; Batterham et al., 2003).

Ghrelin, synthesised by enteroendocrine cells in the gastric mucosa (Kojima et al., 1999), stands alone as the only circulating hormone to increase food intake (Wren et al., 2000). Therefore, it is of great interest to understand how ghrelin promotes orexigenic behaviours - including the neural substrates and pathways engaged - since this information is expected to provide new insights into the aetiology of diseases associated with over- or under-eating behaviours and it may also pave the way towards novel treatments.

A great deal is already known about the mechanisms and sites of action of ghrelin in controlling food-linked behaviours (see reviews Muller et al., 2015 and Yanagi et al., 2018). The work presented in this thesis is built around gaps in knowledge, especially concerning less studied brain areas where the ghrelin receptor is expressed as well as novel aspects of ghrelin-linked behaviours not previously reported.

NEURONAL PATHWAYS REGULATING FEEDING BEHAVIOURS

In order to deconstruct feeding into its various components, in terms of behaviour involved and neuroanatomical substrates engaged, food intake is often described as either homeostatic or non-homeostatic. *Homeostatic* implies that food intake is driven by states of negative energy balance, a function often designated to areas in the hypothalamus and brainstem. *Non-homeostatic* is often used to describe food intake that can surpass metabolic need, reflecting environmental and cognitive aspects of feeding as well as the palatability and pleasure of eating available foods. In this case, areas linked to 'liking' (opioid pathways in the nucleus accumbens (NAcc)) and

'wanting' of food (mesolimbic dopamine system) alongside cortico-limbic pathways are attributed a role (Berridge et al., 2009). This division has been used to make it easier to identify and study feeding circuits and their corresponding food-linked behaviours (Zheng and Berthoud, 2007). In reality, however, the hypothalamus and brainstem are also engaged when eating for pleasure and reward circuits are also engaged when we seek to re-gain energy balance. For instance, the midbrain dopamine system is heavily engaged in situations of energy deficit, increasing the motivational salience of foods (see review by Lockie and Andrews, 2013). This helps restore energy balance in the short term but also ensures over-consumption of available foods. Moreover, as we shall see, many brain areas linked to the homeostatic eating also drive reward-linked and other more complex food-linked behaviours.

Thus, a key milestone in feeding research was the discovery of leptin and its central signalling pathways. This work paved the way to our current understanding of the key brain areas involved in the regulation of feeding. As will become apparent, many of the brain targets for leptin's anorexigenic effects are relevant also for ghrelin's orexigenic effects (Skibicka and Dickson, 2011).

The importance of leptin for uncovering neural pathways engaged in energy homeostasis

Early studies, involving brain lesion, identified a role for specific hypothalamic areas in controlling body weight: the ventromedial nucleus (VMH) was designated the *satiety centre* since lesion caused excessive eating and profound obesity, whereas the lateral hypothalamus (LatH) was identified as the *hunger centre* as animals bearing lesions of this area starved to death (Anand and Brobeck, 1951). In the 1950s, parabiosis studies indicated the presence of a circulating factor that signals to these brain areas to control feeding behaviours and body weight (Hervey, 1959). The first identified satiety factor was leptin, identified through positional cloning (Zhang et al., 1994). Leptin is an adipocyte-derived hormone that is secreted in proportion to fat mass and acts to suppress feeding and promote energy expenditure in animals and humans (Caron et al., 2018). Its discovery was a major breakthrough in energy balance research and also a promising potential treatment for obesity at the time. Yet, research soon revealed that

patients suffering from obesity as well as obese animals already had high circulating leptin levels and that they had developed resistance to leptin (Considine et al., 1996; Frederich et al., 1995; Halaas et al., 1997). Nevertheless, leptin and the neurocircuits mediating its effects have been extensively studied over the years. Leptin's effects on food intake and energy homeostasis appear to target especially the hypothalamus and, in particular, the arcuate nucleus (ARC), VMH and the dorsomedial hypothalamus (DMH), which are known to form connections with other brain regions such as the midbrain and brainstem (Wilson and Enriori, 2015; Pandit et al., 2017; Munzberg and Morrison, 2015). It is now known that leptin's effects are not limited to these hypothalamic and brainstem targets. It was shown, for example, that leptin-deficient human subjects have a heightened striatal (reward system) activation by pictures of food, an effect that could be suppressed by leptin administration (Farooqi et al., 2007).

Hypothalamus

Arcuate nucleus

The ARC is often considered an 'entry point' to the homeostatic feeding circuits because of its proximity to the portal blood circulation, and its responsiveness to many circulating hormones influencing feeding including leptin, ghrelin and insulin (Scott et al., 2009; Zigman et al., 2006; Varela and Horvath, 2012). In relation to food intake, two opposing cell groups in the ARC have been the centre of much investigation: the anorexigenic proopiomelanocortin (POMC)-expressing neurones that also contain cocaine- and amphetamine-related transcript (CART) and the orexigenic agouti-related protein (AgRP)/neuropeptide Y (NPY)/gamma amino butyric acid (GABA)-co-expressing neurones. POMC neurones are activated by leptin (Cowley et al., 2001) and inhibited by energy deficit associated with elevated ghrelin levels (Mizuno et al., 1998; Tschöp et al., 2000), whereas AgRP/NPY/GABA neurones are activated by ghrelin (Dickson and Luckman, 1997; Hewson and Dickson, 2000; Cowley et al., 2003) and inhibited by leptin and insulin (Korner et al., 2001; Niswender et al., 2004). POMC is cleaved into α-melanocyte stimulating hormone (α-MSH) which binds to melanocortin-4 receptors (MC4R) expressed in the paraventricular nucleus (PVN), the LatH and other areas outside the hypothalamus, thereby reducing food intake and increasing energy expenditure (Gantz et al., 1993;

Cone, 2005). On the other hand, NPY acts independently of the melanocortin system at the level of the PVN and LatH, while AgRP acts as an inverse agonist on the MC4R, thereby potentiating the orexigenic effects of NPY (Loh et al., 2015; Ollmann et al., 1997). Moreover, GABA contained in AgRP/NPY neurones, exerts a tonic inhibition of POMC neurones, further reducing their anorexigenic effects (Cowley et al., 2001). AgRP/NPY neurones are also known to send a inhibitory GABAergic projection to the lateral parabrachial nucleus (see page 8) to induce feeding (Wu et al., 2009).

Both of the aforementioned populations of ARC neurones have been shown to play an essential role in the regulation of feeding. Indeed, POMC and MC4R deficiency leads to hyperphagia and obesity in both humans and mouse models (Krude and Gruters, 2000; Martinelli et al., 2011; Yaswen et al., 1999; Sutton et al., 2006). In addition, while neonatal loss of NPY/AgRP neurones has no effect in adulthood, acute ablation of these neurones in adulthood blunts appetite and leads to starvation in mice (Luquet et al., 2005; Gropp et al., 2005). Furthermore, optogenetic studies revealed that 24h stimulation of the POMC neurones reduces food intake (although not seen for more acute stimulation), while stimulation of AgRP/NPY neurones acutely increased consumption of food over a 1h period in fed mice (Aponte et al., 2011).

Although primarily known as a homeostatic feeding centre, recent studies have shown the ARC to have a key role in the conditioned aspects of feeding. The group of Zachary Knight found that the activity of AgRP and POMC neurones in response to food detection are modulated by energy status as well as food palatability, denoting the modulation of ARC neurones by predictive food cues as well as hedonic information (Chen et al., 2015). Moreover, Scott Sternson and colleagues revealed that optogenetic inhibition of AgRP neurones conditions both a place and a flavour preference, indicating a role for AgRP neurones in avoidance. Deep-brain calcium imaging further showed that reduction in activity of AgRP neurones was triggered by exposure to food-related cues as well as by food itself (Betley et al., 2015). Later studies additionally revealed that consumption of novel low-energy foods reduces AgRP neural activity at a much slower rate compared to consumption of familiar caloric foods (Su et al., 2017). Together, these findings indicate that the feeding-related activity of AgRP neurones is regulated by feeding, by hedonic information and by previously learned experiences.

As we shall discover, the NPY/AgRP neurones in the ARC represent one major population of cells activated by ghrelin and ghrelin mimetics that are important for ghrelin's orexigenic effects (see page 15).

Second order brain regions

Among the hypothalamic regions receiving projections from the ARC, the PVN and the LatH appear to be the most relevant for feeding behaviours.

In the PVN, single-minded 1 (Sim1)-expressing neurones, receiving projections from AgRP neurones, have been attributed an anorexigenic role, as both chemogenetic inhibition and targeted ablation of these neurones produced hyperphagia (Atasoy et al., 2012; Xi et al., 2013). On the other hand, chemogenetic activation of the PVN MC4R-expressing neurones, downstream projection site of both AgRP and POMC neurones, and more specifically optogenetic stimulation of the MC4R-positive projections from the PVN to the PBN both suppress feeding (Garfield et al., 2015).

As for the LatH feeding pathways, the focus has been on neurones expressing melanin-concentrating hormone and orexin, which are considered orexigenic (Barson et al., 2013). However, the LatH is now known to contain diverse neuronal populations that receive/send projections from/to many areas in the central nervous system (CNS) and that have roles beyond sole homeostatic feeding (see review by Rossi and Stuber, 2018). Indeed, the LatH is a key integrative node linking the homeostatic and hedonic circuits that control food intake (Berthoud, 2011). The LatH orexin and melanin-concentrating hormone neurones not only act as metabolic sensors, but also send axonal projections to circuits involved in energy homeostasis (brainstem and ARC), hedonic pleasure, reward seeking (e.g. ventral tegmental area (VTA) and NAcc) and cognition (e.g. hippocampus and 'ingestive cortex').

Supramammillary nucleus (SuM)

One less studied hypothalamic area in the context of feeding control is the SuM. This nucleus is located in the posterior hypothalamic area, just

anterior to the VTA and dorsal to the mammillary bodies. Historically, the SuM has been established as an area involved in reward processes (Ikemoto and Bonci, 2014) and modulation of the hippocampus (Pan and McNaughton, 2004). Recent evidence linking the SuM to metabolic control include the observations that (i) there is an abundance of ghrelin binding sites in this area (Cabral et al., 2013), (ii) it expresses receptor for GLP-1, an anorexigenic peptide, (Lopez-Ferreras et al., 2019) and (iii) GLP-1 receptor agonist, exendin-4, (Lopez-Ferreras et al., 2019) as well as a GLP-1-oestrogen conjugate molecule (Vogel et al., 2016) affect appetitive and consummatory behaviours when injected at this site. Moreover, its location between the classically-termed homeostatic and reward centres as well as its widespread connections with regions known to regulate feeding (e.g. LatH, VTA, nucleus of tractus solitarius) suggest that the SuM could be an area important in integrating food-related information and driving complex feeding behaviours (Pan and McNaughton, 2004), a notion that we explore in **Paper II**.

Brainstem

Evidence for the importance of brainstem circuits in the control of fundamental feeding behaviours come from studies of decerebrate rats and anencephalic human neonates (with brains not developed beyond the midbrain). It was found that in these conditions, where the communication between the forebrain and caudal brainstem is inexistent, taste-driven oral-motor responses are identical to those of intact rats and normal neonates, respectively (Grill and Norgren, 1978; Kaplan and Grill, 1989; Steiner, 1973).

Nucleus of tractus solitarius (NTS)

Among the multitude of brain regions that contribute to the regulation of energy homeostasis, the NTS probably receives and processes the largest amount of neuronally mediated and circulating energy-associated signals. Indeed, in addition to the responsiveness of NTS neurones to leptin and ghrelin (Patterson et al., 2011; Zigman et al., 2006), this region also receives vagal glutamatergic and serotonergic transmission of gastric distension

(Ritter, 2004) as well as vagal-mediated signalling from many intestine-derived satiation signals such as CCK, serotonin, PYY_{3-36} and GLP-1 (Moran, 2006; Chaudhri et al., 2006).

Furthermore, the NTS receives inputs from many brain regions involved in feeding control. For example, as much as one quarter of the orexin-expressing neurones of the LatH have been shown to project to brainstem areas including the NTS suggesting a strong influence of the LatH neuronal activity on NTS neurones (Ciriello et al., 2003; Zheng et al., 2005). In fact, medial (m)NTS neurones are inhibited and the excitatory signalling from gastric distension to mNTS neurones reversed by electrical stimulation of LatH neurones (Jiang et al., 2003). The NTS neurones also densely express MC4R (Kishi et al., 2003) and receive α-MSH from the ARC POMC neurones (as well as from NTS POMC neurones) (Zheng et al., 2010). NTS delivery of a MC4R agonist decreases food intake and increases core temperature, whereas antagonist delivery has the opposite effects (Skibicka and Grill, 2009; Williams et al., 2000). Surprisingly, NTS MC4R signalling appears to contribute to the pathways mediating the anorexigenic effects of hypothalamic leptin signalling as NTS injection of a MC4R antagonist reduces the feeding effect of ARC leptin delivery (Zheng et al., 2010).

Parabrachial nucleus (PBN)

Within the PBN there exists a population of neurones that powerfully suppress food intake, hence, this area has been attributed an anorexigenic role. The PBN is separated in two parts based on their position relative to the superior cerebellar peduncle (scp); the medial (m)PBN is medial to the scp and the lateral (l)PBN lateral to the scp. The lPBN receives inhibitory inputs from the AgRP neurones of the ARC to allow feeding to occur. Indeed, acute elimination of GABA signalling from AgRP neurones induces an aberrant activation of PBN neurones and leads to starvation (Wu et al., 2009). Works from the group of Richard Palmiter have identified the lPBN neurones expressing calcitonin gene-related peptide (CGRP) and projecting to the central nucleus of the amygdala (CeA) as the population that mediate appetite suppression. Acute activation of these neurones inhibits feeding and induces aversion (Carter et al., 2013; Carter et al., 2015). The excitatory inputs to the PBN CGRP cells that lead to appetite suppression have been shown to originate from NTS glutamatergic neurones as well as from caudal serotonergic neurones (Wu et al., 2012). Yet, the lPBN also receive a

glutamatergic projection from PVN MC4R neurones, which upon optogenetic stimulation suppresses food intake as well (Garfield et al., 2015).

Surprisingly, considering its potent role in appetite suppression, however, the lPBN markedly express the receptor for the orexigenic hormone ghrelin (Zigman et al., 2006). The role of the ghrelin receptor at this site remains to be explored and forms an important question that we address in **Paper III** and **Paper IV** of this thesis.

It is established that the PBN also has a critical role in taste processing in rodents, although it seems to be bypassed in primates. It receives gustatory information from the rostral part of the NTS, making the PBN the second-order gustatory relay in rodents (Norgren and Leonard, 1973; Norgren and Pfaffmann, 1975).

Reward system in feeding

Food is a natural reward that becomes especially salient when hungry. Incentive motivation for all attractive stimuli including natural rewards such as food engages the mesolimbic dopamine pathway that originates in the VTA and projects to the NAcc in the ventral striatum. Dopamine release in the NAcc is involved in the neural mediation of the rewarding properties of foods and consumption of palatable food results in increased extracellular dopamine levels in the NAcc (Hernandez and Hoebel, 1988). In addition to these food-related effects, NAcc dopamine release can also be evoked by conditioned cues associated with food reward, underscoring the role of dopamine in the control of conditioned incentive motivation (Roitman et al., 2005; Schultz, 1998). While opioid signalling within the NAcc encodes the hedonic value or 'liking' of food, dopamine is thought to be crucial for motivation or 'wanting' of food. Berridge and colleagues defined this dopamine driven motivation as the incentive salience associated with a stimulus, i.e. the motivational drive generated in the brain to reward-predicting food (Berridge et al., 2009). Dopamine neurones also appear to encode reward prediction error; their activity increases when the reward is greater than expected, remains unchanged when the reward matches the prediction and decreases when the reward is less than predicted (see review by Schultz, 2016). This means that the firing of dopamine neurones is most pronounced when foods are novel or more pleasurable than expected.

Overall, the role of the midbrain dopaminergic neurones appears to be to increase approach behaviour for salient stimuli.

Dopamine itself is essential for feeding as dopamine-deficient mice are severely aphagic (Zhou and Palmiter, 1995). In contrast, when NAcc dopamine was selectively depleted, the willingness of rats to exert effort to obtain food was drastically reduced while normal food intake was unaltered (Aberman and Salamone, 1999) supporting the idea that dopamine is key for incentive salience for food but not for food consumption itself.

Evidence that the dopamine system is engaged during energy deficit includes studies in rats showing that chronic food restriction potentiates LatH self-stimulation that rats find rewarding. The key metabolic signal here could be leptin since it attenuated this effect when delivered by intracerebroventricular (i.c.v.) injection (Fulton et al., 2000). Indeed, immunohistochemical studies showed expression of the leptin receptor in the midbrain dopamine system (Figlewicz et al., 2003). In addition, reduction of leptin receptor expression at this site revealed a regulatory role of leptin signalling in midbrain dopamine cells on food motivation and NAcc dopamine release (Davis et al., 2011). On the other hand, ghrelin, associated with short-term energy deficiency, stimulates dopamine release in the NAcc (Jerlhag et al., 2006) and enhances phasic dopaminergic neuron activity as well as NAcc dopamine levels evoked by food-related cues (Cone et al., 2015). The effect of ghrelin on the midbrain dopamine system will be expanded on page 15.

The VTA dopaminergic neurones are known to receive inputs from the LatH (Zheng et al., 2007), the bed nucleus of the stria terminalis (BNST; Georges and Aston-Jones, 2002), the laterodorsal tegmental nucleus (LDTg; Cornwall et al., 1990) and the PBN (Coizet et al., 2010) and send dense projections to the prefrontal cortex (Morales and Root, 2014) and the CeA (Leshan et al., 2010) in addition to the NAcc (Beier et al., 2015).

Limbic pathways

Hippocampus

The hippocampus has recently been recognised as a brain region that integrates the environmental and internal context as well as memory and cognitive information to control feeding behaviours. In particular, the

hippocampus is important in the creation of episodic meal-related memories and conditional learnt associations. Moreover, hippocampal sub-regions express the receptor for key endocrine signals linked to feeding (e.g. leptin, ghrelin, GLP-1) and are well connected to neurocircuits controlling feeding (see review by Kanoski and Grill, 2017).

Extended amygdala

The amygdala is a key brain area linking feeding with reward and emotion (Murray, 2007). As reviewed by Hans-Rudolph Berthoud, the amygdala is closely connected to the hypothalamus, the midbrain, striatum as well as limbic and cortical pathways involved in feeding regulation (Berthoud, 2007). Of relevance for this thesis, certain nuclei of the amygdala express the ghrelin receptor, as seen by *in situ* hybridisation, and mediate the ghrelin-induced orexigenic response, as shown by intra-amygdala ghrelin injections (Alvarez-Crespo et al., 2012).

The brain regions involved in the regulation of feeding behaviours are presented in **Figure 1**.

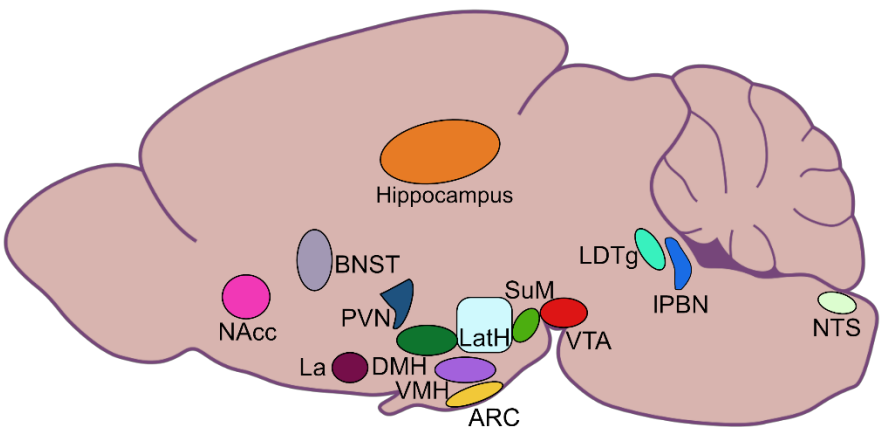

Figure 1. Simplified representation of the brain regions involved in feeding control. *ARC: hypothalamic arcuate nucleus; BNST: bed nucleus of the stria terminalis; DMH: hypothalamic dorsomedial nucleus; La: lateral amygdaloid nucleus; LatH: lateral hypothalamus; LDTg: laterodorsal tegmental area; NAcc: nucleus accumbens; NTS: nucleus of tractus solitarius; lPBN: lateral parabrachial nucleus; PVN: hypothalamic paraventricular nucleus; SuM: supramammillary nucleus; VTA: ventral tegmental area.*

GUT-BRAIN AXIS

The presence of a "gut-brain axis", a term coined to describe the bi-directional interaction between the gut and the brain, was established many years ago (Almy, 1989). Studies from the past centuries revealed a brain to gut regulation of gastrointestinal (GI) function by cognition, emotions and stress alongside a gut to brain route that mediates many information about the state of the GI tract (e.g. gastric distension) and the ingested food including potential toxins (see Al Omran and Aziz, 2014). The known gut signals relayed to the brain consist of nutrients, metabolites, taste, microbial products, cytokines, immune cells as well as hormones, all transmitted by vagal afferents, the blood circulation or the spinal cord (see review by Mayer, 2011).

Of all these gut signals, the gut-derived peptides and taste-related signals are thought to be the most relevant for the regulation of feeding behaviours. The transmission of taste-related information is briefly described on page 9 so this section will focus on gut hormones. Since the 1900s, more than forty regulatory peptides have been identified in the GI tract (Rehfeld, 2014) with some carrying a satiety or satiation signal (CCK, GLP-1, PYY_{3-36}) and ghrelin carrying a hunger signal (Al Omran and Aziz, 2014). Although all of these signals are important and have diverse roles in feeding control, I will limit further introduction to ghrelin, since my experimental work focused in depth on this hormone.

Ghrelin

Ghrelin was discovered in 1999 by the group of Kenji Kangawa, who identified it as the first (and as yet only) known endogenous ligand for the growth hormone secretagogue receptor 1A (GHSR-1a) (Kojima et al., 1999). It is a circulating 28 amino acid peptide hormone produced and secreted mainly by the stomach. At the time of its discovery, the physiological role attributed to ghrelin was the stimulation of growth hormone (GH) secretion, since much work preceding its discovery documented potent growth hormone-releasing effects of synthetic GHSR-1a ligands, the so-called "GH secretagogues" (GHS). The GH-releasing effect of these compounds involves both a direct pituitary action (Bowers et al., 1984) and also a direct effect on the hypothalamic GH-releasing hormone neurones (Dickson et al., 1993). Given that GH is lipolytic, it was a surprising discovery that chronic

treatment with GHS (Lall et al., 2001) and ghrelin (Tschöp et al., 2000) increased fat mass in rodents. Ghrelin is orexigenic in humans and rodents (Tschöp et al., 2000; Wren et al., 2001b; Wren et al., 2001a), exerting potent effects especially after i.c.v. injection (Wren et al., 2001b).

Ghrelin secretion is increased by fasting (Tschöp et al., 2000) and weight loss (Cummings et al., 2002) and its blood levels fluctuate during the day with an increase before meals and a reduction afterwards, suggesting a role in meal initiation (Cummings et al., 2001). The orexigenic effect of ghrelin, via GHSR-1a binding, requires the ghrelin molecule to be acylated by the ghrelin-O-acyl-transferase (GOAT), thereby resulting in the presence of two forms in the blood: the nonacylated, accounting for up to 90 % of circulating ghrelin and the acylated, known as the active form.

Growth hormone secretagogue receptor 1a

The GHSR-1a is a G protein-coupled receptor that was identified in 1996 in the anterior pituitary and hypothalamus as the receptor for GH secretagogues known to stimulate pituitary GH release (Howard et al., 1996). In the hypothalamus, GHSR-1a is abundantly expressed in the ARC (notably on orexigenic AgRP/NPY neurones (Willesen et al., 1999)) but also in other areas of relevance for feeding control such as the VMH, LatH and DMH (Guan et al., 1997; Zigman et al., 2006). It is also expressed in midbrain regions linked to reward including the VTA (particularly on dopaminergic cells (Abizaid et al., 2006)) and the LDTg (Guan et al., 1997). Discrete populations of GHSR-1a-expressing cells have also been found in the hippocampus (Guan et al., 1997; Zigman et al., 2006), where it has been attributed a role in memory formation (Diano et al., 2006) as well as in food motivation and cue-induced feeding (Kanoski et al., 2013) and in the amygdala where a role in anxiety-like behaviour has been proposed (Alvarez-Crespo et al., 2012). In the brainstem, GHSR-1a is expressed by cells of the area postrema, NTS as well as cells of the PBN (Zigman et al., 2006), the latter of which were specifically studied in this thesis in the context of feeding. Peripheral organs, namely the pancreas, adrenal gland, thyroid and myocardium, also express GHSR-1a (Gnanapavan et al., 2002).

Although primarily known as the receptor for ghrelin, GHSR-1a displays a uniquely high constitutive activity (signalling at ~50 % of its capacity in the absence of ghrelin (Holst et al., 2003)), meaning that GHSR-1a transmits

signals independently of ghrelin binding to it. This was shown to be of importance in energy homeostasis by studies revealing that central administration of inverse agonists that block GHSR-1a constitutive activity was sufficient to reduce both food intake and body weight in rats (Petersen et al., 2009; Els et al., 2012). Thus, it should be kept in mind while reading this thesis that GHSR-1a signalling alone appears to regulate energy balance and that ghrelin amplifies a signalling pathway that is already active thereby boosting its effect on feeding behaviours and body weight regulation.

In humans, a truncated non-functional isoform (GHSR-1b) is also transcribed from the gene encoding GHSR-1a (Gnanapavan et al., 2002) and cellular studies suggested the possibility that GHSR-1a and GHSR-1b exist as heterodimers, formation of which appears to impair both ghrelin signalling at the GHSR-1a and GHSR-1a constitutive activity (Leung et al., 2007). GHSR-1a is also known to form homodimers as well as heterodimers with other receptors important in feeding-related behaviours, including the dopamine receptor subtypes 1 (Jiang et al., 2006) and 2 (Kern et al., 2012) and the melanocortin-3 receptor (Rediger et al., 2011), which alters the receptors signalling. Heterodimerisation could turn out to be relevant for controlling the activity of ghrelin-responsive neurones including those studied in this thesis. But caution should be exerted since, although the concept of heterodimerisation is conceptually appealing, it remains to be demonstrated whether it occurs *in vivo* in situation relevant for ghrelin's effects.

Feeding effects and CNS targets for ghrelin

Ghrelin influences many aspects of food-related behaviours, stimulating both the appetitive and consummatory phases of feeding. The rise and decline of ghrelin blood levels before and during meals, respectively, points to the idea that ghrelin secretion is important in mechanisms that lead to meals as well as in the actual consumption of the food (Cummings et al., 2001). Notably, ghrelin injection impacts on meal patterns by decreasing the latency to feed and inducing a meal shortly after administration, without altering meal size or daily food intake (Faulconbridge et al., 2003).

Consistent with a role in meal patterns, ghrelin is of importance in food anticipation for both standard chow (Verhagen et al., 2011) and palatable food (chocolate; Merkestein et al., 2012) as well as in stimulation of food

foraging and hoarding, known as typical appetitive and food-motivated behaviours (Keen-Rhinehart and Bartness, 2005).

Furthermore, not long ago, studies revealed that ghrelin can influence food choice by increasing the intake of chow when animals are given access to a free choice diet consisting of lard, sucrose, chow and water (Schéle et al., 2016). This effect was even remarkably reproduced in animals that were trained to binge on a high-fat diet for 2 hours daily in addition to having *ad libitum* access to chow (Bake et al., 2017).

Early work with growth hormone secretagogues revealed that they activate sub-populations of ARC cells including GH-releasing hormone neurones (Dickson et al., 1993) and also the orexigenic NPY neurones (Dickson and Luckman, 1997) that we now know co-express AgRP (Broberger et al., 1998). It is widely accepted that these NPY/AgRP neurones are a major target for ghrelin's orexigenic effects, since ghrelin-induced food intake is blunted by NPY and AgRP antagonists (Nakazato et al., 2001) and completely abolished in mice deficient in both NPY and AgRP (Chen et al., 2004). In addition, almost all NPY/AgRP neurones express GHSR-1A mRNA (Willesen et al., 1999). However, it should be pointed out that ghrelin injection at many brain regions expressing GHSR-1a can induce a feeding response, including many hypothalamic and brainstem areas (Wren et al., 2001b; Faulconbridge et al., 2003; Zigman et al., 2006).

Besides, ghrelin also induces a feeding response when delivered to key nodes of the reward system, the VTA and NAcc (Naleid et al., 2005). In addition, ghrelin increases the activity of the VTA dopaminergic neurones as demonstrated by electrophysiological recordings from brain sections and peripheral ghrelin injection was shown to stimulate dopamine release within the NAcc (Abizaid et al., 2006; Jerlhag et al., 2006). The neurocircuitry engaged by ghrelin here is complex, however, the VTA→NAcc pathway appears to be important for ghrelin's effects on food motivation, rather than food intake *per se* (Skibicka et al., 2013). A potent effect of ghrelin on food reward and motivation, in accordance with the stimulation of the mesolimbic dopamine system by ghrelin, has indeed been reported in many studies using different paradigms, hence making clear that ghrelin increases the incentive salience or 'wanting' for rewarding foods (Skibicka et al., 2011b; Skibicka et al., 2012; Perello et al., 2010; Menzies et al., 2013). Yet, recent work showed that ghrelin also increases the motivation of rats to receive normal chow (Bake et al., 2019) and ghrelin may even act directly at the level of the VTA to alter food choice (Schéle et al., 2016).

Other parts of the limbic system are also important targets for ghrelin, in particular the hippocampus and the amygdala (Carlini et al., 2004). In the hippocampus, ghrelin improves memory retention while increasing meal frequency (Kanoski et al., 2013). At the level of the amygdala, its role appears to be to reduce anxiety-like behaviours and increase food intake (Alvarez-Crespo et al., 2012).

Within the brainstem, the exact function of GHSR-1a is poorly understood. This thesis, therefore, explored the role of ghrelin and GHSR-1a signalling within the PBN specifically on feeding behaviours.

Thus, ghrelin appears to engage most of the central regions important for feeding control (**Figure 2**), further underlying its key role in the regulation of energy balance.

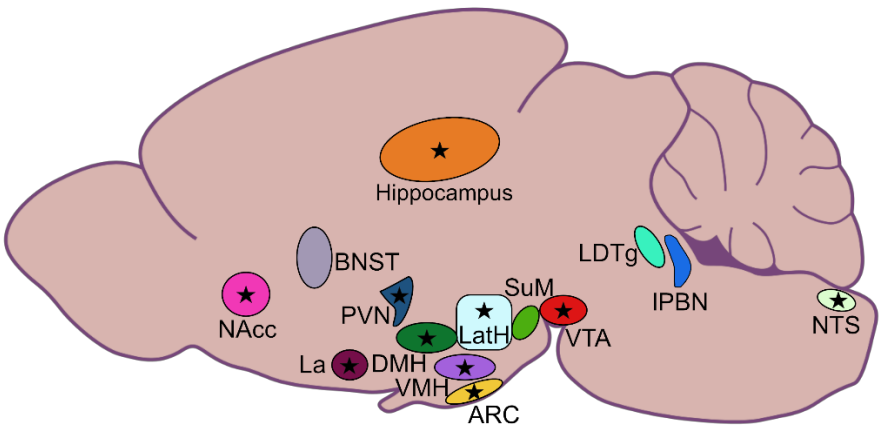

Figure 2. Simplified representation of the brain regions targeted by ghrelin for its effects on feeding (marked with a black star). *ARC: hypothalamic arcuate nucleus; BNST: bed nucleus of the stria terminalis; DMH: hypothalamic dorsomedial nucleus; La: lateral amygdaloid nucleus; LatH: lateral hypothalamus; LDTg: laterodorsal tegmental area; NAcc: nucleus accumbens; NTS: nucleus of tractus solitarius; lPBN: lateral parabrachial nucleus; PVN: hypothalamic paraventricular nucleus; SuM: supramammillary nucleus; VTA: ventral tegmental area.*

Ghrelin's access to the brain

The transport of ghrelin through the blood-brain barrier has been shown by early studies to be quite complex (Banks et al., 2002; Banks et al., 2008). Indeed, in mice, it was found that nonacylated mouse ghrelin and human ghrelin (only differing by 2 amino acids with mouse ghrelin) were both transported from the blood into the brain, but that acylated mouse ghrelin was only transported in the brain-to-blood direction (Banks et al., 2002). Peripheral ghrelin has since been shown to access areas of the brain particularly sensitive to circulating signals (due to a more permeable blood-brain barrier), including the ARC and area postrema (Schaeffer et al., 2013; Cabral et al., 2014), yet, not reaching deeper brain regions that are protected by the conventional blood-brain barrier (Cabral et al., 2014). Recent studies have, however, challenged the idea of limited ghrelin transport into the brain and suggested that ghrelin could enter the brain independently of the GHSR-1a (Rhea et al., 2018) or through the blood-cerebrospinal fluid barrier (Uriarte et al., 2019). Another interesting possibility, given that nonacylated ghrelin enters the brain more easily than the acylated form (Banks et al., 2002), is that the GOAT enzyme might activate the ghrelin molecule directly in the brain. This could especially be the case since GOAT expression is present in the hypothalamus (Gahete et al., 2010), its knockdown reduces body weight in rats fed a high-fat diet (Wellman and Abizaid, 2015) and food restriction as well as fasting (associated with elevated ghrelin levels (Ariyasu et al., 2001; Cummings et al., 2001; Drazen et al., 2006)) increase hypothalamic GOAT mRNA expression (Wellman and Abizaid, 2015). Furthermore, the question of whether or not ghrelin is synthesised in the brain itself has long been and still is a subject of debate (see reviews by Cabral et al., 2017 and Edwards and Abizaid, 2017).

Valence of ghrelin

Recent studies suggest that hunger feelings carry a negative valence (emotion) and that appetitive and food-seeking behaviours are motivated by the learnt alleviation of the negative valence when food is consumed

(Betley et al., 2015; Sternson and Eiselt, 2017). The negative valence signal of hunger appears to be transmitted by activity of the AgRP neurones of the ARC (Betley et al., 2015). Since AgRP neurones are also targeted by ghrelin, it follows that ghrelin could also confer a negative valence signal.

However, one of the other target populations for ghrelin, the midbrain dopamine neurones of the VTA, has an opposite impact on valence. Indeed, optogenetic studies revealed that phasic photostimulation of these neurones is positively reinforcing for rodents as seen by a preference for the chamber paired with the phasic stimulation in a conditioned place preference paradigm and dopamine release (Tsai et al., 2009). Hence, ghrelin action at this site would be expected to confer a positive valence signal.

As a result, ghrelin seems to increase food intake via activating discrete neuronal pathways that carry opposing reinforcing properties (**Figure 3**). While one circuit may induce feeding motivated by the relief of negative valence, the other might do so by signalling anticipation of a positive valence and reward (via the mesolimbic dopamine pathway).

In rodents, valence testing can be performed by conditioned place preference/avoidance (CPP/CPA) studies in which animals return to or avoid a chamber previously coupled to a given stimulus. Using this paradigm, a number of studies in mice have explored the valence signal carried by ghrelin. Curiously, the results are not in agreement. One study reported that systemic ghrelin administration induced a CPP (Jerlhag, 2008), whereas the other study showed that it induced a CPA (Lockie et al., 2015). In this thesis (**Paper I**) we ought clarity on this important issue and our hypothesis favoured a role for ghrelin as a negative valence signal, consistent with its role as a circulating hunger hormone.

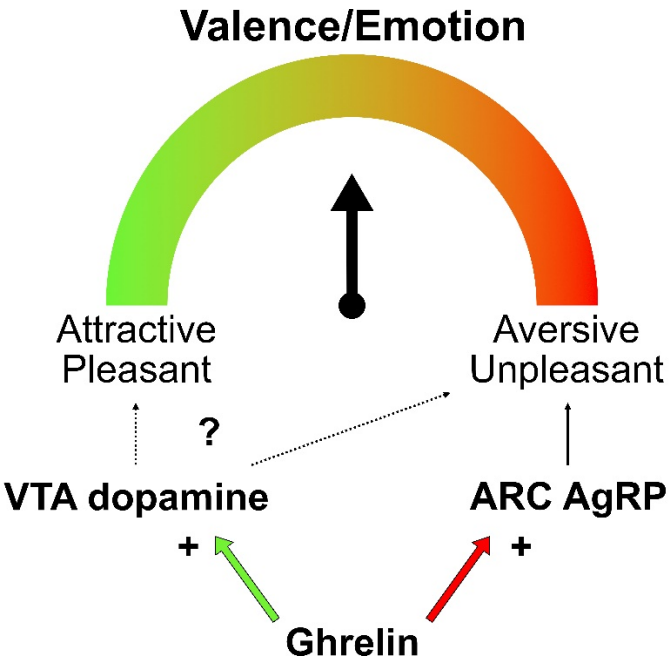

Figure 3. Simplified diagram of the two pathways engaged by ghrelin through which it could affect valence/emotion. *Activation of the ARC AgRP neurones is known to be aversive (Betley et al., 2015), whereas stimulation of the VTA dopaminergic neurones was shown to be positively reinforcing (Tsai et al., 2009). Thus, the VTA dopaminergic neurones are thought to carry a positive valence signal, while ARC AgRP neurones appear to carry a negative valence signal. Since, ghrelin activates both neuronal populations (Abizaid et al., 2006; Dickson and Luckman, 1997; Nakazato et al., 2001), the valence signal it carries could be either positive or negative but research has not provided a clear answer to this question. Work included in this thesis clarifies the reinforcing properties of ghrelin.*

Aims

The overall aims of this thesis were (i) to define the valence (emotion) signal carried by the hunger hormone ghrelin and (ii) to investigate unexplored neuronal populations responsive to ghrelin with regards to their neurochemical identity, circuitry and role in food-linked behaviours.

The specific aims were:

Paper I To determine whether central administration of ghrelin carries a positive- or a negative-valence signal in rats and in mice.

Paper II To investigate the influence of physiological states associated with elevated ghrelin blood levels as well as peripheral ghrelin administration on the activity of SuM neurones and to explore the effect of intra-SuM ghrelin delivery on feeding.

Paper III To test whether ghrelin action at the lPBN alters food-linked behaviours and whether peripheral ghrelin administration impacts on the activity of lPBN neurones.

Paper IV To study the role of GHSR-expressing neurones of the lPBN in food-linked behaviours and to determine their neurochemical identity.

Methodological considerations

Animals

The work of this thesis is based on studies carried out using male rats and mice. Specifically, Sprague-Dawley rats (**Papers I**, **II** and **III**) as well as C57Bl/6J (**Paper I**) and *Ghsr-IRES-Cre* (**Paper IV**) mice were used.

The *Ghsr-IRES-Cre* mouse line is a recently designed line in which the expression of Cre recombinase (Cre) is driven by the *Ghsr* promoter. These mice were obtained from Monash Animal Research Platform at Monash University (Australia). The generation of this mouse line is described in the work by Mani, Zigman and colleagues (Mani et al., 2017). As thereby explained, the *Ghsr-IRES-Cre* mouse line, when crossed to reporter mice, displays a pattern of Cre activity that is consistent with that seen using *in situ* hybridisation histochemistry for *Ghsr* (Guan et al., 1997; Zigman et al., 2006) and another *Ghsr* transgenic reporter mouse line (Mani et al., 2014). Yet, the *Ghsr-IRES-Cre* mouse line even appears to report more accurately and sensitively the expression pattern of GHSR-1a in the CNS compared to other techniques previously mentioned (Mani et al., 2017).

One limitation of this mouse line, however, could come from the potential fluctuations in GHSR-1a expression, and therefore Cre activity, during development. For instance, a strong temporary expression of GHSR-1a during fetal stages may induce strong Cre-induced changes in the brain that persist into adulthood, despite lower GHSR expression at that stage, an element that could question the fidelity of the *Ghsr-IRES-Cre* mouse line (Mani et al., 2017). We have ourselves noticed some disparities in Cre expression in some brain regions between mice of the same genotype and even litter. Yet, this complexity was not seen at the level of the lPBN where, in the contrary, Cre expression, as visualised via crossing *Ghsr-IRES-Cre* mice with ZsGreen reporter mice (from The Jackson Laboratory), was very consistent between mice.

As for the sex of the animals used in the projects presented herein, only male rats and mice were included as introducing more complexity into the already-complex projects was not favourable. In the case of the *Ghsr-IRES-Cre* mice especially, no work has been done using female mice yet, hence

too much was still unknown to decide to include females in our work at this stage. Working on female animals is always complicated by the oestrus cycle and potentially could require many more experimental groups.

All studies were approved by the local committee for animal welfare at either the Institute of Experimental Biomedicine at the University of Gothenburg (**Papers I**, **II**, **III** and **IV**), in accordance with the UK Home Office Animals Scientific Procedures Act 1986 in the UK (**Paper I** and **Paper II**) or at the Institute of Experimental Medicine at the Hungarian Academy of Sciences (**Paper III**) and in accordance with legal requirements of the European Commission. The specific ethical permits can be found in the relevant papers.

Drugs

Ghrelin administration

Rat ghrelin, purchased from Tocris (1465; Bristol, UK), was used for all ghrelin injection studies.

Peripheral injections of ghrelin

Intraperitoneal (i.p.) ghrelin injections at a dose of 100 µg/kg were carried out in rats in **Paper II** in which we sought to determine whether peripheral ghrelin administration induces a Fos response in SuM neurones. This dose of ghrelin i.p. was selected based on its ability to induce a feeding response in rats (Wren et al., 2000).

Ghrelin was administered intravenously (i.v.) in rats in **Paper II** and **Paper III** to find out the effect of peripheral ghrelin on mean spontaneous firing rate of SuM cells and on Fos expression in lPBN neurones, respectively. In **Paper II**, 10 µg ghrelin diluted in 100 µl saline was injected, which is an i.v. ghrelin dose known to induce Fos expression in the ARC of rats (Hewson and Dickson, 2000). In **Paper III**, 20 µg ghrelin diluted in 200 µl saline was injected. The dose was doubled in **Paper III** to make it more likely to see an effect.

Central injections of ghrelin

In **Paper I**, intracerebroventricular (i.c.v.) injections of ghrelin were performed to explore the nature of the valence signal carried by ghrelin. Ghrelin was also administered by i.c.v. injections in **Paper III** to check if such ghrelin injections induce a Fos response in lPBN cells. In both studies, 2 µg of ghrelin was injected i.c.v. in rats, a dose that has previously been shown to increase food intake in rats (Wren et al., 2000). These ghrelin injections were also performed in mice in **Paper I** to check the conservation of ghrelin valence signal in rodents. In mice, a dose of 1 µg ghrelin was used based on previous evidence that this dose engages the mesolimbic dopamine system (Jerlhag et al., 2006).

Ghrelin, diluted in artificial cerebrospinal fluid (aCSF) was also injected directly in the SuM and the lPBN in **Paper II** and **Paper III**, respectively, to investigate the effects of ghrelin action at these sites on feeding behaviours. In both studies, a low and a high dose (0.5 µg and 1 µg) of ghrelin were administered based on previous evidence that injection of the high dose intra-VTA as well as intra-NAcc induces an orexigenic effect (Naleid et al., 2005). In **Paper II**, the volume of injection intra-SuM was 0.3 µl whereas 0.5 µl was used intra-lPBN in **Paper III**. The intra-lPBN injection volume was based on the volume used for intra-VTA injections in previous work (Skibicka et al., 2013). The volume for intra-SuM ghrelin administration was reduced to 0.3 µl to minimize the diffusion of the injected ghrelin to neighbouring brain regions including the VTA.

Ghrelin application on brain sections

In **Paper III**, loose-patch clamp electrophysiology was used to record action currents of cells in the lPBN from brain sections and identify the effect of ghrelin on the activity of these cells. After 4 min of recording in control conditions, a single bolus of 4 µM ghrelin was added to the aCSF in the recording chamber. This dose had been used in a previous electrophysiological study (Alvarez-Crespo et al., 2012).

GHSR-1a antagonist, JMV2959

JMV2959, a GHSR-1a antagonist, was obtained from Aeterna Zentaris GmBH (AEZS-123; Frankfurt, Germany). All JMV2959 injections were

administered in overnight fasted rats (**Paper III**) to test the effect of this antagonist in a situation when ghrelin circulating levels are high (Tschöp et al., 2000). Rats received i.c.v. injections of JMV2959 at a dose of 10 µg in 1 µl aCSF based on previous studies (Salomé et al., 2009) and intra-lPBN JMV2959 injections of 1 µg or 2 µg in 0.5 µl aCSF according to intra-VTA doses used previously (Skibicka et al., 2011b). For loose-patch clamp electrophysiology, 10 µM JMV2959 was added to the aCSF bath as described previously (Alvarez-Crespo et al., 2012).

Surgical procedures

Surgeries were performed in both rats and mice in order to deliver either a drug/hormone or a viral vector in specific areas of the brain or a drug/hormone peripherally.

Anaesthesia

Rats

For surgeries performed at the University of Gothenburg (**Paper I**, **Paper II** and **Paper III**), the rats were anaesthetised by i.p. injection of a combination of Rompun® vet. (10 mg/kg; Bayer, Leverkusen, Germany) and Ketaminol® vet. (75 mg/kg; Intervet, Boxmeer, Netherlands).

For surgeries carried out at The University of Edinburgh (**Paper II**), the animals received an i.p. injection of either sodium pentobarbitone (200 mg/kg) for the experiments measuring c-Fos expression or ethyl carbamate (1.3 g/kg) for electrophysiological experiment.

At the Hungarian Academy of Sciences (**Paper III**), for loose-patch clamp recordings on brain sections, rats were anaesthetised using isoflurane inhalation and the brain was removed quickly.

Mice

For surgeries performed at the University of Manchester (**Paper II**), mice were anaesthetised by inhalation of 3 % isoflurane (Abbot Abbvie Ltd., Maidenhead, UK) in oxygen (1500 ml/min).

In **Paper IV**, the *Ghsr-IRES-Cre* mice received an i.p. injection of a combination of Sedastart vet.® (1 mg/kg; Produlab Pharma B.V., Raamsdonksveer, The Netherlands) and Ketalar® (75 mg/kg; Pfizer AB, New York City, USA).

Intracranial surgeries

I.c.v. cannulation (rats and mice)

In **Paper I** and **Paper III**, rats were implanted unilaterally with an i.c.v. cannula targeting the lateral ventricle. The animals were placed in a stereotaxic frame, the skull was exposed and bregma was identified. Holes were drilled in the skull for placement of the guide cannula and the anchoring screws. A 26-gauge cannula was positioned according to the stereotaxic coordinates and fixed in place with anchoring screws and dental cement (Dentalon, Heraeus Kulzer, Hanau, Germany). The following coordinates were used: 0.9 mm posterior to bregma, 1.6 mm lateral to the midline and 2.5 mm ventral of the skull surface. After surgeries, the rats were injected subcutaneously (s.c.) with an analgesic (Rimadyl; Orion Pharma Animal Health, Sollentuna, Sweden). The length of the injector was later adjusted (between 1.5 - 2.5 mm extension below cannula) to target the lateral ventricle, by injecting the animals i.c.v. with 20 ng angiotensin II (1158; Tocris, Bristol, UK) and checking for dipsogenic response (immediate water drinking) (Epstein et al., 1970).

Mice underwent the same procedure in **Paper I** with the following coordinates used: 0.4 mm posterior to bregma, 1 mm lateral to the midline and 1.2 mm ventral to the skull surface. A 23-gauge cannula was placed and fixed with an anchoring screw and dental cement (Simplex Rapid Powder, Kemdent, Swindon, UK; methyl methacrylate, Metrodent, Huddersfield, UK). The injector used was 0.5 mm longer than the cannula to target the lateral ventricle.

Intra-SuM cannulation (rats)

In **Paper II**, rats were implanted with a cannula directed at the SuM. The procedure was the same as for i.c.v. cannulation, the only difference being the coordinates used: 4.8 mm posterior to bregma, 0.7 mm lateral to the midline and 6.5 mm ventral to the skull surface with injector extending 2.5

mm below the cannula, resulting in a final depth of 0.9 mm. The injection placement was checked by visualising ink injected just prior to sacrifice.

Intra-lPBN surgeries (rats and mice)

Rats, in **Paper III**, were implanted unilaterally with a cannula targeting the lPBN using the following coordinates: 9.5 mm posterior to bregma, 2.0 mm lateral to midline and 4.5 mm ventral to skull surface aiming for a final depth of 6.5 mm with a 2.0 mm injector projection (Skibicka et al., 2011a, Richard et al., 2014). Correct placement was also verified by visualising inject ink post-mortem.

In **Paper IV**, *Ghsr-IRES-Cre* mice (heterozygous in TetoxLC group and wild-type in control group) were injected with a Cre-dependent viral vector (AAV-DIO-TetoxLC-EGFP; see next section for details) intra-lPBN using the following coordinates: 5.34 mm posterior to bregma; 1.3 mm lateral to the midline; 3.7 mm ventral of the skull surface at bregma. Once fully anaesthetized, the mice were placed in a stereotaxic frame and the skull was exposed. After the application of a local anaesthetic (Xylocaine 10 %, AstraZeneca, Cambridge, UK), two holes were drilled and the TetoxLC-expressing viral vector was injected (0.4 µl, 1.8 x 10^{12} particles/ml) bilaterally into the lPBN using a 31 gauge stainless steel needle (Coopers Needle Works Ltd., Birmingham, UK) connected via vinyl tubing to a Hamilton syringe in an infusion pump. After injection, the injection needle remained in place for an additional 7 min and was then retracted slowly to ensure full diffusion from the needle tip. Once the surgery was complete, animals were injected with the sedation-reversing Sedastop vet.® (2.5 mg/kg s.c.; Produlab Pharma B.V., Raamsdonksveer, The Netherlands), individually housed and allowed to recover for at least a week.

Cre-dependent viral vector: AAV-DIO-TetoxLC-EGFP

To decipher the role of the $GHSR^{lPBN}$ neurones in feeding behaviours, these cells were permanently silenced by intra-lPBN injection of a Cre-dependent viral vector expressing tetanus toxin light chain (AAV1-CBA-DIO-EGFP-TetoxLC; Carter et al., 2015) in *Ghsr-IRES-Cre* mice. This means that only the GHSR-expressing cells of the lPBN expressed tetanus

toxin light chain (TetoxLC). TetoxLC impairs synaptic vesicle exocytosis thereby blocking synaptic transmission (Yamamoto et al., 2003).

Jugular vein catheterisation

In **Paper III**, rats were anaesthetized and a small incision made below the neck. The jugular vein was exposed and blood flow clamped with sutures. A plastic catheter consisting of a soft tubing (4 cm) overlapping (0.5 - 0.75 cm) with a PE-50 tubing (20 cm) was inserted through a small hole in the jugular vein and secured in place with sutures. The catheter was then exteriorised at the nape of the neck and again maintained in place with sutures (Luckman et al., 1999). Finally, both incisions were stitched up and the catheter was plugged with a metal clock pin to prevent blood loss. After recovery and before injections, the catheters were flushed 3 to 4 days to maintain their patency.

Behavioural testing

Conditioned place preference test

Conditioned place preference/avoidance is a standard behavioural paradigm based on the principles of Pavlovian conditioning, that is used to study the rewarding or aversive properties of a treatment or drug (Prus et al., 2009). In the context of this thesis, the CPP/CPA apparatus consisted of two chambers with distinct visual and tactile qualities separated by a guillotine door as shown in **Figure 4** (Med Associates Inc., St Albans, VT, USA). CPP/CPA testing was performed in **Paper I** in rats and mice and in **Paper III** only in rats.

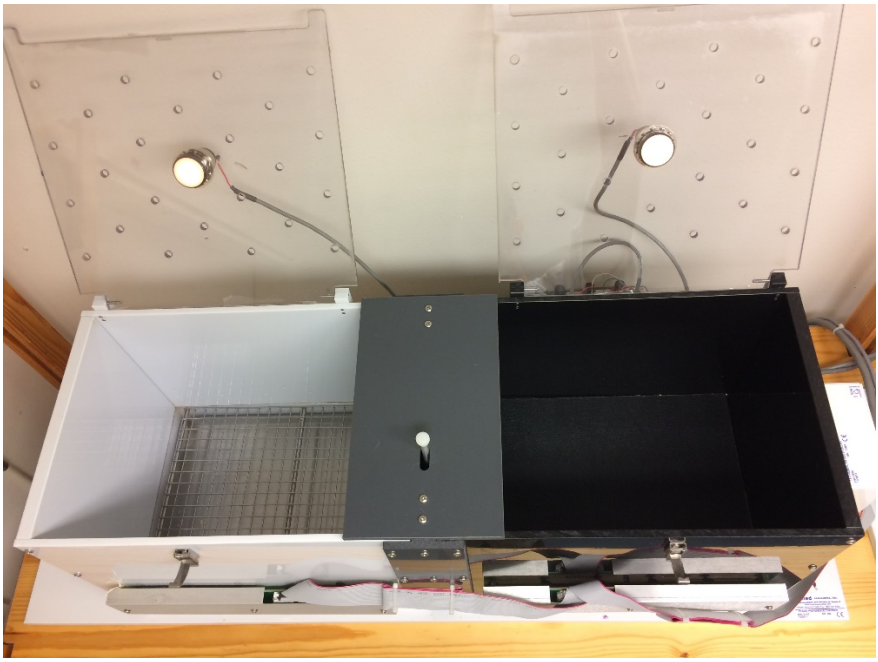

Figure 4. *Standard two-chamber conditioned place preference apparatus.*

The CPP/CPA paradigm was used in **Paper I** to test the nature (reinforcing or aversive) of the valence signal carried by central (i.c.v.) ghrelin injections. On day 1 of the study, a pre-test was performed where rats were placed in the apparatus with free access to both chambers for 20 min to determine the initial chamber preference of each animal. Then, a semi-unbiased conditioning design was used in which an i.c.v. injection of ghrelin was paired with the most preferred chamber for half of the rats and paired with the least preferred chamber for the other half. This design was used as we did not know whether ghrelin would condition a CPP or a CPA prior to the experiment. Conditioning sessions were performed on days 2-5 with one session in the morning and one in the afternoon (total of 8 sessions), during which rats received an injection of either ghrelin or vehicle (aCSF) and were placed immediately after in the corresponding chamber for 20 min. Ghrelin was injected in the morning and vehicle in the afternoon or *vice versa*. On day 6, a test session was performed where rats, like in the pre-test, had free access to both chambers for 20 min and time spent in each chamber was recorded. The schedule was based on a previous study (Jerlhag, 2008). The animals had access to normal chow and water in their home cages but no food was placed in the CPP/CPA apparatus.

To investigate whether the CPP/CPA outcome would be different with food present during all sessions, a second cohort of rats was trained in the CPP/CPA apparatus with food placed in the chambers and following a biased conditioning design, this time, with ghrelin paired with the most preferred chamber. The conditioning design was changed to biased based on the reinforcing properties observed in the first cohort.

In **Paper III**, the CPP/CPA paradigm was used to investigate whether ghrelin direct action at the level of the lPBN affects food reward. In this study, after determination of initial chamber preference at pre-test, a biased conditioning design was used where a palatable food (chocolate pellets) was provided in, and therefore paired with, the least preferred chamber and the most preferred chamber was paired with normal chow. Rats were trained on 20 conditioning sessions of 20 min over 10 days (2 sessions a day; Egecioglu et al., 2010, Dickson et al., 2010, Vogel et al., 2017). On the day following the last conditioning session, the test session was carried out during which half of the animals received intra-lPBN ghrelin and the other half received intra-lPBN vehicle 10 min prior to being placed in the apparatus with free access to both chambers for 20 min. The time spent in each chamber was recorded.

Conditioned flavour preference test

Conditioned flavour preference/avoidance (CFP/CFA) is based on the same principle as CPP/CPA, the only difference being that a treatment or drug is paired with a flavour instead of a place. CFP/CFA testing was carried out in rats in **Paper I** based on the protocol of Betley et al. (2015) in order to determine the rewarding/aversive properties of central ghrelin injections in an additional paradigm. At pre-test, rats were offered free access to two differently flavoured (strawberry and orange) low calorie (0.04 kcal/g) gelatin-based gels (Jell-O Sugar Free, Kraft Heinz Foods Company, Chicago, IL, USA) for 1 h in the morning and 1 h in the afternoon. The positions of the two gels were swapped after 30 min to control for preference of place in the cage. The gels consumption was measured and the initial flavour preference obtained by calculating the average preference over the two sessions. Ghrelin i.c.v. injection was subsequently paired with the most preferred flavour and i.c.v. vehicle with the least preferred flavour since ghrelin was seen to condition avoidance in the CPP/CPA experiments (see

Paper I under Results section). Conditioning consisted of one session in the morning and one in the afternoon for 4 days (8 conditioning sessions in total). Ghrelin was injected in the morning and vehicle in the afternoon or *vice versa*. On the following day (test session), the animals were offered free access to both flavoured gels again for 1 h in the morning and the amount consumed was measured. The two gels were again swapped after 30 min.

Operant conditioning

Operant conditioning is a well-established paradigm used to study food motivation, in which animals press a lever to obtain a food reward (Hodos, 1961). In the case of this thesis (**Paper III**), sucrose-induced operant conditioning training and testing was performed by rats in rat conditioning chambers (Med Associates Inc., St Albans, VT, USA). Each chamber, contained in a light- and sound-attenuated cubicle, had a metal grid floor, a house light, two retractable levers with a light bulb above each and a food pellet dispenser that delivers 45 mg sucrose pellets to the food tray (1811251; TestDiet, Sandown Scientific, Hampton, UK) as shown in **Figure 5**. The procedure used for operant conditioning was adapted from la Fleur et al., 2007, Tracy et al., 2008 and Skibicka et al., 2011b. The conditioning was carried out under mild food restriction (15 g of chow per day) with, first, 30 min fixed ratio (FR) 1, FR3 and FR5 sessions during which the rats had to lever-press one, three or five times, respectively, to obtain one sucrose pellet. Then, once the animals had learnt to press for sucrose pellets, they underwent 120 min progressive ratio (PR) sessions, whereby the amount of lever-press required to obtain a sucrose pellet increased during the session according to the following series: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328. The PR sessions were carried out without food restriction and until the animals' performance was stable, which was defined as the difference in pellets earned not being greater than 15 % for three consecutive sessions. The intra-lPBN injections of ghrelin and JMV2959 therefore commenced after a total of 3 weeks of operant conditioning training. The rats received all injections 10 min prior to the start of the PR session and in a latin square design (i.e. each animal received every treatment), with at least one wash-out day between injections.

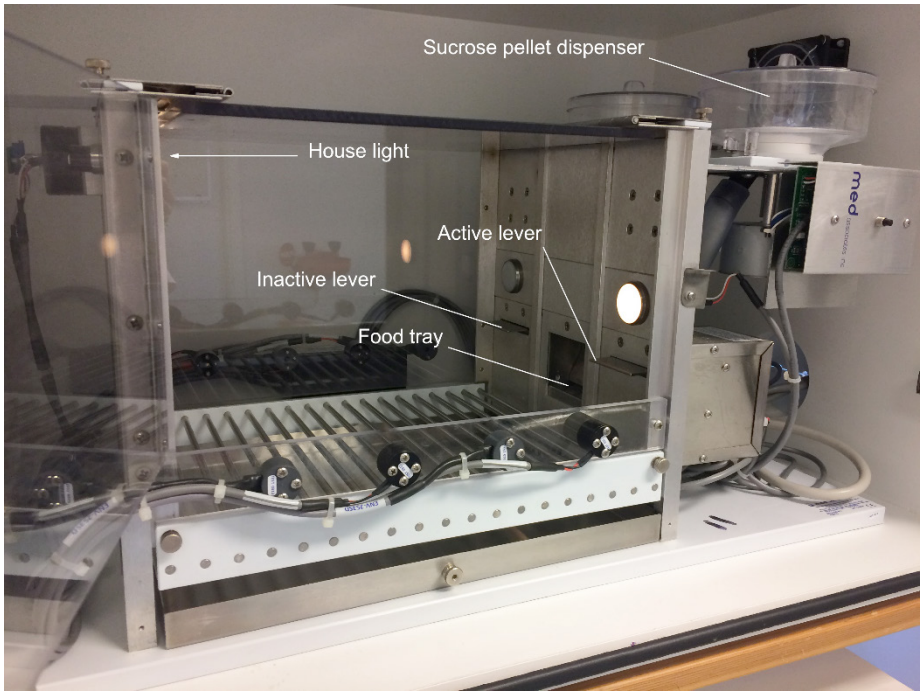

Figure 5. *Two-lever sucrose-induced operant conditioning apparatus.*

Pica response

Figure 6. *Kaolin pellets*

One possible cause of CTA is a state of malaise or nausea. As rats are incapable of vomiting, these animals usually consume non-nutritive mineral-based substances such as clays when experiencing malaise (Mitchell et al., 1976; Takeda et al., 1993). In **Paper I**, to check whether the ghrelin-induced effect in the CPP and CTP tests was due to a feeling of nausea, non-nutritive kaolin pellets (Research Diets Inc., New Brunswick, USA; **Figure 6**) were offered to the rats in addition to the chow they had in their home cage for 3 days. After an overnight habituation period to the kaolin, the animals received an i.c.v. injection of either ghrelin (same dose as used in the CPP/A and CFP/A tests) or vehicle and the consumption of both chow and kaolin was measured 3 h, 6 h and 24 h after injection.

Diets and feeding paradigms

Scheduled feeding

Two scheduled feeding paradigms were used in **Paper II**: the first one was scheduled feeding of standard chow diet for 3 h per day during the light phase (13:00-16:00 or 14:00-17:00) and the second one was scheduled access to a palatable food (sweetened condensed milk) for 15 min per day from 13:00 in *ad libitum*-fed rats. As rats are known to be able to anticipate periods of access to food (Patton and Mistlberger, 2013), these two paradigms were used to test whether anticipation of access to standard food or to a palatable meal alter the activation of SuM neurones. This was particularly relevant for the action of ghrelin since it was shown that ghrelin might mediate both anticipatory behaviours (Verhagen et al., 2011; Merkestein et al., 2012). In both paradigms, the anticipatory response was distinguished from the response to food by having a group of animals receiving access to the food at the expected time and another group at an unexpected time.

Sweetened condensed milk (SCM)

Sweetened condensed milk (**Figure 7**) was chosen as an energy-rich palatable food in **Paper II** at a 1:2 dilution in water as used previously (Hume et al., 2016; Hume et al., 2017; Kosheleff et al., 2018).

Figure 7. *Sweetened condensed milk (Nestlé, Gatwick, UK)*

High fat diet (HFD)

The high fat diet used in **Paper III** comprised of 20 % protein, 20 % carbohydrate and 60 % fat by energy supply (i.e. 5.24 kcal/g; **Figure 8**) and was obtained from Research Diets (D12492, New Brunswick, NJ, USA).

Figure 8. *High fat diet*

High-fat high-sugar (HFHS) free choice diet

The HFHS free choice diet consisted of standard chow pellets, sucrose (pellets for rats, 9 % solution for mice), lard (saturated animal fat; Dragsbæk, Thisted, Denmark) and water (**Figure 9**), as described in our previous study (Schéle et al., 2016). This diet paradigm was used in **Paper III** and **Paper IV** to investigate the effect of different treatments on food choice.

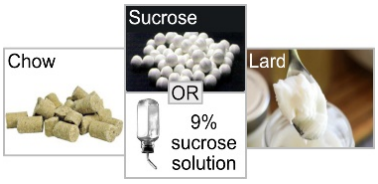

Figure 9. *High-fat high-sugar free choice diet*

Electrophysiological recording

In vivo extracellular recording

For the recording of rat SuM neurones in **Paper II**, a ventral approach was used: the animals were tracheotomised, the ventromedial surface of the hypothalamus was exposed by transpharyngeal surgery and a glass microelectrode (tip with 1 µm diameter) filled with 1.5 % neurobiotin in 0.25 mol/L sodium chloride was lowered into the tissue using the following coordinates: <1 mm posterior to the pituitary, on the midline and 2.5 mm dorsal to the surface of the arachnoid tissue. These coordinates were optimised in pilot experiments and the location of the recorded cells (in the SuM) was confirmed by juxtacellular labelling, a well-established method first described two decades ago (Pinault, 1996).

For each recorded cell, the mean spontaneous firing rate was recorded for a minimum of 10 min under basal conditions before ghrelin was administered. The basal firing rate was then compared with the firing rate over 20 min from 5 min to 25 min after injection. Activation and inhibition thresholds were set to +0.5 spikes/sec and -0.3 spikes/sec, respectively, based on our collaborators' previous study (Sabatier and Leng, 2008). The significantly responsive cells were defined as responding above those thresholds and with a significance of $P < 0.0001$ when comparing the mean and standard deviation firing rate in 30-sec bins.

Interspike interval histograms were made using SPIKE2 (CED, Cambridge, UK) and then used to construct hazard functions to study how the excitability of a neurone evolves with time since the last spike, as previously described (Sabatier et al., 2004).

In vitro loose-patch clamp recording

Loose-patch clamp electrophysiological recordings were performed to measure action currents (**Paper III**) with slight modifications from a previously-described protocol (Farkas et al., 2010). Pipette potential was held at 0 mV, pipette resistance 1-2 MΩ and loose-patch seal resistance 7-40 MΩ. The pipette solution contained (in mM): NaCl 123, KCl 3.5, $CaCl_2$ 2.5, $MgCl_2$ 1.3, HEPES 10 and glucose 10 (pH of 7.3). Large bipolar cells of the external part of the lPBN were chosen for recordings based on previous morphological studies of cells in this nucleus (Herbert and Bellintani-Guardia, 1995). An initial basal recording of 4 min was carried out before ghrelin or JMV2959 was added to the recording chamber, then the measurements continued for 11 min more.

The detection of events was carried out using the Clampfit module of the PClamp 10.4 software (Molecular Devices Co., San Jose, CA, USA). Change in the firing rate of cells upon ghrelin administration was expressed as the percentage ratio of the firing rates during the period with ghrelin (11 min) and the period in control conditions (4 min) in the recording.

Biochemical and imaging techniques

mRNA expression

For the study of mRNA expression in the lPBN after ghrelin or JMV2959 i.c.v. injections (**Paper III**), the lPBN was dissected 90 min after treatment, then frozen and stored at -80 °C. The RNeasy Mini Kit (74106; Qiagen, Hilden, Germany) was used to extract the mRNA from the tissue followed by cDNA synthesis (4322171; Applied Biosystems, Foster city, CA, USA). Messenger RNA expression in the lPBN was assessed by TaqMan® low density arrays using TaqMan® gene expression assays (4331182; Applied Biosystems). Analysis of the resulting data was then performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Data was normalised using two endogenous control assays: ACTB (beta actin; NM_001101; Applied Biosystems) and HMBS (hydroxymethybilane synthase; NM_000190; Applied Biosystems).

Immunohistochemistry

Immunohistochemical stainings were performed to identify cells expressing the Fos protein (indicating neuronal activation; **Papers II** and **III**) or CGRP (**Paper IV**). Rats and mice were transcardially perfused with heparinised saline followed by 4 % paraformaldehyde (PFA), the brains were dissected and post-fixed overnight in 4 % PFA containing 15 % sucrose and then incubated in 0.1 M phosphate buffer containing 30 % sucrose for cryoprotection until sectioning. Coronal sections, 30 µm-thick, were produced using a cryostat and stored in cryoprotectant.

Immunohistochemical experiments were carried out on free-floating sections to ensure optimal access of the antibody to the epitopes, using the following primary antibodies: anti c-Fos rabbit antibody (1:100 000; 226 003; Synaptic Systems, Göttingen, Germany) for the experiments performed at the University of Edinburgh in **Paper II** (Hume et al., 2017), anti c-Fos rabbit antibody (1:20 000; Ab-5 (4-17) PC38; Calbiochem, San Diego, CA, USA) for stainings carried out at the University of Gothenburg in **Papers II** and **III** (D'Anna and Gammie, 2006; Raineki et al., 2012) and anti CGRP goat antibody (1:40; ab36001; Abcam, Cambridge, UK) in **Paper IV**.

Binding of the primary antibody to the epitope was detected using either a fluorochrome-conjugated secondary antibody or a method using 3,3'-diaminobenzidine (DAB) reaction. In **Paper II**, Alexa Fluor 488 goat anti-rabbit antibody was used (1:250; A-11008; Invitrogen, Carlsbad, CA, USA), and in **Paper IV**, Alexa Fluor 594 chicken anti-goat antibody (1:200; A21468; Invitrogen) was used. In **Paper II**, the detection of Fos immunoreactivity employed at the University of Edinburgh used a DAB-hydrogen peroxidase method comprising a biotinylated horse anti-rabbit IgG antibody (1:500; BA-1100; Vector Laboratories, Burlingame, CA, USA), an avidin-biotin complex (PK-6100; Vectastain Elite ABC kit, Vector Laboratories) and a DAB, nickel and hydrogen peroxide solution as used previously (Hume et al., 2017). At the University of Gothenburg, a nickel-intensified DAB reaction was used which included a horseradish peroxidase goat anti-rabbit antibody (1:200; PI1000, Vector Laboratories) and a nickel-intensified DAB (D5905; Sigma-Aldrich, St. Louis, MO, USA) solution. For every immunohistochemical staining, some sections that were not treated with the primary antibody were incubated with the secondary antibody to check for its specificity.

The sections were then counterstained with DAPI for 5 min (1:5000; D1306; Thermo Fisher Scientific, Waltham, MA, USA) and cover-slipped with Prolong Diamond Antifade mounting medium (P36970, Thermo Fisher).

RNAscope (fluorescent *in situ* hybridisation)

In **Paper IV**, detection of the EGFP co-expressed by the AAV-DIO-TetoxLC using immunohistochemistry proved difficult. Although we were able to visualise the EGFP in positive control sections from rats, the same antibody did not produce a specific signal in mice. Therefore, RNAscope was used to visualise the injections sites and exclude mice with injections not contained within the lPBN. For RNAscope, tissue processing was essentially the same as for immunohistochemistry with the exception that all solutions used from cryosectioning onwards were autoclaved for sterilisation and the coronal sections cut were 15 µm-thick. All reagents were purchased from Advanced Cell Diagnostics (ACD, Hayward, CA, USA) if not otherwise stated.

On the day before the experiment, every sixth lPBN sections was mounted on SuperFrost Plus slides (631-9483; VWR, Radnor, PA, USA), dried at room temperature, briefly rinsed in autoclaved Milli-Q purified water, air-dried again and baked overnight at 60 °C using the ACD HybEz II hybridization system (321462).

The *EGFP* probe used (400281) contained 13 oligo pairs and targeted the 628-1352 region of the *EGFP* transcript (Acc. No. U55763.1). Three-plex positive and negative control probes recognizing PolR2A, cyclophilin and Ubiquitin (320881), and bacterial dihydrodipicolinate reductase, Dap B (320871), respectively, were processed in parallel with the target probes to ensure integrity of the RNA and optimal assay performance.

On the day of the experiment, slides were incubated for 7 min in hydrogen peroxide (322335), submerged in Target Retrieval (322001) for 7 min at 98.5 - 99.5 °C, followed by two brief rinses in autoclaved Milli-Q purified water. The slides were then quickly dehydrated in 100 % ethanol and allowed to air dry for 5 min. During this time, a hydrophobic barrier was drawn around the sections using an ImmEdge hydrophobic barrier pen (310018). The sections were then incubated with Protease Plus (322331) for 30 min for the detection of *EGFP*. Hybridization of the probes as well as

amplification and detection steps were performed according to the manufacturer's protocol for the tyramide-based RNAscope® Multiplex Fluorescent v2 Assay (323100) that labelled the probe with Opal 520 (1:500; Akoya Biosciences, Menlo Park, CA, USA). Finally, as for immunohistochemistry, the sections were counterstained with DAPI and cover-slipped with Prolong Diamond Antifade mountant and stored at 4 °C until imaging.

Imaging

For the work of this thesis, imaging of fluorescence or bright-field signals was performed using simple fluorescent or light microscopes.

For the co-localisation work carried out in **Paper IV**, a LSM 700 inverted confocal microscope was used at the Centre for Cellular Imaging core facility at the University of Gothenburg using 3 x 2 tiling settings. The images were then stitched together in ImageJ/Fiji (NIH, Bethesda, MD, USA) and the GHSR- and CGRP-positive cells as well as co-localisations were counted using the 'Cell counter' plug-in.

Results

Paper I

In this paper, we investigated whether central administration of ghrelin carries a positive or a negative valence signal. This was tested using conditioned preference/avoidance tests. **Figure 10** presents the key findings of **Paper I**.

We found that i.c.v. injection of ghrelin conditioned both a place (CPA) and a flavour avoidance (CFA) in rats. Remarkably, the CPA remained even when chow was provided in the chambers during the pre-test, conditioning and test sessions, demonstrating that food consumption is not sufficient to elevate the negative feeling carried by i.c.v. ghrelin. This effect was not linked to a feeling of malaise as shown by the fact that i.c.v. ghrelin had no effect on kaolin intake (pica test). Moreover, we showed that i.c.v. ghrelin also conditioned a place avoidance in mice.

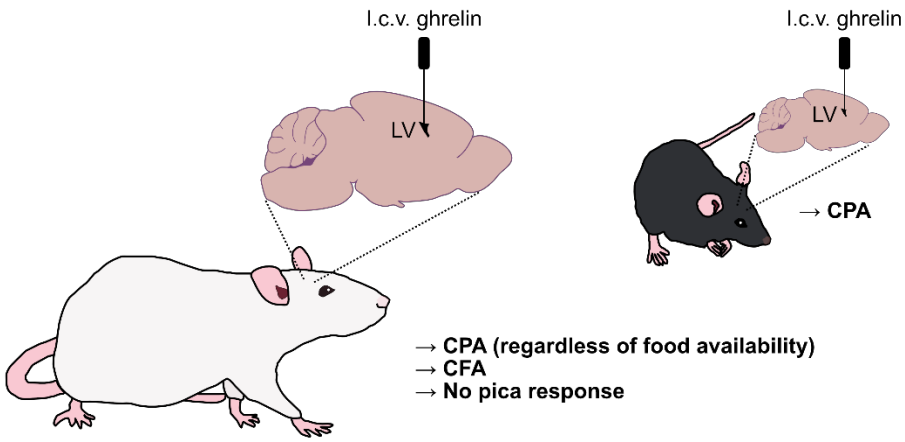

Figure 10. Schematic illustration of the key findings of Paper I. *Intracerebroventricular (i.c.v., into the lateral ventricle (LV)) ghrelin injection induced a conditioned place avoidance (CPA) in both mice and rats. Food availability in rats did not alter the CPA. In rats, i.c.v. ghrelin administration also induced a conditioned flavour avoidance (CFA). Ghrelin i.c.v. injection did not produce any malaise/nausea as seen by the absence of a pica response.*

Paper II

In this work, we sought to determine whether the SuM is a target area for ghrelin in rats based on evidence that there are ghrelin binding sites in this region (Cabral et al., 2013). **Figure 11** presents the key findings of **Paper II**.

We discovered that the number of cells expressing the Fos protein (denoting neuronal activation) in the SuM was increased by peripheral ghrelin injection as well as by two situations associated with elevated ghrelin blood levels, namely in food-restricted rats anticipating access to standard food and in fed rats anticipating the consumption of a palatable energy-dense meal (SCM). Interestingly, the Fos response in food-restricted rats anticipating chow was comparable whether chow was omitted, given at the expected time or given at an unexpected time. Similarly, in fed rats anticipating a palatable meal, access to SCM at the expected time or at an unexpected time both increased the number of Fos-positive cells in the SuM.

In addition, *in vivo* electrophysiological studies revealed that peripheral ghrelin administration induced predominantly an excitatory response in SuM cells. There was, however, heterogeneity in the response of SuM cells to peripheral ghrelin and therefore, the SuM neurones were classified according to the different firing patterns of cells (oscillatory, broad or doublet). Peripheral ghrelin injection increased specifically the firing rate of the vast majority of the oscillatory SuM neurones.

Finally, we also demonstrated that ghrelin's orexigenic effect can be driven at the level of the SuM. Indeed, intra-SuM administration of ghrelin increased food intake for up to 6 hr post-injection.

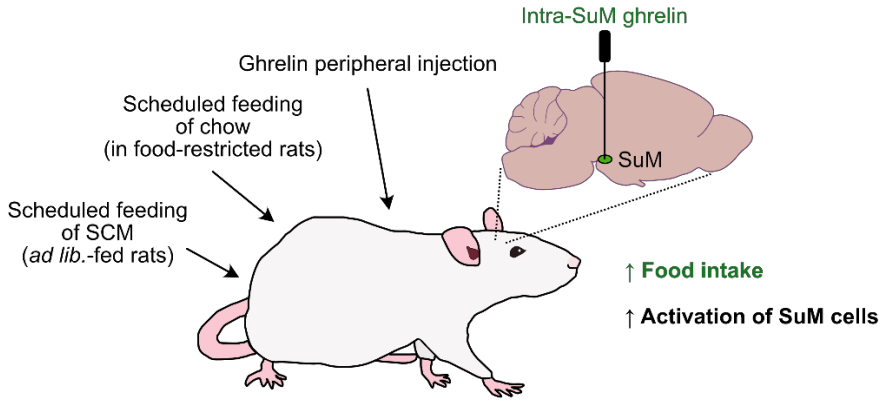

Figure 11. Schematic illustration of the key findings of Paper II. *Scheduled feeding of chow in food-restricted rats and scheduled feeding of sweetened condensed milk (SCM) induced a Fos response (neuronal activation) in the supramammillary nucleus (SuM). Peripheral ghrelin activated SuM cells (seen as Fos response and increased firing rate). Intra-SuM ghrelin injection produced an orexigenic response.*

Paper III

In this paper, we explored the lPBN as a potential new target for ghrelin's behavioural effects in rats based on studies showing abundance of receptor for ghrelin in this area in both mice and rats (Zigman et al., 2006). **Figure 12** presents the key findings of **Paper III**.

Our immunohistochemical studies showed that both i.v. and i.c.v. ghrelin injections in rats increased the number of lPBN cells expressing Fos, whereas our electrophysiological recordings from external lPBN cells in brain sections displayed a ghrelin-induced decrease in firing rate. This discrepancy in effect might come from the fact that Fos-expressing cells are not the same cells as the ones recorded from.

Moreover, we found that intra-lPBN ghrelin administration increased the consumption of both chow and high fat diet, while intra-lPBN injection of the ghrelin receptor antagonist JMV2959 decreased only chow intake. Intra-lPBN ghrelin injection also increased the amount of chow consumed for up to 24 hr when the rats had free access to chow, sucrose, lard and water without altering the intake of sucrose or lard. By contrast, food motivation assessed by sucrose-induced operant conditioning, and food reward tested

by conditioned place preference for chocolate, were not affected by intra-lPBN ghrelin injections.

Finally, mRNA expression analyses revealed that i.c.v. ghrelin injection led to a decreased expression of cocaine and amphetamine-related transcript prepropeptide and bombesin-like receptor 3, whereas i.c.v. JMV2959 injection increased the expression of interleukin 1 beta, interleukin 6, tumor necrosis factor alpha, glutamate receptor N-methyl D-aspartate 2b and adrenergic alpha-1a receptor.

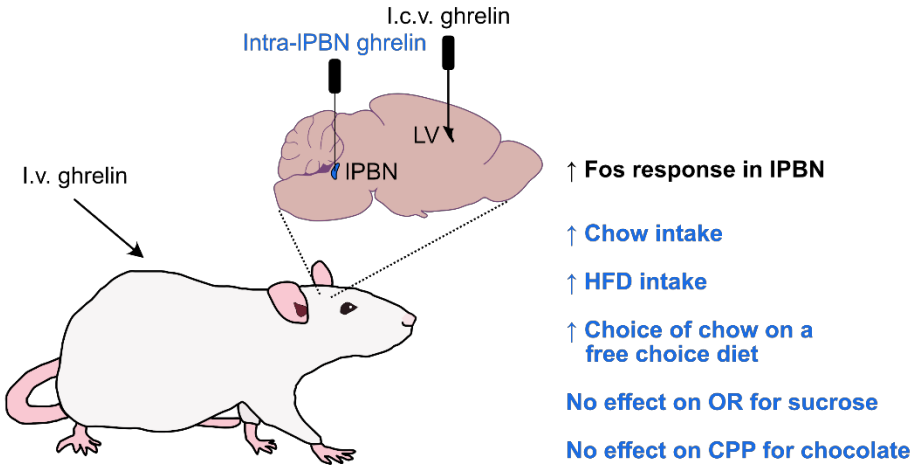

Figure 12. Schematic illustration of the key findings of Paper III. *Intracerebroventricular (i.c.v., into the lateral ventricle (LV)) and intravenous (i.v.) administration of ghrelin both produced a Fos response in the lateral parabrachial nucleus (lPBN). Intra-lPBN ghrelin injection increased consumption of both chow and high fat diet (HFD) while increasing only the amount of chow consumed when rats were given a free choice diet (chow + lard + sucrose). No effect was seen of intra-lPBN ghrelin administration on operant responding (OR) for sucrose (food motivation) or conditioned place preference (CPP) for chocolate (food reward).*

Paper IV

Following the results of **Paper III**, we aimed to determine specifically the role of the $GHSR^{lPBN}$ cells in feeding behaviours using *Ghsr-IRES-Cre* mice in which these cells were silenced by TetoxLC. **Figure 13** presents the key findings of **Paper IV**.

Silencing the GHSR^{lPBN} cells suppressed diet-induced body weight gain and adiposity, which could be explained by a reduced food intake as caloric efficiency was unaffected.

In addition to a reduced food intake, mice with silenced GHSR^{lPBN} cells also consumed less sucrose solution compared to controls when given free access to chow, lard, 9 % sucrose solution and water, while there was no difference in their consumption of chow, lard and water. Silencing the GHSR^{lPBN} neurones did not affect saccharin preference, thereby suggesting that the reduced intake of sucrose solution is not due to a reduced ability to taste sweetness.

Our immunohistochemical studies revealed that GHSR- and CGRP-expressing neurones in the lPBN are distinct cell populations with only a few cells expressing both molecules.

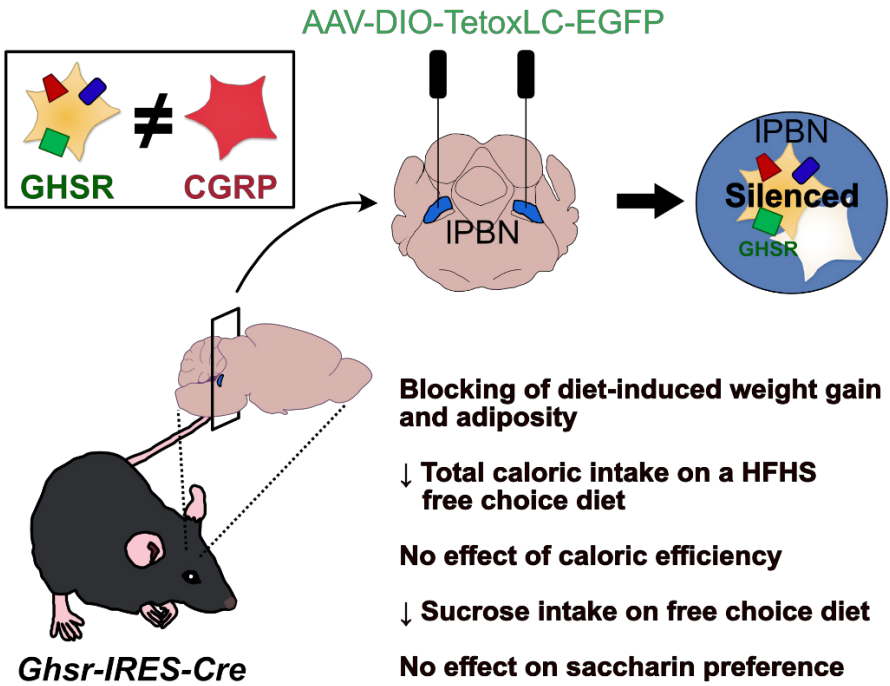

Figure 13. Schematic illustration of the key findings of Paper IV. *Silencing of the GHSR^{lPBN} cells by injecting a Cre-dependent viral vector expressing tetanus toxin light chain (TetoxLC) in the lPBN of Ghsr-IRES-Cre mice prevented diet-induced weight gain and adiposity. This effect was caused by a reduced total caloric intake as caloric efficiency was unaltered when mice were fed a high-fat high-sugar (HFHS) free choice diet. Silencing of GHSR^{lPBN} cells also altered food choice by*

reducing the amount of sucrose solution consumed, while not having any effect on saccharin preference. GHSR^{lPBN} cells were also shown no to overlap much with the CGRP-expressing cells of the lPBN.

Discussion

Ghrelin has been shown to engage specific brain regions for its role in the regulation of food-linked behaviours (Skibicka and Dickson, 2011) including the ARC, where AgRP/NPY neurones carry a negative valence signal (Betley et al., 2015), and the VTA, containing dopamine cells that signal reward (Jerlhag et al., 2006). The studies comprising this thesis demonstrated that (i) central ghrelin carries a negative valence signal, (ii) the SuM and lPBN are newly identified targets for ghrelin's effects on feeding and (iii) $GHSR^{lPBN}$ cells have a role in feeding and body weight control.

The valence signal of ghrelin

Our first study demonstrated that central ghrelin injection carries a negative valence signal in both rats and mice, consistent with its role as a hunger hormone. This effect was seen both in CPP/CPA tests, in which animals avoided a chamber previously paired with central ghrelin injection, and in CFP/CFA tests in which the flavour paired to ghrelin injection was also avoided.

These avoidance-conditioning effects of ghrelin resonate with those of Betley and colleagues, who found similar results by optogenetic activation of AgRP neurones in the ARC; mice dislike when their AgRP neurones are activated and start to eat, since feeding suppresses activation of these neurones (Betley et al., 2015). Given that ghrelin activates AgRP/NPY neurones of the ARC, it may be that ghrelin routes through the ARC for our observed effects on valence, although this is difficult to test directly. Indeed, as inhibiting AgRP/NPY neurones itself conditions a preference (Betley et al., 2015) and blocking GHSR signalling in these neurones suppresses ghrelin's orexigenic effects (Wu et al., 2017), determining whether this population is necessary for ghrelin to induce avoidance appears to be complex.

To then determine whether the consumption of food can suppress the negative valence signal carried by ghrelin, we repeated the same CPA test in rats with food available at all times (during conditioning and during the

test). We found that ghrelin still conditioned place avoidance despite food being available and consumed, meaning that the negative reinforcing properties of ghrelin are independent of food intake. This is in contradiction with the fact that food delivery alone can decrease the activity of the AgRP neurones and that this reduction in activity is maintained during food consumption (Betley et al., 2015; Chen et al., 2015). If the AgRP/NPY neurones transmit ghrelin's negative valence signal, we would imagine that availability of food during conditioning would at least reduce this signal. One possibility might be that the activation of AgRP/NPY neurones induced by central ghrelin injection has not yet been reduced enough by food availability to suppress the CPA. Indeed, AgRP/NPY neuronal activity was shown to still be increased 20 min after a peripheral ghrelin injection in *ad libitum*-fed mice (Betley et al., 2015), suggesting that a central ghrelin injection would have at least an effect of a similar length, if not longer. Yet, an alternative explanation could be that ghrelin recruits pathways that bypass the AgRP/NPY neurones to induce a CPA. Studies that could help test this alternative include injection of a GHSR antagonist or inverse agonist in distinct brain area known to be ghrelin-responsive before each CPP conditioning session to see if ghrelin-induced CPA is suppressed by the blockade of GHSR signalling in any of the regions, although like for AgRP/NPY neurones, the known effects of GHSR blockade in these regions should be controlled for. The recruitment of downstream pathways not responsive to ghrelin *per se* could also be tested in a similar manner by inhibiting neuronal activity directly using chemogenetics for example.

Before the current study, two studies had explored the reinforcing properties of ghrelin in CPP/CPA tests in mice and reported disagreeing results. In the earliest study, peripheral ghrelin administration induced a CPP (Jerlhag, 2008), while in the other, the same injection induced a CPA (Lockie et al., 2015). However, the second study described that systemic ghrelin administration caused a CPP when food was available during conditioning and test sessions (Lockie et al., 2015). Different elements could account for the differences and similarities seen between these studies and our current work. The route of administration (peripheral in previous studies compared to central in our study) or the doses used could, for instance, have a differential effect on the ability ghrelin has to access or activate the AgRP/NPY neurones of the ARC. The transport of ghrelin through the blood-brain barrier has indeed been shown to be quite complicated and somewhat species-dependent; in mice, mouse acylated ghrelin was found to be more easily transported from brain-to-blood than

in the other direction, while nonacylated mouse ghrelin was shown to diffuse only in the blood-to-brain direction and human active ghrelin (differing by only two amino acids from mouse ghrelin) to be transported in both directions (Banks et al., 2002).

Another explanation for the discrepancies seen in the effect of ghrelin in CPP/CPA tests could reflect differences in the engagement of the dopamine system since peripheral and central ghrelin injections produce different dopamine release in the NAcc. Indeed, although both routes of ghrelin administration are known to stimulate dopamine release in the NAcc, the magnitude of the release is difficult to compare between studies (Abizaid et al., 2006; Jerlhag, 2008). Whilst the ability of peripheral ghrelin to access the brain is clear (Schaeffer et al., 2013), the degree to which peripheral ghrelin can access the VTA compared to central ghrelin remains to be established.

Given that there is disagreement in the literature concerning ghrelin's effects on CPP/CPA, it was important that we also confirmed its avoidance-conditioning effects in a completely different paradigm, the CFA test. Similar to the CPA seen in the previous paradigm, rats learnt to avoid the non-nutritive gel paired with central ghrelin injection. This result demonstrates that in addition to visual and tactile cues (in the CPP paradigm), the cues paired to the negative valence signal of central ghrelin also include olfactory and gustatory ones. Likewise, Betley and colleagues showed that photo-stimulation of AgRP/NPY neurones in the ARC induced a flavour avoidance in a similar non-nutritive gel-based paradigm (Betley et al., 2015).

The fact that rodents find central ghrelin injection aversive might be due to a feeling of malaise. As rodents cannot vomit, the amount of kaolin they ingest was measured (see the section on pica response on page 33). We found that rats that received central ghrelin injection at a dose that increased chow intake (same as used in our CPP and CFP tests) did not ingest more kaolin than the ones injected with vehicle. Therefore, we can conclude that the place and flavour avoidance conditioned by central ghrelin injection is not due to a feeling of malaise caused by the treatment.

This study, hence, demonstrates that ghrelin injected in the ventricular system of the brain conditions avoidance involving visual and tactile as well as olfactory and gustatory cues, without inducing any malaise/nausea. Central ghrelin might, therefore, contribute to the unpleasant feeling of hunger and drive food-seeking and consummatory behaviours via negative reinforcement.

The SuM, part of the neurocircuit engaged by ghrelin

In our second study, we revealed the SuM to be an area that is activated by physiological states associated with elevated circulating ghrelin levels, namely scheduled feeding of chow in food-restricted rats (Verhagen et al., 2011) and scheduled feeding of sweetened condensed milk in *ad libitum*-fed rats (Merkestein et al., 2012) and after peripheral ghrelin injections. Interestingly, the increase in the level of activation (i.e. number of Fos-positive cells) in the SuM was similar whether the food was omitted, offered at the expected time or offered at an unexpected time. Electrophysiological recording of SuM also showed an overall excitatory effect of peripheral ghrelin and allowed us to classify SuM neurones into different populations depending on their firing pattern. Finally, we demonstrated that ghrelin also exerts an orexigenic effect when delivered directly to the SuM.

To determine whether metabolic state and access or anticipation of access to standard or palatable food affects Fos expression in the SuM, we scheduled-fed chow to food-restricted rats and gave scheduled access to an energy-dense food (SCM) to *ad libitum*-fed rats. In our first experiment, omission of chow at the scheduled time in food-restricted rats significantly increased the number of Fos-positive cells in the SuM compared to *ad libitum*-fed controls. Offering chow at the scheduled-time or at an unexpected time induced a Fos response comparable to that seen with omission. Similarly, in our second experiment, *ad libitum*-fed rats trained to anticipate access to SCM showed a significant increase in the number of Fos-positive cells in the SuM compared to controls, regardless of whether rats were anticipating access to SCM or not. The number of SuM Fos-positive cells was greater in rats offered SCM at an unexpected time compared to those receiving it at the expected time, although this was not significant. Nevertheless, these results may point to the potential role of the SuM in appetite-related behaviours (Vogel et al., 2016) and motivated behaviours (Ikemoto, 2005; Shin and Ikemoto, 2010). In addition, to mimic an endocrine aspect of the hungry state, we peripherally administered ghrelin in satiated rats and looked at Fos expression in the SuM. Peripheral ghrelin injection, compared to saline injection, resulted in an increase of the number of Fos-positive cells in the SuM in fed rats. Collectively, these data show that the number of Fos-positive SuM cells is relatively low in the fed state but that SuM cells are activated by actual or anticipated food access

and by peripheral ghrelin administration. However, whether the same SuM neurones are Fos-positive after food access or during anticipation is not clear. In the same way, it is unclear whether the SuM cells Fos-positive after ghrelin injection are the same as the SuM cells expressing Fos in rats anticipating food.

Historically, the neuroanatomy of the SuM has not been very well-defined. Yet, some studies showed that SuM cells are small to medium in size, spherically-shaped and that they are more densely packed in the medial region of the SuM compared to the lateral region (Geeraedts et al., 1990; Shepard et al., 1988). The SuM is adjacent to multiple nuclei such as the LatH, the posterior hypothalamic area and the VTA, from which the SuM has a distinct cellular organisation, making it a defined separate nucleus (Geeraedts et al., 1990). Previous immunohistochemical studies revealed that neurones of the SuM express tyrosine hydroxylase, CCK and substance P among other peptides (Seroogy et al., 1988). Nevertheless, SuM neurones are not the only ones to display this pattern of expression and no specific marker has been found for SuM cells so far.

The sensitivity of the SuM to hormones is not so well-characterised either. One neurohormone known to affect feeding is oxytocin (Leng and Sabatier, 2017). Previous studies showed that gavage of SCM increases the electrical activity of magnocellular oxytocin cells in the rat supraoptic nucleus (Hume et al., 2017) and that the SuM receives oxytocin-positive inputs (Cumbers et al., 2007) and contains oxytocin binding sites (Kremarik et al., 1995). Moreover, oxytocin receptor agonists were seen to activate approximately 50 % of the SuM cells tested in an acute brain slice preparation (Cumbers et al., 2007). It would, therefore, be of interest to find out whether oxytocin signalling is involved in the SuM Fos response seen after food consumption, especially SCM consumption.

Since ghrelin has a well-established role in food anticipation, it could be that ghrelin mediates the Fos responses observed in the SuM of rats anticipating food. Thus, we used *in vivo* electrophysiology to study directly the effect of peripheral ghrelin on neuronal excitability of SuM neurones. Our study provides a preliminary characterisation of the different firing patterns found in the SuM, leading to our classification of SuM cells as oscillatory, broad or doublet cells. We found that peripheral ghrelin administration induced an excitatory response in 78 % of the oscillatory cells recorded from. This cell type might be particularly interesting as they exhibit a rhythmic firing pattern that is consistent with an underlying

sinusoidal oscillation in excitability in the low theta range and there is evidence for a role of the SuM in driving rhythmic theta activity in the hippocampus. Hippocampal theta oscillations are linked to many cognitive and behavioural functions such as learning, spatial and temporal memory, locomotion and emotion (Korotkova et al., 2018). SuM neurones are known to project to the dentate gyrus and CA2 area of the hippocampus (Vertes, 2015) and hippocampal theta rhythm is disrupted by pharmacological blockade of the SuM in both conscious and anaesthetised rats (Aranda et al., 2008). Studies in urethane-anaesthetised rats showed that SuM neurones display at least four patterns of rhythmic activity, each of which has a phase-locked relationship, either in- or out-of-phase, with hippocampus theta field activity (Kocsis and Vertes, 1997). In addition, *in vivo* recordings from the SuM and mammillary body of urethane-anaesthetised rats found that 17 % of cells fired synchronously with hippocampal theta (Kocsis and Vertes, 1994). Taken together, these observations suggest that the SuM might provide or relay a rhythmic input to the hippocampus that modulates its theta oscillations.

Previous studies simultaneously recording from hippocampal CA1 area and medial prefrontal cortex (mPFC) suggested a role of theta rhythms in information processing in the brain (Jones and Wilson, 2005). Supporting the idea that the SuM enhances functional connectivity in the brain, previous work demonstrated that, in rats navigating a T-maze, information about planned decision (encoded by the mPFC) is integrated with spatial maps (in the hippocampus) via a mPFC-thalamic nucleus reuniens (NR)-hippocampal CA1 circuit (Ito et al., 2018). Just prior to a decision point, the firing pattern of mPFC and NR cells is coordinated with hippocampal CA1 theta rhythm and coordination with CA1 theta rhythm is also seen in SuM neurones. The same study showed that optogenetic inhibition of SuM cells reduces temporal coordination in the mPFC-NR-CA1 circuit, suggesting that the SuM might enable appropriate information flow in the mPFC-NR-CA1 circuit. Hence, since ghrelin is also involved in memory processes (Diano et al., 2006), it would be of interest to determine the role of the ghrelin-sensitive oscillatory cells in the SuM, identified in our study, in learning and memory.

The *in vivo* electrophysiological technique used in our study requires the recorded cells to be spontaneously active, allowing observation of both excitatory and inhibitory responses. Obviously, this inevitably biases against recording neurones with very little or no spontaneous activity. Similarly to the neurones of the ARC, also responsive to ghrelin (Dickson et al., 1993),

we found heterogeneity in the response of SuM cells. Nonetheless, SuM responses were significant, long lasting and comparable in magnitude to the responses of ARC cells to ghrelin receptor agonists *in vivo* (Dickson et al., 1993), identifying the SuM as a relevant ghrelin-responsive area of the hypothalamus.

The fact that peripheral ghrelin alters the electrical activity of SuM neurones of anaesthetised rats and increases the number of Fos-expressing cells in the SuM provides compelling evidence that the SuM is a significant target of ghrelin as it is also activated in situations when endogenous ghrelin levels are elevated. Although the exact mechanism through which peripheral ghrelin accesses the brain are not fully understood, it was shown to bind to the ARC (Schaeffer et al., 2013) and the hippocampus (Diano et al., 2006). Since centrally administered ghrelin binds to the SuM (Cabral et al., 2013), it is possible that peripheral ghrelin access the SuM to activate these neurones directly, although no evidence supporting a direct action of ghrelin in the SuM has been reported previously.

In addition, since intra-SuM ghrelin injection could drive feeding in our study, it might be that the SuM contains cells expressing GHSR-1a, which can contribute to the orexigenic effect of ghrelin. SuM neurones could be activated directly by ghrelin reaching this area or by other mechanisms such as heterodimerisation of GHSR-1a with other receptors (Schellekens et al., 2013) or LEAP2 (Ge et al., 2018). However, the feeding data should be interpreted with some caution due to the potential diffusion of intra-SuM injected ghrelin to neighbouring regions, notably the LatH and VTA, which are known targets of ghrelin for its orexigenic effects. Interestingly, recent work found that the SuM is highly interconnected with other ghrelin-responsive sites including the LatH, VMH, DMH and VTA (Plaisier et al., 2020), providing concrete evidence that the SuM might be an integrated part of the circuits regulating feeding.

In summary, our study identifies the SuM as a brain region of potential importance in metabolic and feeding control. Ghrelin might reach the SuM directly to exert its neurobiological effects, although it may also act indirectly by engaging afferent pathways. Further studies are needed to determine the neurochemical identity of the SuM ghrelin-responsive cells as well as their projections and the inputs they receive from other areas. As both the SuM and ghrelin have a role in reward, feeding and memory, further investigation may highlight the SuM as a region that receives and

integrates information related to motivation, memory and food intake to drive complex behaviours.

The role of lPBN ghrelin signalling in feeding

In this study, we found that, in rats, both systemic and central injection of ghrelin increases the number of Fos-positive cells in the lPBN, while reducing the firing rate of large bipolar cells of the external lPBN. Our studies further showed that ghrelin injected directly into the lPBN increases the consumption of both standard chow and high fat diet separately and stimulates only chow intake when the rats are given a free access to chow, lard, sucrose and water. Food motivation and food reward tested by operant conditioning and conditioned place preference, respectively, were not altered by intra-lPBN ghrelin administration in rats.

Our analysis of Fos expression in rats after peripheral and central ghrelin injection demonstrates that the lPBN is part of the neurocircuit activated by ghrelin. Ghrelin might target the lPBN directly (since it contains cells that express GHSR-1a (Zigman et al., 2006)) and/or engage afferent pathways originating from other brain areas (as central ghrelin injection also induced a Fos response). Ghrelin also altered the electrical activity of lPBN cells recorded in slice preparation where distant inputs were severed, although the effect was mainly inhibitory in this case. Thus, ghrelin appears to have contradictory effects on the electrical activity of lPBN cells (i.e. express the activity marker Fos but are electrically inhibited). The dogma that Fos is a ubiquitous marker of neuronal excitation has been challenged in other studies, such as those showing that oxytocin cells of the supraoptic nucleus express Fos in response to MC4R agonists but are electrically inhibited, consistent with the effects of these compounds to inhibit oxytocin release (Sabatier et al., 2003). Thus, it may be that ghrelin-responsive cells in the lPBN also express Fos when inhibited. Another explanation could be that the Fos-positive cells and the cells recorded from, in our study, are different populations, which is very plausible since Fos expression was seen throughout the lPBN while the patch clamp recordings were carried out only in the cells of the external lPBN (where the anorexigenic CGRP-expressing cells reside).

Acute stimulation of food intake is one of the best-described effects of ghrelin. As a matter of fact, it has been shown that ghrelin can drive feeding

when injected into many different brain regions, including the ARC (Wren et al., 2001b), PVN (Olszewski et al., 2003), dorsal vagal complex (Faulconbridge et al., 2003), VTA (Naleid et al., 2005), NAcc (Naleid et al., 2005) and amygdala (Alvarez-Crespo et al., 2012). Our study reveals that the lPBN is also an area of relevance for ghrelin's orexigenic effects as intra-lPBN injection of ghrelin resulted in an acute feeding response, seen for foods with different palatability (chow and high fat diet).

The effect we reported of intra-lPBN ghrelin injection on food choice is somewhat comparable to the effect of intra-VTA administration of ghrelin, where intake of chow and lard (but not of sucrose) was increased 3 hr and 6 hr after injection, resulting in an increased total energy intake (Schéle et al., 2016). Prior to any treatment, rats liked the three foods equally in the choice paradigm. When ghrelin was injected into the lPBN, its orexigenic effect was not evenly distributed across the different foods with only the intake chow increased to more than double compared to vehicle injection, but no effect on lard and sucrose intake. It appears, therefore, that intake of palatable food is increased by intra-lPBN ghrelin only when rats are offered this food without a choice (in high fat diet).

A role of the lPBN in food intake and/or food reward has been suggested by studies showing that hormones and neurotransmitters such as GLP-1 (Alhadeff et al., 2014), endocannabinoids (DiPatrizio and Simansky, 2008), GABA (De Oliveira et al., 2011), glutamate (Wu et al., 2012) and melanocortin (Skibicka and Grill, 2009) alter food intake and/or food motivation when delivered to this site. The effects of systemic and central ghrelin administration on food motivation are well known (Skibicka et al., 2011b; Skibicka et al., 2012; Perello et al., 2010; Bake et al., 2019) and the VTA is thought to be the principal target of ghrelin for these effects (Egecioglu et al., 2010; Skibicka et al., 2011b). In our study, we did not find any effect of intra-lPBN ghrelin injection on food-motivated behaviours (in progressive ratio sucrose-induced operant responding) or food reward (in CPP for chocolate). Thus, our data suggest that ghrelin signalling at the level of the lPBN contributes to the feeding effects of ghrelin but not to its effects on food motivation, probably involving different cell populations to those regulating food-motivated behaviours.

Central ghrelin injection did not alter the expression of most of the candidate genes we chose on the basis of potential links to energy balance, in the lPBN, with a possible exception being cocaine and amphetamine-related transcript prepropeptide which as an anorexigenic role (Aja et al.,

2001) and was down-regulated by ghrelin, thereby possibly contributing to the orexigenic effect of ghrelin. Central delivery of a GHSR antagonist upregulated interleukin1-beta, interleukin 6, tumour necrosis factor alpha, glutamate receptor N-methyl D-aspartate 2b and adrenergic alpha-1a receptor mRNA expression in the lPBN, which would be coherent with an anorexigenic response.

In summary, this study reveals that peripheral and central ghrelin engage the lPBN and that this region is relevant for ghrelin's orexigenic effects (both on chow and high fat diet), but not for ghrelin's effects on food motivation and reward. We further found that intra-lPBN ghrelin injection alters food choice by increasing only chow intake when rats are offered a choice diet including chow, lard, sucrose and water.

The relevance of the $GHSR^{lPBN}$ cells in feeding

The last study presented in this thesis demonstrates that the $GHSR^{lPBN}$ cells have a role in the body weight homeostasis of mice fed an obesogenic HFHS diet. We provide evidence that silencing the $GHSR^{lPBN}$ neurones prevents HFHS diet-induced body weight gain and reduces adiposity by decreasing total caloric intake.

Global *Ghsr*-null mice were found to also be resistant to diet-induced obesity when fed a high-fat diet from an early age (Zigman et al., 2005). This effect was reproduced by ablation of GHSR specifically in neurones (Lee et al., 2016) and to some extent in AgRP neurone-specific GHSR knockout mice (Wu et al., 2017). Surprisingly considering the well-known role of the AgRP neurones in promoting food intake (Krashes et al., 2011; Essner et al., 2017), the body weight phenotype displayed by the AgRP GHSR knockout mice was not explained by reduced food intake but rather by increased energy expenditure via upregulation of non-shivering thermogenesis (Wu et al., 2017). By contrast, in our study, the reduction in body weight and body fat (compared to controls) resulting from silencing $GHSR^{lPBN}$ cells was clearly caused by decreased food intake. Indeed, despite not measuring energy expenditure directly, we found no effect on caloric efficiency. $GHSR^{lPBN}$ cells appear, thus, to be more relevant for diet-induced hyperphagia than for energy expenditure.

Silencing of the $GHSR^{lPBN}$ neurones was achieved by delivering tetanus toxin light chain into the lPBN via injection of a Cre-dependent viral vector into *Ghsr-IRES-Cre* mice. This treatment produced a phenotype, meaning

that the $GHSR^{lPBN}$ cells have a role in this phenotype. However, we do not know if GHSR or GHSR signalling is the important signal in these cells that explains the outcome observed since the cells lost the capacity to respond to any afferent signal. The idea that GHSR might be the critical signal here is nevertheless supported by our previous data showing that intra-lPBN delivery of ghrelin in rats increased food intake and altered food choice (**Paper III**). Other receptors known to drive a feeding response when activated in the lPBN include benzodiazepine receptors (Higgs and Cooper, 1996; Söderpalm and Berridge, 2000), μ-opioid receptors (Wilson et al., 2003) and cannabinoid 1 receptors (DiPatrizio and Simansky, 2008). It would certainly be of interest to determine whether the $GHSR^{lPBN}$ cells co-express any of these receptors. Besides, glutamatergic neurones have been identified in the lPBN that project to orexin-expressing neurones of the hypothalamus in rats (Niu et al., 2010). Orexin peptides are known to promote food intake (Sakurai et al., 1998) and it is believed that ghrelin can stimulate hypothalamic orexin-containing neurones directly (Yamanaka et al., 2003). Yet, these neurones might be activated by ghrelin-responsive inputs coming from another brain area. Hence, finding out whether $GHSR^{lPBN}$ cells also express glutamate would be very informative with regard to the identity of the $GHSR^{lPBN}$ neurones.

Since, in our study, mice were fed a HFHS free choice diet, we could study their dietary food choice. Mice with silenced $GHSR^{lPBN}$ cells consumed less sucrose solution compared to controls. This effect was not accompanied by a change in preference for the non-caloric sweetener, saccharin, indicating that the reduction in sucrose intake was not a result of impaired sweet taste sensation. The PBN is a well-established relay for taste/gustatory information (Norgren and Leonard, 1973; Norgren and Leonard, 1971; Norgren and Pfaffmann, 1975). Moreover, the lPBN is involved in the hedonic valuation of food (Hajnal and Norgren, 2005; Scott and Small, 2009) and in the regulation of palatable food intake (De Oliveira et al., 2011; Rodriguez et al., 2019). Hence, the $GHSR^{lPBN}$ neurones studied in this thesis are more likely part of the neuronal pathways controlling consumption of palatable food (notably sucrose) and food choice than part of the neurocircuit sensing and relaying sweet taste information.

Finally, since the CGRP neurones are the best-described cell type of the lPBN with a very potent role in feeding control (Carter et al., 2013; Campos et al., 2016), we used immunohistochemistry to determine whether CGRP colocalize with GHSR in the lPBN. We found that $GHSR^{lPBN}$ and $CGRP^{lPBN}$ cells are different neuronal populations with only a small proportion of cells

expressing both GHSR and CGRP. Consequently and not surprisingly, the majority of the orexigenic $GHSR^{lPBN}$ cells are not part of the anorexigenic circuit containing CGRP. Hence, more studies are needed to determine the neurochemical identity of the $GHSR^{lPBN}$ neurones.

On a technical note, visualisation of the viral vector injection site required using RNAscope to detect *EGFP* mRNA expression. Despite successful immunohistochemical detection of EGFP protein in EGFP-positive sections from rats, we failed to detect it in lPBN-containing sections from the TetoxLC mice. *EGFP* mRNA was present in all lPBN cells, nicely defining the viral vector injection site, but was of course not found specifically in the $GHSR^{lPBN}$ cells. The lack of protein staining could have multiple explanations. It could be, for instance, that the translation machinery of the $GHSR^{lPBN}$ cells of *Ghsr-IRES-Cre* mice cannot carry out the expression of the proteins encoded by the viral vector for as long as 5 weeks after injection (when we terminated the study). Another explanation could reflect the fact that when *Ghsr-IRES-Cre* mice are bred with the Cre-inducible ZsGreen reporter line, it only takes Cre to be expressed once during development to induce recombination and expression of ZsGreen permanently, as described for *Nts*-Cre;GFP mice (Schroeder et al., 2019). This would influence the fidelity with which ZsGreen in our mice reflects Cre activity and GHSR expression. However, we are confident regarding the validity of GHSR expression within the lPBN in our study as it perfectly resembles the known distribution of the receptor revealed by *in situ* hybridisation studies in adult mice (Zigman et al., 2006).

To sum up, our results show that $GHSR^{lPBN}$ neurones are of importance for the development of diet-induced hyperphagia and body weight gain. These cells also appear to have a role in food choice and possibly hedonic sucrose consumption. Although we already know that the majority of the $GHSR^{lPBN}$ neurones do not express CGRP, more studies are needed to determine the neurochemical identity of the $GHSR^{lPBN}$ cells involved in dietary obesity.

Conclusion

This thesis provides novel information about different aspects of ghrelin signalling within the CNS in the context of feeding control. First, we show that central ghrelin administration carries a negative valence signal, in both rats and mice, independently of food availability and malaise. Then, the SuM is identified as a brain region that is activated in situations associated with elevated circulating ghrelin levels and from which ghrelin can drive an

orexigenic response. The second part of this thesis demonstrates that the lPBN is a relevant target for ghrelin's feeding effects but not for its effects on food motivation or reward, and that the $GHSR^{lPBN}$ neurones, a distinct cell population from the $CGRP^{lPBN}$ cells, are important in diet-induced hyperphagia and body weight gain as well as in the regulation of food choice. **Figure 14** gives a schematic representation of the novel information presented in this thesis.

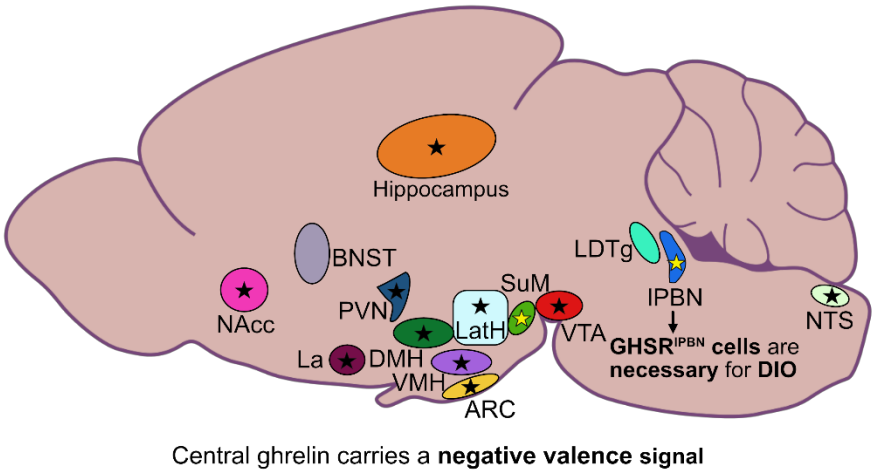

Figure 14. Schematic representation of the new knowledge provided in this thesis. *Work presented herein revealed that central ghrelin administration carries a negative valence signal. The brain regions found in this thesis to be novel targets for ghrelin's effects on feeding are marked by a yellow star, namely the supramammillary nucleus (SuM) and the lateral parabrachial nucleus (lPBN). More specifically, $GHSR^{lPBN}$ cells were identified as being necessary for the development of diet-induced obesity (DIO).*

Future perspectives

During my thesis, I have been involved in the introduction of *Ghsr-IRES-Cre* mice to our research group. The presence of Cre in all GHSR-expressing neurones opens up a host of interesting possibilities to study the function of specific sub-populations of ghrelin-responsive neurones in discrete brain areas. To resolve the issue of which populations of ghrelin-responsive cells are responsible for ghrelin's aversive effects, for example, we could use a chemogenetic approach and determine whether activation of ARC (or indeed, VTA) GHSR-expressing cells is aversive, in conditioned place and flavour avoidance studies. This would involve the injection of a Cre-dependent viral vector that delivers DREADDs (Designer Receptor Exclusively Activated by Designer Drugs) to a given area; all cells in that area that express Cre (here, the GHSR-expressing cells) will express the designer receptor, enabling us to specifically control their activity using a designer drug (e.g. clozapine N-oxide). Likewise, it may be possible to use inhibitory DREADDs or silencing viral vectors to inhibit these neurones and then explore the ensuing phenotype.

The tetanus toxin light chain silencing approach used in **Paper IV** provided a phenotype, namely that the mice had reduced body weight gain when fed a high-fat high-sugar diet (due to reduced food intake). Of note, we do not know whether this phenotype is specifically due to loss of GHSR signalling in those cells (as they likely also receive other afferent signals). It would, therefore, be of interest to deliver activating DREADDs to the $GHSR^{lPBN}$ cells and determine whether the opposite phenotype is observed (i.e. weight gain and increase food consumption).

Besides, investigating the identity and function of the downstream neuronal circuits engaged by the GHSR-expressing cells of interest (e.g. in the ARC, VTA or lPBN) would also be very informative. Mapping Fos expression after chemogenetic or optogenetic stimulation of the ghrelin-responsive neurones studied would for instance allow us to find out which brain regions are activated by these GHSR-expressing cells. We could then take control of sub-populations of the Fos-positive cells using the Fos-TRAP technology (Guenthner et al., 2013; DeNardo et al., 2019) and further define their role in feeding. In addition, other viral vectors (Cre-inducible or not) and tracers could be used to visualise the projections (excitatory and inhibitory) of different cell populations. This would allow us to reconstruct the full circuit

engaged by the activity of the GHSR-expressing cells at specific sites in the brain.

Future studies are also needed to explore the neurochemical identity of the ghrelin-responsive cells in the SuM and in the lPBN, which could be achieved using immunohistochemistry or RNAscope, for example.

Finally, another gap in knowledge that needs to be addressed is whether ghrelin reaches the SuM and lPBN directly or indirectly. If ghrelin's action is direct, the mechanism through which it accesses these brain areas should be determined. If, on the other hand, ghrelin indirectly engages these regions, the afferent inputs mediating ghrelin's action at these sites should be identified.

The knowledge generated by such studies would significantly broaden our understanding of how ghrelin acts in the CNS to generate specific feeding behaviours and contribute to the identification of potential neuronal targets for future pharmacotherapies against obesity and other feeding behaviours.

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